SPIN-LATTICE RELAXATION TIMES FOR ^{13}C IN ISOTOPE-ENRICHED GLYCINE ACCUMULATED IN FROG MUSCLE

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ABSTRACT Spin-lattice relaxation times (T_1's) of ^{13}C-enriched glycine accumulated in frog muscles were determined at 1°C by the inversion-recovery (180°-τ-90° pulse sequence) method and compared with the values obtained in free solution. The value of T_1 for the α-^{13}C nucleus of glycine in the tissue was 50% of that obtained in free solution. The observed value for T_1 in the tissue was not concentration-dependent, and no difference in chemical shift was observed between tissue and free solution. Quantification of the area under the glycine peak suggested that the observed signal represents at least 80% of the intracellular glycine. An average nuclear Overhauser enhancement of 2.83 for intracellular glycine indicates that the relaxation mechanism within the cell is predominantly dipolar, as in free solution. The value of T_1 for the $^{13}C'$ nucleus of glycine in the tissue was 67% of that in a solution of similar concentration. A quantitative analysis of the findings suggests that the observed difference in the value of T_1 between tissue and free solution results from a difference in viscosity. The data provide no evidence either for special organization of intracellular water or for glycine binding. It is proposed that intracellular diffusion coefficients may be determined from measurements of ^{13}C T_1's of ^{13}C-enriched intracellular solutes.

INTRODUCTION

The nuclear magnetic relaxation rate depends on the presence of fluctuating magnetic fields in the environment of a particular nucleus. In liquids in the absence of paramagnetic species, such fluctuating fields arise primarily from random rotational and diffusional motion of the molecules in which the nucleus is located (Carrington and McLachlan, 1967). For this reason nuclear magnetic relaxation (NMR) measurements are a valuable tool for probing molecular motion in both physical and biological systems.

The finding that relaxation rates from protons of water in living systems are often at least an order of magnitude greater than those measured in pure water (see Walter and Hope, 1973, for review) added fuel to a controversy about the state of the organization of the small molecular weight constituents of the cytoplasm. Ling (1962)

proposed that the cell water is so organized that its solvent properties are different from those of the water in a dilute salt solution. Furthermore, he hypothesized that most of the small molecular weight components of the cytoplasm are specifically bound to cellular macromolecules (see also Neville, 1973). From an NMR standpoint the controversy centered about the question of whether the proton NMR relaxation rates in intracellular water were faster than in free solution because of structuring of the bulk of the cell water or because of exchange between a small number of highly structured water molecules (bound, say to intracellular macromolecules) and a bulk phase of relatively normal water (Cooke and Wien, 1973).

Many investigators have approached this problem by examining aspects of the proton relaxation behavior of cell water (Civan and Shporer, 1975; Hazlewood, et al., 1974; Block and Maxwell, 1974; Abetsedarskaya et al., 1968; Outhred and George, 1973; Finch et al., 1971). We felt that a valuable alternative approach to the study of both water organization and solute binding would be to examine the motion in the cytoplasm of a small molecular weight substance other than water. The finding that the intracellular relaxation behavior of such a substance could be explained solely on the basis of viscosity differences between the cytoplasm and a dilute salt solution would provide strong evidence (*a*) that the organization of the bulk of the cell water does not differ from that of the water in a dilute salt solution and (*b*) that the substance was not bound to cytoplasmic constituents of the cell.

Because one of us (Neville, 1973) had suggested that amino acid accumulation results from intracellular binding, we chose to examine the longitudinal relaxation behavior (T_1) of ^{13}C nuclei in glycine accumulated in frog muscle. This system presented several advantages: (*a*) glycine is accumulated against a concentration gradient in frog muscle under certain conditions (Neville, 1973); (*b*) at temperatures near 0°C glycine metabolism in frog muscle is very slow so that metabolism of the compound during the relatively long NMR measurements could be prevented; (*c*) procedures for eliminating glycine from both extracellular space and sarcoplasmic reticulum are available[1] allowing behavior of intracellular solute to be examined; (*d*) relaxation of the C nucleus of [α-^{13}C]glycine was found to be predominantly by dipolar interaction with protons, both in free solution and within the cells, so that effects of viscosity could be predicted (see below); and (*e*) both 90% enriched [α-^{13}C]glycine and [$^{13}C'$]glycine are commercially available, allowing the muscles to be loaded with a compound whose signal could be distinguished from the naturally abundant ^{13}C in the organic constituents of the tissue. A preliminary report of the results of these studies has been given (Neville and Wyssbrod, 1975).

THEORY

To infer a special organization of the cytoplasmic constituents from relaxation measurements, it is necessary to show that any differences between relaxation of ^{13}C nuclei in glycine in solution and in the cell do not result simply from increased cytoplasmic

[1]Neville, M. C., and S. White. 1976. The sarcoplasmic reticulum and solute compartmentalization in frog sartorius muscle. In preparation.

viscosity. Fortunately a firm theoretical formulation for the relationship between viscosity and the longitudinal relaxation time T_1 exists. When the major mechanism of relaxation of the ^{13}C nucleus is dipolar interaction with nearby ^1H nuclei (as we show below for [α-^{13}C]glycine), the relation between T_1 and the rotational correlation time (τ_c) is given by (Solomon, 1955; Kuhlmann et al., 1970):

$$\frac{1}{T_1} = N \frac{\gamma_C^2 \gamma_H^2 \hbar^2}{r^6} \tau_c, \tag{1}$$

where N is the number of ^1H nuclei attached to the ^{13}C nucleus; γ_C and γ_H are the magnetogyric ratios for the carbon and hydrogen nuclei, respectively; $\hbar$ is Planck's constant/2π; and r is the distance between the ^{13}C and ^1H nuclei.

Eq. 1 is valid only in the "extreme narrowing condition" or when

$$\tau_c \gg (\omega_C + \omega_H)^2, \tag{2}$$

where ω_C and ω_H are the precession frequencies of the ^{13}C and ^1H nuclei, respectively. In the Bruker HX-90 spectrometer used in these experiments (Bruker Scientific, Inc., Elmsford, N.Y.), $\omega_C = 1.42 \times 10^8$ rad/s and $\omega_H = 5.65 \times 10^8$ rad/s.

We obtain a theoretical value for τ_c as follows: From the theory of Debye (Carrington and McLachlan, 1967), the correlation time of a sphere of Stokes radius a is related to the viscosity η by

$$\tau_c = \frac{4\pi\eta a^3}{3kT} \tag{3}$$

where k is the Boltzman constant, and T the absolute temperature. As a first approximation, we assume that the nonspherical glycine molecule can be treated as if it were a sphere embedded in a continuous fluid. In this case the Stokes radius a can be obtained from the translational diffusion coefficient D by the Stokes-Einstein relationship:

$$a = \frac{kT}{6\pi\eta D}. \tag{4}$$

At 25°C, D for glycine in aqueous solution is 1.06×10^{-5} cm^2/s and η for pure water is 0.8904 cp (Weast, 1973). Substituting these values into Eq. 4, a is found to be 2.31×10^{-8} cm. This value is a slight overestimation because the contribution of the glycine per se to the viscosity was not taken into account. At 1°C, the temperature at which the experiments reported in this paper were performed, η for pure water is 1.711 cp (Weast, 1973). Substituting this value and the value for a calculated above into Eq. 3, we find that τ_c is approximately 2.3×10^{-11} s. Clearly the condition stated by Eq. 2 holds and Eq. 1 gives a valid estimate for T_1, not only in pure water but under conditions of greatly increased viscosity.

It is now possible to combine Eqs. 1 and 3 to obtain an inverse relation between T_1 and η at constant temperature:

$$\frac{1}{T_1} = \mathbf{K}\eta, \text{ where } \mathbf{K} = N \frac{4\pi a^3 \gamma_C^2 \gamma_H^2 \hbar^2}{3kTr^6} \tag{5}$$

Thus, if intracellular glycine is not bound and the bulk of the cell water is structured normally, the intracellular relaxation time should be predictable from the intracellular viscosity.

METHODS

Materials

[α-^{13}C]glycine (92% ^{13}C) was obtained from Koch Isotopes, Inc., Cambridge, Mass. and [^{13}C']glycine (90% ^{13}C), from Bio-Rad Laboratories, Richmond Calif. All other chemicals were reagent grade. Frogs (*Rana pipiens*, northern) were obtained from NASCO, Fort Atkinson, Wis. Composition of Ringer solution was NaCl, 105 mM; KCl, 2.5 mM; $NaHCO_3$, 6.6 mM; NaH_2PO_4, 1.4 mM; Na_2HPO_4, 0.7 mM; $CaCl_2$, 0.72 mM; $MgSO_4$, 1.5 mM; glucose, 5 mM. Up to 150 mM of glycine were added to this solution for incubation. Although the muscles shrank temporarily in the hypertonic solution, as glycine entered they gradually regained their initial volume.

Muscle Preparation

Small leg muscles were dissected intact and preincubated for 6 h at 25°C in frog Ringer solution containing 0.1 U/ml insulin to increase glycine accumulation (Neville, 1973). They were then incubated in frog Ringer solution containing a measured concentration of either [α-^{13}C](92% ^{13}C) or [^{13}C']glycine (90% ^{13}C). In most experiments trace amounts of radioactive [U-^{14}C]glycine were added to provide a measure of intracellular glycine. Incubation protocols were designed to load both intra- and extracellular spaces (4–10 days at 0°C), extracellular space and sarcoplasmic reticulum (2–3 h at 0°C), or intracellular spaces only (overnight at 25°C, followed by 0°C wash in a large volume of solution for 2–3 h). Efflux analysis[1] showed that the latter procedure removed glycine from both extracellular space and sarcoplasmic reticulum with little loss from the intracellular compartment of the muscle. Thin layer chromatography of glycine extracted from muscles after overnight incubation with 100 mM glycine at 25°C indicated that 98% of the isotope present migrated with the glycine peak. After completion of the NMR measurements, the muscles were removed from the NMR tube and weighed, and the intracellular glycine was extracted with 5% trichloracetic acid (wt/vol). Aliquots of the extract were counted with Bray's scintillation fluid (1960) in a Picker Liquimat β-scintillation counter (Picker Corp., Cleveland, Ohio). The average concentration of glycine in the tissue was calculated from the following expression:

$$[\mathrm{Gly}]_{\mathrm{tis}} = \frac{(A - B) \times D}{W \times S}, \tag{6}$$

where $[\mathrm{Gly}]_{\mathrm{tis}}$ = average tissue concentration of glycine (μmol/g tissue), A = radioactivity of aliquot of tissue extract (counts/min), B = radioactivity background (counts/min), D = aliquot dilution factor (unitless), W = total sample weight of muscle (gram tissue) and S = specific activity of the sample (counts/min/μmol) = [radioactivity of supernatant incubation fluid (counts/min/μl)]/total chemical concentration of glycine (M).

Sample Configuration for NMR Studies

1 g of muscle was placed in a 10-mm outside diameter Wilmad tube (Wilmad Glass Co., Inc., Buena, N.J.) with Teflon spacers to maintain the sample within the transmitter coil of the spectrometer. In early experiments a capillary containing C_6F_6 was placed at the center of the sample to provide a lock signal on ^{19}F. In later experiments the spectrometer was locked on the ^{19}F resonance in an external capillary of C_6F_6. In these experiments an internal

capillary contained neat $^{13}CH_3OH$ to provide a reference for quantitation of the [^{13}C]-glycine signal. The NMR tube was kept in ice until placed in the probe, which was maintained between 1 and 3°C to prevent metabolic utilization of accumulated glycine.

NMR Measurements

NMR measurements were made at 22.62 MHz on the Bruker HX-90 spectrometer (Bruker Instruments, Inc., Billerica, Mass.), located in the Department of Chemistry at Colorado State University with a ^{13}C probe and Digilab software (Digilab, Inc., Cambridge, Mass.). With exception of experiments to determine nuclear Overhauser enhancement (NOE), protons were irradiated throughout all measurements at a level of power sufficient for maximal enhancement of the ^{13}C signal. The proper pulse durations for the 90° and 180° pulses were determined with either 1.5 or 0.5 M [α-^{13}C]glycine in Ringer solution.

Quantitation of [α-^{13}C] Glycine Signal

The amount of [α-^{13}C]glycine in the muscle sample was determined by comparing the area under the signal from the [α-^{13}C]glycine with the area under the signal from a reference solution of $^{13}CH_3OH$ in a capillary in the center of the sample. The free induction decay (FID) from a sufficiently large series of 90° pulses to obtain a satisfactory signal to noise (S/N) ratio was accumulated. The absorption mode spectrum (see inset, Fig. 1, for example) was obtained by Fourier transformation of the FID and plotted on a scale on the abscissa of 20–50 Hz/cm to obtain peaks that could be cut out and weighed to obtain the relative areas. To obtain reference values, the ratio of the area under the [α-^{13}C]glycine peak to the area under the $^{13}CH_3OH$ peak was determined for [α-^{13}C]glycine solutions of varying concen-

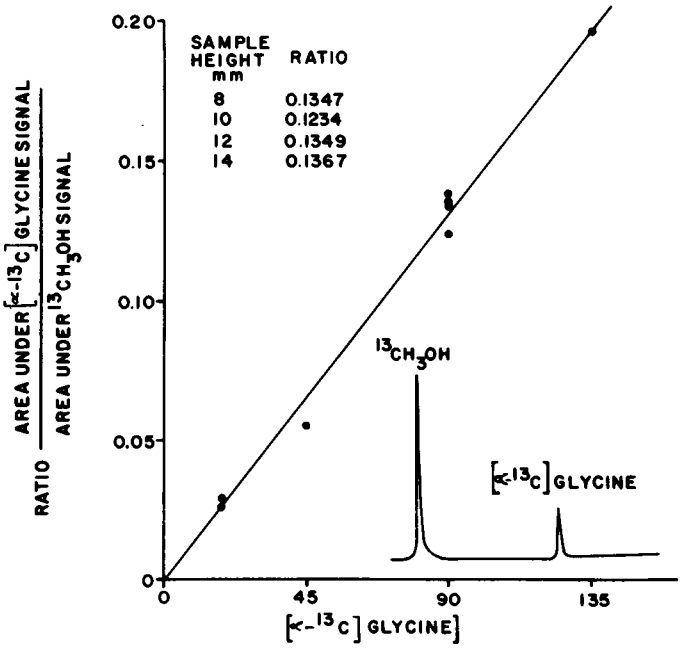

FIGURE 1 Quantitative of area under [α-^{13}C]glycine signal. Ratios obtained from spectra from solutions of known composition. Lower right: spectrum from solution containing 135 mM [α-^{13}C]glycine. Upper left: ratios and sample heights from 90 mM [α-^{13}C]glycine solution.

tration. The standard curve obtained is plotted in Fig. 1. The linear relationship and the correlation coefficient of 0.995 suggest that the technique is satisfactory. Our concern that small variations in the height of the sample would affect the results was allayed by the finding that a variation in the height of the standard solutions in the NMR tube from 8.5 to 14 mm did not appreciably affect the relative peak areas (upper inset, Fig. 1).

Measurement of Nuclear Overhauser Enhancement (NOE)

The NOE was measured by the gated decoupling method of Freeman et al. (1972). The areas under the peaks from spectra obtained under coupled and fully decoupled conditions were measured by weighing as above.

Measurement of T_1

T_1's of the ^{13}C nuclei were measured by the inversion-recovery (180°-τ-90° pulse sequence) method (Hahn, 1949; Vold et al., 1968). In all cases the FID was accumulated after the 90° pulse and enough scans to obtain a satisfactory S/N ratio were collected. The absorption-mode spectrum was obtained by Fourier transformation of the FID and peak height relative to noise computed. The proper phase correction for the absorption-mode spectrum was chosen for the first measurement of each series (the fully inverted spectrum obtained when τ = 0.01 or 0.1 s) and used for subsequent spectra. A delay of at least 4 × T_1 was allowed between successive pairs of pulses.

The value of T_1 was computed from the following expression:

$$S(\tau) = S_\infty - (S_\infty - S_0) \exp(-\tau/T_1) \quad (7)$$

where τ is the time delay between the 180° and 90° pulses (in seconds) and $S(\tau)$, S_0, and S_∞ are the observed absorption-mode signal heights for the time delays τ, zero, and infinity, respectively. Values for T_1, S_0, and S_∞ were obtained by use of the program NNLSQ, a nonlinear least squares data-fitting routine on the CDC 6400 computer (Control Data Corp., Minneapolis, Minn.) at the University of Colorado in Boulder, based on an algorithm proposed by Marquardt (1963) and which weights each datum in proportion to its absolute value. For controls, T_1's of solutions of ^{13}C-enriched glycine in Ringer solution were measured by the same inversion-recovery method.

RESULTS

T_1 Measurements for [α-^{13}C]Glycine

Spectra at various values of τ were obtained for muscles containing [α-^{13}C]glycine. Base-line noise arose mainly from the naturally abundant (~ 1.1%) ^{13}C of organic material within the tissue. Nevertheless, the S/N level after 80 scans was adequate to obtain reasonable estimates of the spin-lattice relaxation time (T_1). Pertinent data from these spectra as well as for free solutions of [α-^{13}C]glycine are given in Table I.

If the durations of the 180° and 90° pulses are sufficiently accurate, the absolute value of S_0, which essentially corresponds to a 270° pulse, should equal the value of S_∞, which essentially corresponds to a 90° pulse. That this is not the case (see Table I) suggests that the pulse durations deviated slightly from those necessary to produce true 180° and 90° rotations of the macroscopic magnetization vector. It can be shown, however, that the value of T_1 is relatively immune to slight deviations in the pulse durations when Eq. 7 is used for the calculations (Jones and Sternlicht, 1972; Freeman

TABLE I

SPIN-LATTICE RELAXATION TIMES (T_1's) OF THE α-^{13}C NUCLEUS OF [α-^{13}C]GLYCINE

Sample	[α-^{13}C]glycine concentration	T	N	S_0		S_∞		T_1
				Observed	Computed	Observed	Computed	
Glycine solutions, mM		s						s
	450	8	20	−0.501	−0.524	0.671	0.674	1.93 ± 0.23
	150	10	1	−0.557	−0.508	0.653	0.638	2.07 ± 0.23
	100	10	5	−0.477	−0.505	0.728	0.735	2.24 ± 0.14
	50	10	9	−0.218	−0.201	0.252	0.242	1.91 ± 0.19
	20	10	50	−0.053	−0.049	0.066	0.067	2.09 ± 0.27
								av 2.05 ± 0.06
Frog muscle, $\mu mol/g$								
All compartments loaded								
	48.3 ± 1.7	6	80	−0.517	−0.562	0.728	0.735	1.13 ± 0.14
	14.8 ± 1.3	6	160	−0.282	−0.285	0.327	0.318	1.18 ± 0.15
								av 1.15
ECS and SR loaded								
	40.3 ± 2.5	10	50	−0.046	−0.040	0.078	0.071	0.92 ± 0.28
	28.9 ± 2.6	10	200	−0.048	−0.068	0.142	0.165	1.53 ± 0.89
								av 1.23
Cytoplasm loaded								
	30.2 ± 1.0	4	50	−0.050	−0.052	0.107	0.107	1.00 ± 0.10
	27.5 – 35*	4	50	−0.148	−0.151	0.216	0.215	1.05 ± 0.13
								av 1.03
Cytoplasm loaded, hypertonic wash								
	37–47*	4	100	−0.141	−0.148	0.197	0.209	0.81 ± 0.04

T is the time between repeated pulse sequences; N is the number of pulse sequences; S_0 and S_∞ are the signal heights in arbitrary units when τ = 0.01 s and ∞, respectively; the technique for loading the various compartments is described in the text. All ± values are SEM.

*From quantitation of NMR signals. All other muscle [α-^{13}C]glycine concentrations obtained from [^{14}C]glycine analysis.

et al., 1972). In computing T_1 by the nonlinear least squares technique, it was found that the value obtained depended strongly on the computed value of S_∞ (the correlation coefficient between T_1 and S_∞ computed by NNLSQ was 0.77). For this reason it is gratifying that the computed and observed values of S_∞ are in good agreement (see Table I).

For free solutions of [α-^{13}C]glycine, we obtained 2.05 s for the value of T_1. This value, which did not vary significantly over the range 20–500 mM, was obtained from measurements made at 1.3°C. It compares favorably with the 3-s value reported by Armitage et al. (1974) for the α-^{13}C nucleus of [α-^{13}C]glycine (90% ^{13}C) in D_2O solutions, presumably at room temperature.

To obtain muscles containing as much [α-^{13}C]glycine as possible, in early experiments we incubated them with the isotope at 0°C for 7–10 days before the NMR experiment. T_1 values obtained from the [α-^{13}C]glycine in such muscles are shown in Table I under the heading "all compartments loaded". The average value of about 1.1 s is about one-half that obtained from glycine in free solution. It should be noted there was no difference in chemical shift for the [α-^{13}C]glycine nucleus between free solution and muscle and no significant effect of concentration.

Muscles loaded with [α-^{13}C]glycine in the above manner contain a sizeable amount of [α-^{13}C]glycine in the extracellular space (ECS) and sarcoplasmic reticulum (SR) as well as in the intracellular compartment.[1] Since the viscosities of these compartments are likely to differ, we felt our results could be interpreted more accurately if we examined the relaxation behavior of [α-^{13}C]glycine selectively located in either the cytoplasm or the ECS and SR.

We have shown elsewhere[1] that both the ECS and SR are freely accessible to small molecular weight components of the bathing medium, equilibrating in less than 1 h at 0°C. On the other hand, glycine enters the cytoplasm very slowly at this temperature, requiring several days for full equilibration (Neville, 1973). This allowed us to load selectively the SR and ECS by incubating the muscles with [α-^{13}C]glycine for 2–3 h at 0°C before the NMR measurements. Trace amounts of [^{14}C]glycine were added as described in Methods. By keeping muscles cold as they were placed in the NMR tube and keeping the probe temperature low, redistribution of isotope during the NMR measurements, which took up to 10 h, was largely prevented. To demonstrate this, we analyzed a portion of the muscle sample for [^{14}C]glycine activity directly after the NMR measurements. Another portion was washed in a large volume of Ringer solution for 1 h at 0°C before analysis. At least 80% of the radioactivity and thus presumably the [α^{13}C]glycine left the muscles during the 1-h wash, demonstrating that the majority of isotope was located in the ECS and SR during the course of the NMR measurements (Table II).

The T_1 values from these muscles (Table I) were quite variable, but on the average were only slightly higher than the earlier values obtained with all compartments loaded.

To load the cytoplasmic compartment selectively, we took advantage of the large temperature coefficient of amino acid transport into the sarcoplasm (Neville, 1975)

TABLE II

ANALYSIS OF GLYCINE CONTENT OF MUSCLES

Treatment	Major compartments loaded	[α-^{13}C]glycine*		[U-^{14}C]glycine‡	
		Average	Range	Directly after NMR measurement	After 1-h wash
		mM		*μmol/g*	
2–4-h incubation at 0°C with isotopes	SR and ECS	34	28–40	40.3 ± 2.5 (4)	6.7 (2)
Overnight incubation at 25°C with isotopes, followed by 2.5-h wash at 0°C	Sarcoplasm	31.5	24–39	30.2 ± 1.0 (4)	23.9 (2)

*Estimated from area under [^{13}C]glycine peak, as described in text.

‡Estimated from analysis of extracts of muscles as described in text. Results are expressed as mean ±SEM (number of samples).

and loaded the muscles at 25°C overnight. [α-^{13}C]glycine was then removed from the ECS and SR by washing at 0°C for 2–3 h. The intracellular location of the majority of the [α-^{13}C]glycine during the NMR measurements was verified by analyzing the muscles as described above. In this experiment 85% of the radioactivity remained in the muscles after the 1-h wash (Table II).

The T_1 for [α-^{13}C]glycine in the cytoplasm averaged 1.03 s (Table I), exactly one-half the value obtained for glycine solutions at the same temperature. The possibility that this reduction in T_1 can be attributed to intracellular viscosity will be considered in the discussion section.

To determine whether a decrease in cell water would have any effect on T_1, we loaded the muscles by incubating overnight at 25°C with 90 mM [α-^{13}C]glycine, then washed them 2 h in Ringer solution containing 300 mM sucrose. This procedure not only removes [α^{13}C]glycine from the ECS and SR, it also reduces the water content of the cytoplasmic compartment by approximately one-half.[1] Under such conditions we observed a significant decrease in T_1 (Table I).

Quantitation of NMR Signal

To rule out substantial intracellular binding of glycine, it is necessary to show that the observed ^{13}C-NMR signal represents all of the glycine in the tissue, i.e. that there is no tightly bound, slowly exchanging fraction whose signal is so broad that it is obscured by base-line noise. To investigate this, we compared the area under the [α-^{13}C]glycine peak from the tissue with a reference peak whose area had been related to glycine concentration, as described in the Methods section. The method gave low precision, primarily because the background noise in the tissue led to uncertainties about the base-line. To deal with this, an average base-line level was first estimated, and the area measured and related to concentration, with the values shown in Table II as "average." Upper and lower limits for the base line were then estimated and the values reported under "range" in Table II calculated.

TABLE III

NUCLEAR OVERHAUSER ENHANCEMENT

Sample	NOE
Solution: 150 mM [α-^{13}C]glycine	2.95
Muscle: ECS and SR loaded	2.90
Muscle: Sarcoplasm loaded (average of three samples)	2.83

For a sample incubated in such a way as to load mainly the SR and ECS, the [α-^{13}C]glycine peak gave an average concentration of 34 mM, not significantly different from the value of 40.3 mM estimated from radioactive glycine (Table II). For a sample in which the sarcoplasm was the major compartment loaded, the [α^{13}C]glycine peak gave an average concentration of 31.5 mM compared to a value of 30.2 mM estimated from the radioactive glycine. The results of this experiment are consistent with the postulate that all the intracellular glycine is detected in the [α-^{13}C]glycine peak; they set a lower detection limit of 80%. Thus these results rule out a tightly bound, slowly exchanging fraction larger than 20% of the intracellular glycine.

Nuclear Overhauser Enhancement

The data in Table III show the values for the NOE of [α-^{13}C]glycine in both solution and in tissue samples. When compared to the theoretical value of 3.0 for pure dipolar relaxation, the results indicate that, within the error of the method, the α-^{13}C nucleus of glycine relaxes by dipolar interaction with neighboring ^{1}H nuclei.

NMR Measurements of [$^{13}C'$]Glycine

Preliminary measurements were made of the value of T_1 for the ^{13}C′ nucleus of [^{13}C′]glycine (90% ^{13}C) accumulated in frog muscle. Spectra were obtained from a muscle containing 15.9 μmol glycine/g tissue. The data obtained from these spectra are compared in Table IV with data obtained from a free solution containing 20 mM [^{13}C′]glycine (90% ^{13}C). The value of the T_1 for the ^{13}C′ nucleus of glycine in the tissue ($\sim$ 14 s) was about 67% of that in a free solution of similar concentration ($\sim$ 22 s).

TABLE IV

SPIN-LATTICE RELAXATION TIMES (T_1's) OF THE ^{13}C′ NUCLEUS OF [^{13}C′]GLYCINE

Sample	[^{13}C′]glycine concentration	T	N	S_0		S_∞		T_1
				Observed	Computed	Observed	Computed	
		s						s
Glycine solution	20 mM	90	12	−0.190	−0.234	0.333	0.326	22.2 ± 3.1
Frog muscle	15.9 ± 2.3 μmol/g	60	20	−0.262	−0.278	0.305	0.317	14.0 ± 1.4

All comments that apply to Table I also apply to this table, except that the observed S_0 is the signal height in arbitrary units when τ = 0.1 s.

DISCUSSION

These results clearly demonstrate the feasibility of determining ^{13}C T_1 values of ^{13}C-enriched compounds within biological systems. Two limitations of this technique must be recognized. The first is the large amount of time required to obtain adequate data. Muscle from northern *Rana pipiens* as used in these experiments can be preserved sufficiently long near 0°C (4–12 h in the NMR probe) to make meaningful measurements. Other tissues might not withstand this treatment. The second limitation is the sensitivity of the method. We were able to obtain adequate S/N ratios at concentrations as low as 15 μmol/g of 90% ^{13}C-enriched glycine. By extending the time of data accumulation or by increasing the sample size, it should be possible to work at concentrations approaching, say, 5 μmol/g in a spectrometer operating at 22.62 MHz. There is no reason why other intracellular ^{13}C-enriched compounds cannot be studied at this low concentration if they contain ^{13}C nuclei with T_1 values equal to or less than those of the ^{13}C nuclei in glycine.

When Eq. 5 is valid, it should be possible to predict the intracellular diffusion coefficient, D, from T_1. Combining Eqs. 4 and 5, we obtain

$$D = K'T_1, \tag{8}$$

where $K' = 2Na^2(\gamma_C^2\gamma_H^2\hbar^2)/9r^6$.

Eq. 8 shows that when dipolar relaxation is predominant and the "extreme narrowing case" holds, there should be a direct proportionality between the diffusion coefficient, D, and T_1. That this is indeed the case for glycine in free solution is indicated by comparing the value for D that can be calculated from Eq. 8 and the observed value of T_1 (2.05 s) of 1.0×10^{-5} cm^2/s with the published value of 1.06×10^{-5} cm^2/s (Weast, 1973).

The 50% reduction in the α-^{13}C T_1 value in muscle suggests that the diffusion coefficient for glycine is reduced by about 50% in this tissue. This finding is consistent with the measurements of Kushmerick and Podolsky (1969), who found, using a radioisotope diffusion method, that the diffusion coefficients of K^+, Na^+, SO_4^{-2}, ATP, sorbitol, and sucrose in frog muscle fibers were about 50% of those in nonviscous free solution. Further, proton NMR measurements suggest that the diffusion coefficient of water in frog muscle is also reduced by about 50% (Hazlewood et al., 1974). The consistency of all these findings suggests that a single factor, most likely the viscosity, underlies the reduction of diffusivity. From Eq. 5 it can be seen that a doubling of viscosity would account quantitatively for our value of T_1 for the α-^{13}C nucleus of [α-^{13}C]glycine.

A quantitative examination of the T_1 data for the $^{13}C'$ nucleus of [$^{13}C'$]glycine would be premature at this time, inasmuch as the contribution of spin rotation to the relaxation of C′ nuclei has not yet been quantitatively assessed (Armitage et al., 1974). Preliminary measurements of the effect of temperature on the T_1 value for $^{13}C'$ nuclei of [$^{13}C'$]glycine in frog muscle suggest that dipolar interactions predominate here also, and that the observed reduction of T_1 in muscle may also be a result of a viscosity effect on molecular motion.

The data presented here indicate that intracellular viscosity accounts for the reduction in T_1 for [^{13}C]glycine in frog muscle. They are in accord with the conclusions drawn by others that the bulk of the cell water is in a normal state (Cooke and Wien, 1973; Civan and Shporer, 1975; Outhred and George, 1973; Finch et al., 1971) and that the large majority of the glycine accumulated in frog muscle is free in solution (Neville, 1975). These results also suggest that T_1 measurements of ^{13}C-enriched solutes within cells may be a useful method of obtaining the intracellular diffusion coefficient.

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