Microviscosity of human erythrocytes studied using hypophosphite two-spin order relaxation

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ABSTRACT A new ^{31}P NMR method is used to probe the cytoplasmic viscosity of human erythrocytes. The method is based on observing two-spin order relaxation of the ^{31}P atom of the hypophosphite ion. This method is superior to our previous method, using the longitudinal relaxation time of the ion, because random field effects such as intermolecular dipole–dipole relaxation can be separated from intramolecular relaxation. This allows a more accurate determination of the effective reorientational correlation time from the measured intramolecular relaxation because it is now unaffected by random field effects. The new method also provides a means by which to estimate the random field effects. Both two-spin order and proton-decoupled T_1 measurements were conducted on hypophosphite in water solutions at various temperatures, glycerol solutions of various viscosities, and in erythrocyte samples of various cell volumes. The results show that the effective reorientational correlation time of the hypophosphite ion varies from 7.2 to 15.2 ps in the cytoplasm of cells ranging in volume from 102 to 56 fl cells.

INTRODUCTION

The cytoplasmic viscosity of erythrocytes can have broad physiological effects. It can affect red cell deformability (Evans, 1989), which will ultimately affect blood circulation due to the increase in whole blood viscosity. Increased cytoplasmic viscosity will also lead to slower diffusion rates for molecules in the cytoplasm. In the human erythrocyte, the enzymes carbonic anhydrase and glutathione peroxidase are thought likely to be under diffusion control (Hasinoff, 1984; Endre et al., 1983). Thus, increased cytoplasmic viscosity may affect metabolism through effects on diffusion-controlled reactions (e.g., Olea and Thomas, 1989).

Most methods for studying blood viscosity report on only the bulk properties of the blood and it can be difficult to separate membrane and cytoplasmic contributions (Dintenfass, 1968). Methods designed specifically to study intracellular viscosity often involve nonphysiological probe molecules (Morse et al., 1979; Herrmann and Müller., 1986) and so do not report on the forces relevant to small molecules of 'metabolite size'.

NMR provides a convenient, noninvasive means of studying the intracellular milieu. There are two types of NMR method for noninvasively probing intracellular viscosity, the pulsed field gradient method (e.g., Price et al., 1989a; Price et al., 1990; Price and Kuchel, 1990a) and the correlation-time method (e.g., Endre et al., 1983; Endre and Kuchel, 1986; Price et al., 1989b). However, the two methods report on motions on different timescales (Stilbs, 1987), with the correlation-time method giving information on motions occurring on very short timescales of the order of the reorientational correlation time (τ_c) of the molecule. In this study, τ_c refers to an 'effective' reorientational correlation time.

In our previous study of erythrocyte intracellular viscosity using the correlation-time method (Price et al., 1989b), proton-decoupled ^{31}P spin-lattice (longitudinal) relaxation time (T_1) measurements were used to determine τ_c of the probe molecule, hypophosphite ($H_2PO_2^-$; HP). From τ_c and molecular dimensions of HP the intracellular viscosity and translational diffusion coefficient were determined. The PH_2 moiety of HP forms an AX_2 system of spin-½ nuclei and so the proton-coupled ^{31}P spectrum appears as a triplet of resonance lines. In an erythrocyte suspension the intra- and extracellular HP species give rise to distinct ^{31}P NMR resonances. HP is a structural analogue of both the phosphate and bicarbonate ions and diffuses rapidly into the cell via the band 3 protein (Price and Kuchel, 1990b; Price et al., 1991). HP has also been used as a probe molecule in pulsed field gradient NMR studies of human erythrocytes (Price and Kuchel, 1990a) and for determining membrane potential from its transmembrane distribution (Kirk et al., 1988).

In this study, ^{31}P two-spin order ($2I_zS_z$, where the operators I and S are used to denote the ^{31}P and ^1H spins in the PH_2 moiety, respectively) relaxation (Tsai et al., 1991) in conjunction with proton-decoupled ^{31}P spin-lattice measurements are used to determine the effective reorientational correlation time of HP. Measurements

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were conducted in three systems: HP in H_2O solutions, HP in glycerol solutions, and HP in erythrocyte suspensions. Two-spin order relaxation results from chemical shift anisotropy (CSA)-dipole–dipole (DD) cross-interactions.

Selective study of cross-relaxation, which is extremely sensitive to reorientational correlation time, has been shown to be a useful tool for elucidating relaxation mechanisms and molecular dynamics (Hwang et al., 1988; Wang and Hwang, 1988; Wong et al., 1989; Chang et al., 1989, 1990; Chang and Hwang, 1991, Tsai et al., 1991). More importantly, this method allows separation of intramolecular and intermolecular (i.e., random field) contributions to the relaxation mechanism, thus affording more accurate estimates of the reorientational correlation time. In previous in vivo studies, using standard relaxation time measurements, the importance of random field effects was not quantitated and so the value of τ_c determined contained both intramolecular relaxation and unaccounted for intermolecular relaxation contributions.

THEORY

^{31}P NMR relaxation

We have previously shown that, excluding random field effects, the relaxation mechanism for the ^{31}P atom of hypophosphite is a combination of CSA, DD, and spin-rotation (SR) and CSA-DD cross-interactions (Price et al., 1989b; Tsai et al., 1991). Hence, the spin-lattice interaction, Hamiltonian, for this system may be defined by

$$\mathscr{H} = \mathscr{H}_{CSA} + \mathscr{H}_{DD} + \mathscr{H}_{SR}, \tag{1}$$

where $\mathscr{H}_{CSA}$, $\mathscr{H}_{DD}$, and $\mathscr{H}_{SR}$ represent the Hamiltonians for the chemical shift anisotropy, dipole–dipole and spin-rotation interactions, respectively.

For the ^{1}H-decoupled ^{31}P nucleus of HP in solution, the contributions of the individual mechanisms to the overall (extrinsic) relaxation rate constant are additive:

$$1/T_1 = 1/T_{1CSA} + 1/T_{1DD} + 1/T_{1SR} + 1/T_{1RAN}; \tag{2}$$

the individual relaxation rate constants are defined below.

The relaxation rate constant for the ^{31}P CSA interaction is given by (Abragam, 1961),

$$1/T_{1CSA} = 2/15\gamma_P^2 B_0^2 \Delta\sigma^2 \tau_c, \tag{3}$$

where γ_P is the phosphorus magnetogyric ratio, B_0 is the strength of the static magnetic field and $\Delta\sigma$ is the shielding anisotropy ($\sigma_\parallel - \sigma_\perp$).

The ^{31}P relaxation rate constant for the DD interaction, in the extreme narrowing condition, is given by (Abragam, 1961),

$$1/T_{1DD} = n_H(\hbar\gamma_H\gamma_P)^2/r_{PH}^{-6}\tau_c, \tag{4}$$

where n_H is the number of attached protons, $\hbar$ is Plancks constant, γ_H is the proton magnetogyric ratio, and r_{PH} is the phosphorus to proton bond length.

The relaxation rate constant for ^{31}P SR relaxation is given by (Green and Powles, 1965),

$$1/T_{1SR} = 2kTI_{av}C^2\tau_j/(3\hbar^2), \tag{5}$$

where k is the Boltzmann constant, T is temperature, I_{av} is the averaged moment of inertia of the molecule, C is the SR coupling constant of the ^{31}P nucleus, and τ_j is the effective angular momentum correlation-time of HP. The Hubbard relation (Hubbard, 1963) has been used to relate τ_c to τ_j.

The relaxation rate constant for ^{31}P random field relaxation is given by (Bain and Lynden-Bell, 1975),

$$1/T_{1RAN} \equiv 2\beta \equiv \tfrac{2}{3}\gamma_P^2\langle B \cdot B\rangle\tau_c, \tag{6}$$

where β is the isotropic random field term (for ^{31}P in the present case) defined by Bain and Lynden-Bell (1975), and B is the isotropic random magnetic field experienced by the ^{31}P nuclei. The corresponding formula for ^{1}H is

$$1/T_{1RAN}^{^1H} \equiv 2\eta \equiv \tfrac{2}{3}\gamma_H^2\langle B \cdot B\rangle\tau_c, \tag{7}$$

where η is the isotropic random field term defined by Bain and Lynden-Bell (1975), and B now refers to the isotropic random magnetic field experienced by the ^{1}H nuclei.

The CSA-DD cross-interaction gives rise to the two-spin order signal. Here we give only an outline of the theory, a more complete treatment may be found in our earlier publication (Tsai et al., 1991). To model the NMR relaxation process, the populations of the AX_2 system of spin-½ nuclei are described by the expectation values of multi-spin operators (e.g., $\langle I_zS_z\rangle$). Redfield theory (Redfield, 1965) is used to describe the relaxation processes including effects of CSA-DD cross-interactions. The description of the relaxation processes is given in terms of the rate equation,

$$\frac{d\mathscr{O}}{dt} = \mathbf{R}\mathscr{O}, \tag{8}$$

where t is time, $\mathscr{O}$ is an array of spin operators, and $\mathbf{R}$ is the Redfield relaxation matrix. $\mathscr{O}$ is defined by (Tsai et al., 1991),

$$\mathscr{O} = [\mathscr{O}_1, \mathscr{O}_2, \mathscr{O}_3, \mathscr{O}_4, \mathscr{O}_5, \mathscr{O}_6, \mathscr{O}_7], \tag{9}$$

where

$$\begin{aligned}
\mathscr{O}_1 &\equiv S0(0) - I_z^0 \equiv \langle I_z - I_z^0\rangle \equiv \langle \Delta I_z\rangle \\
\mathscr{O}_2 &\equiv U1(1) - S_z^0/\sqrt{2} \equiv \langle S_z - S_z^0\rangle/\sqrt{2} \equiv \langle \Delta S_z\rangle/\sqrt{2} \\
\mathscr{O}_3 &\equiv S0(2) \equiv -[4\langle I_zS_z'S_z''\rangle + 2\langle (S_+'S_-'' + S_-'S_+'')I_z\rangle]/\sqrt{3} \\
\mathscr{O}_4 &\equiv S2(2) \equiv 2[4\langle I_zS_z'S_z''\rangle - \langle (S_+'S_-'' + S_-'S_+'')I_z\rangle]/\sqrt{6} \\
\mathscr{O}_5 &\equiv S1(1) \equiv \sqrt{2}\langle I_zS_z\rangle \\
\mathscr{O}_6 &\equiv U0(2) \equiv -[2\langle S_z'S_z''\rangle + \langle (S_+'S_-'' + S_-'S_+'')\rangle]/\sqrt{3} \\
\mathscr{O}_7 &\equiv U2(2) \equiv [4\langle S_z'S_z''\rangle - \langle (S_+'S_-'' + S_-'S_+'')\rangle]/\sqrt{6},
\end{aligned} \tag{10}$$

and the U and S operators of Bain and Lynden-Bell (1975) have been written in operator form. In the above expressions the operators I and S ($\equiv S' + S''$) are used to denote the ^{31}P and ^{1}H spins in the PH_2 moiety of HP, and the superscript, 0, represents the equilibrium value of the corresponding spin operators.

$\mathbf{R}$ is given in the work of Bain and Lynden-Bell (1975) modified in accordance with the interaction Hamiltonian defined in Eq. 1 and the

initial preparation of the system (Tsai et al., 1991). In our previous study, the HP was dissolved in D_2O thereby minimizing random field effects. In this study the relaxation matrix includes the terms β and η. A completely asymmetric CSA tensor is incorporated into the relaxation matrix formulation as the sum of two axially symmetric CSA tensors. By analogy to the work of Goldman (1984), the CSA terms of the A spin are replaced by the following equations:

$$(\sigma_{\parallel}^{A} - \sigma_{\perp}^{A})^2 \rightarrow (\sigma_x^{A} - \sigma_z^{A})^2 + (\sigma_y^{A} - \sigma_z^{A})^2 - (\sigma_x^{A} - \sigma_z^{A})(\sigma_y^{A} - \sigma_z^{A}) \quad (11)$$

$$(\sigma_{\parallel}^{A} - \sigma_{\perp}^{A})P_2(\cos\delta) \rightarrow (\sigma_x^{A} - \sigma_z^{A})P_2(\cos\theta_{xz'}) + (\sigma_y^{A} - \sigma_z^{A})P_2(\cos\theta_{yz'}), \quad (12)$$

where P is the Legendre function, δ is the angle between the AX internuclear vector and the principal axis of the CSA tensor, while the angles $\theta_{xz'}$ and $\theta_{yz'}$ define the relative orientation between the IS internuclear vector (i.e., z' directed along P-H) with respect to the principal axes x and y of the chemical shift shielding tensor of HP, σ. For HP $\sigma_{11} = -105$, $\sigma_{22} = -7$ and $\sigma_{33} = 112$ ppm (relative to the isotropic chemical shift). σ_{33} is the most shielded element. There is a $6.0 \pm 1.0°$ tilt angle between the σ_{22} direction and the O-P-O plane (i.e., $\theta_{yz'} = 35.5°$) and $\theta_{xz'} = 90°$ (Tsai et al., 1991).

The radiofrequency pulse sequence used for measuring two-spin order relaxation is part of the DEPT pulse sequence (Bulsing and Doddrell, 1985),

$$(\pi/2)_x^{H} - 1/(2J_{PH}) - (\pi/2)_{\phi}^{H} - t - (\pi/2)_y^{P} - (FID),$$

where J_{PH} (Hz) is the spin–spin coupling constant and t is the evolution time. The two proton pulses generate $^1H \rightarrow {}^{31}P$ coherence transfer and the phosphorus pulse converts the two-spin order coherence into single-quantum coherence for ^{31}P observation. Immediately after the generation of coherence transfer the density matrix for the system is given by (Bulsing and Doddrell, 1985),

$$\rho(t = 0) \simeq (1/8)(1 + \hbar\omega_P I_z/kT \pm \hbar\omega_H(2I_zS_z)/kT), \quad (13)$$

where ω_P and ω_H are the ^{31}P and ^1H Larmor frequencies, respectively. The sign of the third term in Eq. 13 depends on the phase (i.e., $\phi = \pm y$) of the second proton pulse. ^{31}P difference spectra (i.e., $\phi = \pm y$), recorded for each value of the evolution time, appear as antiphase doublets of spectral resonances (e.g., see Fig. 1). The difference in the intensity of the two (antiphase) resonances is related to the evolution of the $\langle I_zS_z\rangle$ terms. The time evolution of $\langle \Delta I_z(t)\rangle$ is calculated from Eq. 8 with the initial expectation values of the multispin operators (defined in Eq. 9). The initial expectation values of the multispin operators are calculated from the initial density matrix given in Eq. 13 and the equilibrium distribution. We obtain $\langle \Delta I_z(0)\rangle = 0$, $\langle \Delta S_z(0)\rangle = -\hbar\omega_H/2kT$, $\langle I_zS_z(0)\rangle = \pm\hbar\omega_H/8kT$; the expectation values of other multispin operators vanish. The experimental and calculated results are expressed in terms of $\langle I_zS_z(t)\rangle/I_z^0$, where the superscript 0 denotes the equilibrium value. In practice the equilibrium value is obtained from the spectral resonance acquired using only a very short evolution time, typically 1 ms (e.g., see Fig. 1 *a*).

MATERIALS AND METHODS

Reagents

4,4'-Dinitrostilbene-2,2'-disulfonate (DNDS) was obtained from Tokyo Kasei Kogyo Co., Ltd. Japan. Sodium hypophosphite and glycerol (anhydrous) were obtained from Ferak, Berlin. Lysozyme was from Sigma Chemical Co., St Louis, MO. Human blood was freshly drawn by venipuncture from one donor (William Price).

Solution preparation

Hypophosphorus acid has a pKa of 1.1 (Windholz, 1976), so that at the pH values used in this work (i.e., all above 7), HP is almost totally ionized. Water solutions were prepared by dissolving HP in water to give a concentration of 0.1 M and adjusting the pH to 7.4 with NaOH. Glycerol solutions containing 80 mM HP were prepared by mixing glycerol, water and a small amount of concentrated HP, pH 7.4. EDTA was added to the glycerol solutions to a concentration of 0.1 mM.

A lysozyme solution was prepared by dissolving lysozyme in an aqueous solution of HP to give a final lysozyme concentration of 14.5 mM and HP concentration of 83 mM. The solution had a pH of 7.3

Erythrocyte sample preparation

The blood was collected into centrifuge tubes containing heparin, the buffy coat and plasma were removed by centrifugation. The cells were then washed once in saline (NaCl 154 mM; glucose 10 mM), resuspended in saline, and gassed with carbon monoxide to minimize magnetic susceptibility effects (Fabry and San George, 1983). The cells were then pelleted by centrifugation and then divided into four portions. Each portion was then washed three times in a large excess of HP buffer (HP 80 mM; glucose 10 mM; EDTA 0.1 mM; pH 7.4) containing various sucrose concentrations to adjust the solution osmolality and thereby the cell volume. All the HP buffer solutions were saturated with CO before cell suspension. Finally, the cells were 'blotted' with filter paper to increase the hematocrit to greater than 0.91. The anion transport inhibitor, DNDS, was then added to give a concentration of 1 mM in the extracellular fluid. The ratio of UV-visible absorbance measurements at 353 and 310 nm showed that at least 95% of the DNDS was present as the active *trans*-isomer (Falke et al., 1984).

Erythrocyte cell volumes were calculated from the cell count and hematocrit (Dacie and Lewis, 1975). The cell count was performed using a Coulter particle counter (model T 540; Coulter Electronics, Dunstable, UK). The hematocrit was determined using a Hawksley microhematocrit centrifuge (Hawksley, UK) operated for 8 min. The hematocrits were multiplied by 0.97 to allow for trapped extracellular fluid (International Committee for Standardization in Hematology, 1980).

Bulk viscosity measurements

An Ostwald viscometer (Merrington, 1949) was used in a water bath controlled at 310°K. Deionized water served as a viscosity standard (Weast, 1984). Samples were allowed to equilibrate thermally for at least 10 min before measurement. The results were the average of at least three measurements.

NMR measurements

^{31}P NMR measurements were performed on a Bruker MSL-300 spectrometer operating at 121.5 MHz. An ethylene glycol (Van Geet, 1970) or methanol (Van Geet, 1968) capillary was used to calibrate the temperature. After calibration, the temperature was controlled to within ±0.5°K.

Proton-decoupled ^{31}P T_1 measurements were performed using the inversion recovery pulse sequence (Vold et al., 1968). $2I_zS_z$ order relaxation experiments were performed using the pulse sequence given in the Theory section.

Typical acquisition parameters were: spectral width 2.5 kHz; digi-

tized into 8 k data points; $\pi/2$ pulse length 32 μs; recycle delay of at least $5 \times T_1$. For each time point the ^{31}P signal was averaged over eight scans for proton-decoupled T_1, 24 scans for $2I_zS_z$ spin order relaxation experiments.

The experimental two spin order data was analyzed by simulating it using Eq. 8 and adjusting the parameters τ_c, β, and η. The shape $2I_zS_z$ order relaxation profile has a different dependence for each of τ_c, β, and η. A 'cross-check' of the parameters determined was obtained by comparing the time evolution of the total magnetization obtained using Eq. 8 with that obtained (experimentally) from the inversion recovery experiments.

RESULTS

Examples of two-spin order spectra of HP in H_2O and in erythrocyte suspensions are shown in Fig. 1. The time evolution of $2I_zS_z$ relaxation of HP in H_2O at various temperatures, HP in glycerol solutions of various viscosities and of HP in erythrocyte suspensions with various cell volumes are given in Figs. 2, 3, and 4, respectively. There is ~5% error in the determination of the $2I_zS_z$ relaxation profiles of HP in water and glycerol solutions. Due to the poorer signal-to-noise ratio in the erythrocyte samples, the errors in the determination of the $2I_zS_z$ relaxation profile is ~10%. Error bars are omitted in Figs. 2, 3, and 4 to avoid crowding the figures and thus masking the position of the data points. The parameters used in Eq. 8 to stimulate the experimental $2I_zS_z$ relaxation data for HP in the three systems are given in Tables 1–3. Experimental and simulated proton-decoupled ^{31}P T_1 relaxation times are also given in Tables 1–3. The T_1 relaxation times were simulated with and without the effects of the CSA-DD cross-interaction using Eqs. 8 and 2, respectively. The effects of the ^{31}P random field relaxation on the simulated T_1 values is also given in the tables. Table 3 also contains details of relevant erythrocyte parameters. There is an ~8% error associated with the T_1 measurements. There is ~5% error in the experimentally determined parameters for HP in the water and glycerol solutions and 10% for the parameters obtained in the erythrocyte suspensions.

The ^{31}P NMR linewidths of the HP triplet in the lysozyme solution at 280°K were (from low field to high field) 1.1, 1.2, and 1.4 Hz, respectively.

DISCUSSION

Similar to our previous work (Tsai et al., 1991) we found that there was a nearly negligible change in J_{PH} ($\ll 1\%$) for HP suspended in the different media. This implies that the H-P-H bond angle (and presumably the structure of HP) is not altered (Spitz et al., 1986), thus providing justification for using the same value of C for HP in all cases. DNDS was included in the erythrocyte suspensions to inhibit any effects due to transmembrane HP exchange. DNDS is a potent inhibitor of transmembrane HP transport (Price and Kuchel, 1990b). The erythrocyte suspensions were adjusted to high hematocrit to increase the cytoplasmic volume of the sample.

The CSA contribution to the overall relaxation rate constant is small (Price et al., 1989b), consequently the CSA-DD cross-interaction is also small. Hence, we used selective excitation with a coherence transfer technique to facilitate the two-spin order measurements. It should be noted, however, that if the relaxation is too short [i.e., on the time scale of $1/(2J_{PH})$] then selective excitation with coherence transfer cannot be used. The small CSA-DD cross-interaction in conjunction with the field strength of the present spectrometer means that the accuracy and precision of the two-spin order experiments are not optimal. The signal-to-noise ratio is further compromised in the erythrocyte samples because osmotic pressure limits the maximum HP concentration that may be used, and even then the HP species present is divided between two spectral resonances. This method would be greatly facilitated at higher magnetic fields because the two-spin order effect is directly proportional to B_0 and the concomitant increase in NMR sensitivity. Thus, at double the present field strength, it should be possible to conduct the $2I_zS_z$ relaxation experiment in erythrocyte suspensions with approximately one-quarter of the HP concentration used in this study.

In our previous study (Tsai et al., 1991), the HP ^{31}P two spin-order data in D_2O solutions was fit without considering random field terms. This was possible because paramagnetics were excluded by careful sample preparation, and intermolecular dipole–dipole relaxation was almost negligible due to the lower gyromagnetic ratio of deuterium. However, random field terms were needed for HP in H_2O solutions, HP in glycerol solutions and HP in erythrocyte suspensions. The random field term for ^{31}P is understandably less than that of the protons because, from the structure of HP, the ^{31}P nucleus is more distant from the solvent. In the glycerol solutions paramagnetic effects were excluded by addition of EDTA. It was not possible to exclude paramagnetic ions from the erythrocyte cytoplasm.

From the T_1 data in Tables 1–3 it can be seen that for relaxation measurements *in vivo,* random field effects must be accounted for if the correct τ_c is to be determined. Also due to CSA-DD cross-interaction effects increasing with viscosity, the spin-lattice relaxation of the net magnetization becomes increasingly nonexponential. Comparison of the T_1 values simulated using Eq. 2 with the values obtained using Eq. 8 shows that the CSA-DD cross-interaction increases the relaxation time.

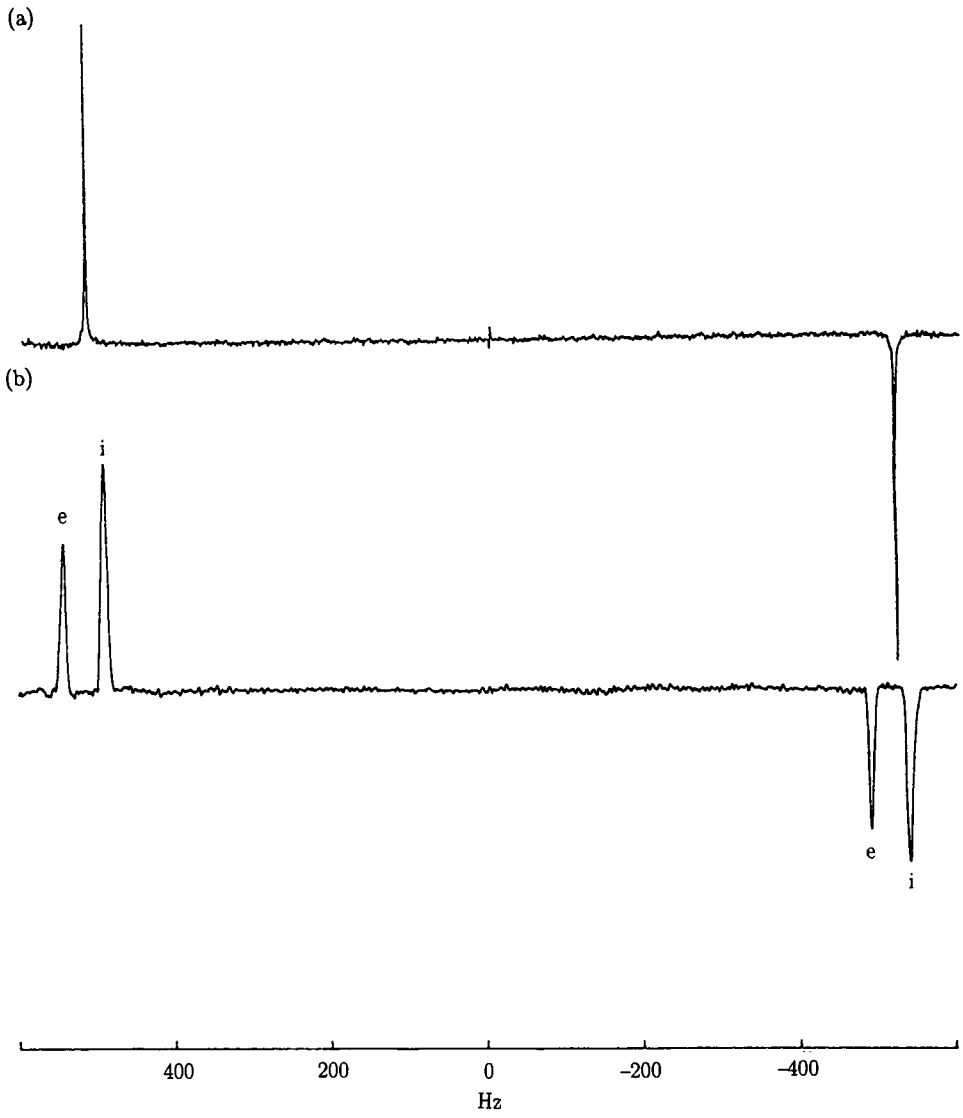

FIGURE 1 (*a*) $2I_zS_z$ spectra 116 mM HP in H_2O solution at 310°K with an evolution time of 1 ms (see Theory section). The spectrum appears as an antiphase doublet of resonances. Because of the very short evolution time, the effects of CSA-DD cross-interaction are not visible and so both resonances have the same absolute intensity. (*b*) $2I_zS_z$ spectra of 80 mM HP in a suspension of 56 fl erythrocytes at 310°K obtained after an evolution time of 0.5 s. The spectrum appears as a pair of antiphase doublets from the intracellular (i) and extracellular (e) species, respectively. Before Fourier transformation the FID was subject to gaussian multiplication. The area of the extracellular resonance is small due to the high hematocrit (0.91) and because the recycle delay only allowed complete relaxation of the faster relaxing, intracellular species. The separation between the intra- and extracellular resonances was 51.9 Hz. The difference in absolute intensity between the resonances of the doublet resulting from the intracellular HP species is due to the effects of CSA-DD cross-interaction.

Our data show (see Table 1) that only in the H_2O solutions, where the random field effects on the ^{31}P nuclei are very small, will no significant error in the value of τ_c result if it is determined from longitudinal relaxation measurements alone. However, the random field effects on the ^1H nuclei are significant. If, at the same temperature, allowance is made for the viscosity difference between D_2O and H_2O (Kell, 1972), then the simulated value of T_1 without random field effects ($T_{1sim\text{-}RF}$) for HP in H_2O (see Table 1) will be, within experimental error, identical to the T_1 in D_2O. In biological systems the random field cannot be 'calibrated out', because among other reasons, ion distributions and ion concentrations are likely to alter with cell volume and or membrane potential changes.

Binding of HP to molecules in the erythrocyte cyto-

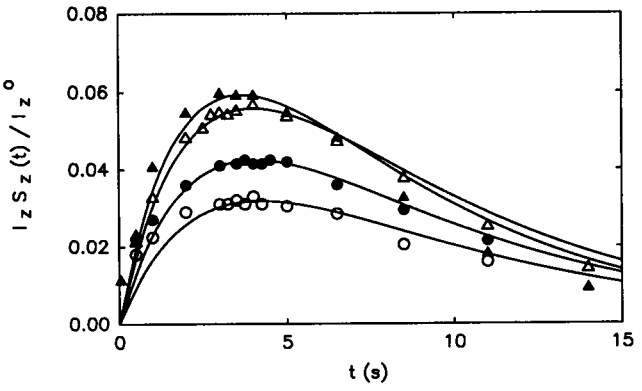

FIGURE 2 $2I_zS_z$ relaxation profiles for 116 mM HP in H_2O at 286.5 (▲), 293.5 (△), 313.0 (●), and 328.0°K (○). τ_c is closely related to the locus of the maximum point of each curve (see Table 1). Since the random field interactions are small the locus of the maximum point (i.e., evolution time) decreases with increasing viscosity and therefore τ_c (see Eq. 8). The depression of the intensity of the maximum point provides a method to quantitate the random field contribution to the overall longitudinal relaxation.

plasm (predominantly hemoglobin) in cells of physiologically relevant volume is thought to be negligible and therefore has no affect on the τ_c values determined. Minor differential line broadening in the proton-coupled ^{31}P HP spectrum was only observed in the erythrocyte suspension of extremely shrunken cells (56 fl); from low field to high field the linewidths were 19.5, 19.5, and 23.2 Hz, respectively. Since differential line broadening only occurs when $\omega\tau_c \approx 1$, where ω is the larmor frequency of the nucleus in question (Wang and Hwang, 1988; Hwang et al., 1988; Wong et al., 1989), the

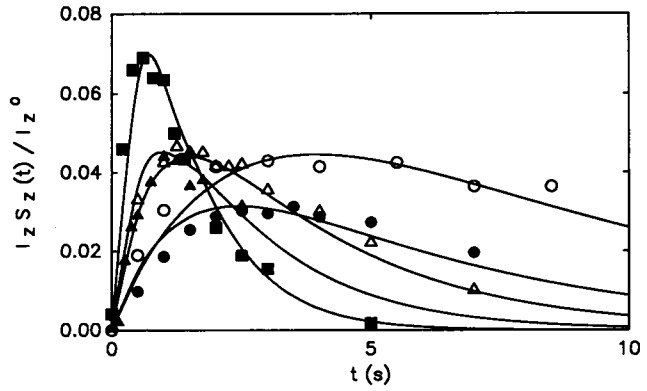

FIGURE 3 $2I_zS_z$ relaxation profile for 80 mM HP in glycerol solutions containing 5 (○), 20 (●), 45 (△), 60 (▲), and 80 (□) glycerol (g/100 g) at 310°K. The trend of the maximum intensity increasing with decreasing values of the evolution time is not as clear as that observed in the water solutions.

absence of differential line broadening in the other erythrocyte suspensions is indicative of the absence of binding. However, a proton-coupled ^{31}P spectrum of HP dissolved in a solution of lysozyme, to which HP is thought to bind (Kirk and Kuchel, 1988), exhibits differential line broadening of ~27% between the satellite peaks of the proton-coupled ^{31}P triplet.

It is likely that much of the random field interaction arises from intermolecular DD relaxation between HP and water (and possibly protein) protons in the cytoplasm (Andree, 1978; Peemoeller et al., 1986). To test this hypothesis we use the formula for a spin, I, being relaxed by a spin, S (Abragam, 1961; Hwang and Freed, 1975), in the extreme narrowing condition (i.e., $\omega\tau_c \gg 1$),

$$1/T^I_{1DD}(\text{inter}) = 64\pi n_S \gamma_I^2 \gamma_S^2 \hbar^2 S(S+1)/(81Dd),$$

where n_S is the number of S spins (i.e., non-HP protons in this case) per unit volume, D is the relative translational self-diffusion coefficient of the molecules containing I (which represents either the proton or phosphorus atoms of HP) and S, and d is the distance of closest approach of the molecules containing spins I and S. In this case, we have assumed that n_S is equal to the number of protons in pure water. If we assume that at 310°K the HP diffusion coefficient in erythrocyte cytoplasm is $\sim 1.5 \times 10^{-6}$ cm^2 s^{-1} (Price et al., 1990a) and that the cytoplasmic water diffusion coefficient is $\sim 4.2 \times 10^{-6}$ cm^2 s^{-1} (Price, 1990), then $D = 5.7 \times 10^{-6}$ cm^2 s^{-1}. d is taken as water molecule radius plus the van der Waals radius of a proton (4.20×10^{-8} cm) for calculating the intermolecular DD relaxation rate for the HP protons or as a water molecule radius plus the van der Waals radius of a proton plus r_{PH} (i.e., 5.64×10^{-8} cm) for calculating the intermolecular DD relaxation rate for the phosphorus atom of HP. The calculation gives values of 0.3 and 0.04 s^{-1} for the intermolecular DD relaxation for the HP protons and phosphorous, respectively. These values are in reasonable agreement with what we have found β and η for HP to be in the erythrocyte suspensions (see Table 3). It has previously been shown that the cytoplasmic microviscosity depends almost exclusively on hemoglobin concentration (Herrmann and Müller, 1986; Johnson, 1989). Scattering studies suggest that appreciable amounts of hemoglobin exist in associated states such as dimers and higher order aggregates of the hemoglobin tetramer within normal erythrocytes (Krueger et al., 1990), and this aggregation is likely to be further enhanced at higher hemoglobin concentrations (see Table 3) such as in the cytoplasm of the shrunken erythrocytes in this study. Thus, as the intracellular viscosity increases, the translational diffusion coefficients will decrease causing greater intermolecular DD effects.

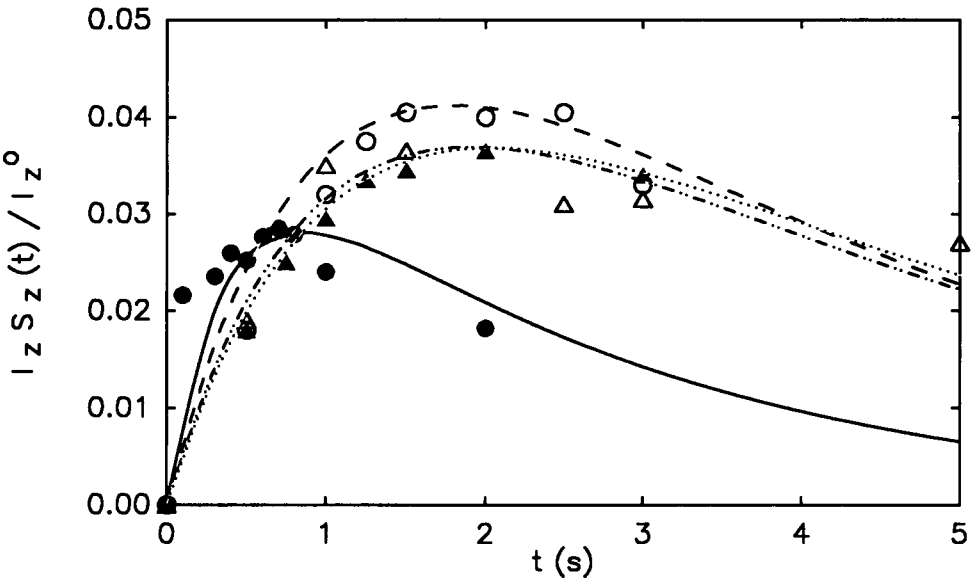

FIGURE 4 $2I_zS_z$ relaxation profiles for 80 mM HP in erythrocyte suspensions of 56 (●), 69 (○), 85 (▲), and 102 fl (△) cells at 310°K. The lines represent the simulated relaxation profiles for the 56 (———), 69 (– – – –), 85 (– · · –), and 102 fl cells (· · · ·). The respective effective correlation times and other relevant relaxation parameters are given in Table 3. The intracellular viscosity increases with decreasing cell volume.

With decreasing cell volume, the fraction of HP being at least partly solvated by the hydration layers of the hemoglobin molecules is likely to increase (N.B. the hemoglobin volume fraction, ϕ, increases as the cell volume and cytoplasmic water content decreases; see Table 3). This could lead to additional complications in fitting the data because the reorientation of the hydration layer is likely to be sufficiently slow (Schreiner et al., 1991 and pertinent references therein) so as to cause the τ_c of HP to be outside the extreme narrowing condition, in which the random field interaction would no longer be

TABLE 1 Effective reorientational correlation times (τ_c) and isotropic random field relaxation rate parameters for ^{31}P (β) and ^{1}H (η) derived from two-spin order measurements and proton-decoupled T_1 measurements (T_{1exp}) at various temperatures for HP in H_2O solutions.

T (K)	286.5	293.5	313.0	328.0
τ_c (ps)	6.8	5.8	4.4	3.1
β (s^{-1})	0.002	0.002	0.003	0.003
η (s^{-1})	0.10	0.09	0.11	0.10
T_{1expt} (s)	5.4	5.8	6.6	6.6
T_{1add} (s)	5.1	5.6	6.4	6.7
$T_{1add+RF}$ (s)	5.0	5.5	6.1	6.5
$T_{1sim+RF}$ (s)	5.3	5.7	6.3	6.6
$T_{1sim-RF}$ (s)	5.5	5.9	6.6	6.8

Proton-decoupled T_1 values were simulated using the (simplistic) Eq. 2, which excludes the effects of the CSA-DD cross-interaction, with ($T_{1add+RF}$) and without ^{31}P random field effects (T_{1add}). The T_1 values were also simulated using Eq. 8, which includes the effects of the CSA-DD cross-interaction, with ($T_{1sin+RF}$) and without random field effects ($T_{1sim-RF}$). The parameters used in the simulations were C = 1.92×10^5 s^{-1}, I_{av} = 79.48×10^{-40} g cm^2, and r_{PH} = 1.44 Å (Tsai et al., 1991). The relevant chemical shift shielding tensor data is given in the Theory section.

TABLE 2. Effective reorientational correlation times and isotropic random field relaxation parameters derived from two-spin order measurements and proton-decoupled T_1 measurements for HP in glycerol solutions at 310°K.

Glycerol (g/100g)	5	20	45	60	80
τ_c (ps)	4.8	5.6	12.5	18.5	41.7
β (s^{-1})	0.003	0.03	0.02	0.04	0.04
η (s^{-1})	0.11	0.24	0.40	0.56	0.56
T_{1expt} (s)	6.4	4.3	3.1	1.9	1.0
T_{1add} (s)	6.2	5.7	3.2	2.2	1.0
$T_{1add+RF}$ (s)	5.9	4.3	2.8	1.9	0.9
$T_{1sim+RF}$ (s)	6.2	4.6	2.9	2.0	1.0
$T_{1sim-RF}$ (s)	6.4	6.0	3.6	2.6	1.2
η_b (mPas)	0.76	1.01	2.22	4.28	17.29

Proton-decoupled T_1 values, T_1 values simulated with and without random field effects as well as the bulk viscosity derived from viscometry measurements are also given. The parameters used in the simulations are given in the caption to Table 1.

TABLE 3 **Effective reorientational correlation times and isotropic random field relaxation parameters derived from two-spin order measurements at 310°K and proton-decoupled T_1 measurements at approximately 312°K for 80 mM HP in erythrocyte suspensions.**

Cell Volume (fl)	56	69	85	102
τ_c (ps)	15.2	10.6	7.9	7.2
β (s^{-1})	0.02	0.02	0.02	0.02
η (s^{-1})	1.2	0.38	0.33	0.28
T_{1expt} (s)	2.8	3.2	4.0	3.8
T_{1add} (s)	2.7	3.7	4.6	4.9
$T_{1add+RF}$ (s)	2.4	3.2	3.9	4.1
$T_{1sim+RF}$ (s)	2.4	3.3	4.1	4.1
$T_{1sim-RF}$ (s)	3.1	4.1	4.9	5.2
[Hemoglobin] (g/ml)	0.47	0.38	0.30	0.25
$\phi_{Hemoglobin}$	0.35	0.29	0.23	0.19

Proton-decoupled T_1 values and T_1 values simulated with and without random field effects are also given. The parameters used in the simulations are given in the caption to Table 1. The concentration and volume fraction (ϕ) for hemoglobin is also given for each cell sample. The partial specific volume for hemoglobin used in calculating the volume fraction was 0.7546 ml/g (Bernhardt and Pauly, 1975).

proportional to the viscosity, and Eqs. 2–7 and the elements of the relaxation matrix are no longer valid.

In all of the experimental systems in this work the Markovian limit holds (i.e., $\tau_c \gg \tau_j$), justifying the use of the Hubbard relation to relate τ_c to τ_j. In this limit, inertial effects are unimportant so other models for interpreting the molecular reorientation of HP, such as 'extended J-diffusion' (McClung, 1977) and the Fokker-Planck-Langevin model (Perry et al., 1981; Powles and Rickayzen, 1977), give the same answer. The simplest means to relate τ_c to solution viscosity is via the Debye equation (Bloembergen et al., 1948; Boeré and Kidd, 1982). However, at least in the case of HP dissolved in erythrocyte cytoplasm, a basic assumption of the Debye equation is violated because the physically small HP cannot 'see' the solvent (mainly hemoglobin and H_2O) as a continuum. Examples of modifications to the Debye equation to account for the discontinuous nature of the solvent and for nonsphericity of the probe molecule may be found in a review by Boeré and Kidd (1982). In our previous work (Price et al., 1989b) we used an experimentally determined Stokes radius of 1.74 Å, although it has been suggested that the best choice for a small molecule is the van der Waals radius (Edward, 1970), which for HP is 2.78 Å. The translational diffusion coefficient can then be calculated from the solution viscosity; two possible methods are the Stokes-Einstein relationship (Stokes, 1856; Einstein, 1956) or a later relationship by Zwanzig (1983).

Although the two-spin order measurements in conjunction with the proton-decoupled T_1 measurements provides a more accurate determination of the reorientational correlation time, the data in Tables 2 and 3 show that the use of T_1 measurements alone is still a good qualitative probe of viscosity changes. The bulk viscosity of the glycerol solutions is shown to enable the sensitivity of the two-spin order experiments to viscosity changes to be visualized. The corresponding bulk measurements were not performed on the erythrocyte suspensions for a number of reasons. First, it has been previously shown for erythrocyte cytoplasm that bulk (i.e., macro) and NMR-determined (i.e., micro) viscosity are essentially different things (Endre and Kuchel, 1986; Price et al., 1989b). Second, it is difficult to get reliable values for the cytoplasmic bulk viscosity due to the inevitable dilution by extracellular fluid upon lysing the cells (it is not possible to get 100% hematocrit) and if on preparing the lysate not all of the cell debris is removed, the flow of the lysate may be non-Newtonian (Cokelet and Meiselman, 1968). Finally, cytoplasmic organization (e.g., cytoskeleton, etc.) may make the viscosity inside the cell greater than that in the lysate (Everhart et al., 1982).

The results of this study have shown that two-spin order relaxation provides a convenient and noninvasive method for accurately determining the reorientational correlation times of HP in erythrocyte cytoplasm. The reorientational correlation times can then be related to the cytoplasmic viscosity. Most importantly the method allows random field effects to be separated from the effects of intramolecular relaxation, thereby permitting the reorientational correlation time to be determined with greater accuracy. Previously, it was not possible to separate random field and viscosity effects. The method also allows the random field effects to be quantitated.

The Hematology Service, Clinical Pathology Department and Dr. W. C. Perng of the Tri-Services General Hospital, Mr. M. L. Lai of the Union Clinical Laboratory, and Professor Y. H. Chen of the Graduate School of Biological Chemistry at National Taiwan University are thanked for assistance.

Support of this work by the National Science Council of the Republic of China is gratefully acknowledged.

Received for publication 3 June 1991 and in final form 7 October 1991.

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