Biophysical Chemistry, 33 (1989) 205–215
Elsevier

BIOCHE 01355

Microviscosity of human erythrocytes studied with hypophosphite and ^{31}P-NMR

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Received 13 June 1988
Revised manuscript received 23 January 1989
Accepted 25 January 1989

NMR, ^{31}P-; Erythrocyte; Microviscosity; Hypophosphite ion; Longitudinal relaxation time; Spin-rotation relaxation; (Human)

A ^{31}P-NMR method, which complements earlier ^{13}C-NMR procedures for probing the intra-erythrocyte microenvironment, is described. Hypophosphite is an almost unique probe of the erythrocyte microenvironment, since it is rapidly transported into the cell via the band 3 protein, and intra- and extracellular populations give rise to distinct resonances in the ^{31}P-NMR spectrum. Relaxation mechanisms of the ^{31}P nucleus in the hypophosphite ion were shown to be spin-rotation and dipole-dipole. Analysis of longitudinal relaxation rates in human erythrocytes, haemolysates and concentrated glycerol solutions allowed the determination of microviscosity using the Debye equation. Bulk viscosities of lysates and glycerol solutions were measured using Ostwald capillary viscometry. Translational diffusion coefficients were then calculated from the viscosity estimates using the Stokes-Einstein equation. The results with a range of solvent systems showed that 'viscosity' is a relative phenomenon and that bulk (i.e., macro-) viscosity is therefore not necessarily related to the NMR-determined viscosity. The intracellular NMR-determined viscosities from red cells, ranging in volume from 65.5 to 100.1 fl, varied from 2.10 to 2.67 mPa s. This is consistent with the translational diffusion coefficients of the hypophosphite ion altering by only 20%, whereas the values determined from bulk viscosity measurements conducted on lysates of these cells are consistent with a 230% change.

1. Introduction

Viscosity of the cytoplasmic medium and thus diffusion rates of substrates of enzymes are potentially important in the regulation of metabolic reactions in cells [1,2]. In the human erythrocyte, the enzymes carbonic anhydrase and glutathione peroxidase are thought likely to be under diffusion control (i.e., the lifetimes of diffusion steps are longer than those of the reaction steps [2–4]).

Several methods have been used to study intracellular viscosity [2,5,6]. We describe here the use of hypophosphite ($H_2PO_2^-$) as a ^{31}P-NMR viscosity probe based on spin-relaxation measurements of the anion. Hypophosphite diffuses rapidly into the cell through band 3, the anion-transport protein of the erythrocyte membrane [7]; in fact, its transmembrane distribution may also be used to infer the membrane potential in these cells [8]. Previous NMR viscosity probes have relied on (expensive) ^{13}C-labelled probe molecules [2,9], but ^{31}P-NMR offers greater sensitivity and utility in the study of energy metabolism in cells and tissues [10], thus hypophosphite provides a

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Abbreviations: CSA, chemical shift anisotropy; DD, dipole-dipole; J_{PH}, phosphorus-proton coupling constant; J_{P^2H}, phosphorus-deuterium coupling constant; ϕ, volume fraction; κ, ν, empirical hydrodynamic interaction parameters; η, viscosity; r_0, Stokes radius; SR, spin-rotation; τ_c, rotational correlation time; τ_{fr}, free rotor correlation time; τ_{SR}, spin-rotation correlation time; T_1, longitudinal relaxation time; $\bar{v}$, partial specific volume.

potentially important alternative probe molecule in the latter studies.

From measurements of longitudinal relaxation times (T_1) and the subsequent analysis, appropriate to the relaxation mechanism [11,12] of the ^{31}P atom in hypophosphite, the rotational correlation time (τ_c) of the ion can be calculated. From the rotational correlation time and molecular dimensions (principally the Stokes radius; r_0) the intracellular viscosity and diffusion coefficient can be determined.

2. Materials and methods

2.1. Materials

Hypophosphite was obtained from Aldrich (Steinheim, F.R.G.) and ^2H_2O (99.75%) was from the Australian Institute of Nuclear Science and Engineering (Lucas Heights, N.S.W.). All other reagents were of A.R. grade. Human blood was obtained from the Red Cross Blood Transfusion Service, N.S.W.

2.2. Solution preparation

Reference solutions of hypophosphite were prepared by diluting the acid in phosphate buffer (0.1 mol/l NaH_2PO_4, 0.1 mmol/l EDTA in $^2H_2O/H_2O$, 20:80) and adjusting to pH 7.4 with concentrated NaOH. The osmolality was measured (Wescor model 5100C vapor pressure osmometer) to be 288 mosmol/kg, the final hypophosphite concentration being 42 ± 3 mmol/l. The solution was then degassed with nitrogen and 3-ml samples were dispensed into 10-mm NMR tubes which were flame sealed. Hypophosphite/glycerol solutions were prepared by mixing water, EDTA and a small amount of a concentrated hypophosphite solution, pH 7.4 (adjusted with concentrated NaOH), to give a total EDTA concentration of 0.67 mg/ml and a hypophosphite concentration of 50 mmol/l.

Solutions containing 0.5 M hypophosphite in 0, 20, 60, 80 and 100% ^2H_2O were prepared by mixing appropriate amounts of neutralized hypophosphite with degassed ^2H_2O and H_2O.

Deuterated hypophosphite was prepared by mixing hypophosphite at high pH (> 11) with ^2H_2O giving a hypophosphite concentration of 1.5 M. This mixture was kept at room temperature for more than 2 weeks to allow deuteration to occur.

2.3. Erythrocyte preparation

Erythrocytes were washed once in 154 mmol/l NaCl containing glucose (10 mmol/l) and twice in phosphate/sucrose buffer at 277 K (NaH_2PO_4, 1.97 mmol/l; Na_2HPO_4, 8.07 mmol/l; sucrose, 245 mmol/l, pH 7.4); osmolality, 290 mosmol/kg measured at room temperature. The cells were gassed with carbon monoxide for 15 min and then centrifuged ($2000 \times g$, 5 min, 277 K). The cell pellet was suspended in sucrose buffers of varying osmolality, all containing glucose (10 mmol/l), EDTA (0.1 mmol/l) and hypophosphite (40 mmol/l). These suspensions were sedimented, the supernatants removed and the cells 'blotted' (with Whatman no. 1 filter paper) to increase the hematocrit to 0.93 or greater. 3 ml of each sample were dispensed into 10-mm NMR tubes. The samples were stored at 277 K and warmed to 310 K prior to the NMR experiment.

2.4. ^{31}P-NMR measurements

T_1 measurements were made at 310 K (unless otherwise stated) with Varian XL-200 and XL/VXR-400 NMR spectrometers, with spectrometer frequencies for ^{31}P of 80.98 and 161.5 MHz, respectively. Typical acquisition parameters were: spectral width, 2 kHz; 90° pulse length, 45 μs; acquisition time, 2 s; free induction decay digitized into 8192 points. High-power WALTZ decoupling of protons [13] was used. The sample temperature was measured using either an ethylene glycol [14] or methanol [15] capillary, thus allowing measurement of, and compensation for, radiofrequency (rf) heating due to the decoupler [16].

T_1 values were measured using the inversion recovery pulse sequence [17]. A delay of $5T_1$ or above was allowed between transients [18]. At least eight transients were averaged for each spectrum.

2.5. Bulk viscosity measurements

An Ostwald viscometer was used in a water bath maintained at 310 K. Deionized water served as the viscosity reference [19]. Samples were allowed to equilibrate thermally for at least 10 min before measurement. Results (unless otherwise stated) were the average of at least three measurements for each sample.

3. Theory

Nuclear magnetic relaxation in liquids is a consequence of molecular motion [20]. From a knowledge of the relaxation mechanisms of a particular solute, it is possible to relate the experimentally observed T_1 value to a rotational correlation time and from this value the translational diffusion coefficient of the probe molecule and the viscosity of the solution can be calculated.

3.1. NMR relaxation

Six nuclear magnetic relaxation mechanisms have been observed for nuclei in solution [21]: (a) dipole-dipole (DD), (b) chemical shift anisotropy (CSA), (c) spin-rotation (SR), (d) quadrupolar, (e) scalar coupling and (f) that resulting from the presence of paramagnetic species. The relaxation rate constants of these mechanisms are additive when the liquid is of low viscosity [22,23]. Thus,

$$1/T_1 = \sum_{m=1}^{6} 1/T_{1m}, \qquad (1)$$

where $1/T_1$ is the overall (extrinsic) relaxation rate constant and the index m refers to one of the above relaxation processes. Of these, quadrupolar relaxation is excluded for ^{31}P nuclei which have spin quantum number 1/2. Also, the scalar coupling mechanism is excluded [24], since none of the atoms which are directly bonded to the phosphorus are quadrupolar or undergo chemical exchange. Paramagnetic species can be complexed by chelators such as EDTA, although the effects of the chelator are not always straightforward [25]. The relaxation rate constant from CSA (R_{1CSA}, corresponding relaxation time T_{1CSA}) is proportional to the square of the external magnetic field [26], and so can be determined from relaxation measurements at different field strengths.

The remaining mechanisms of relaxation are dipole-dipole and spin-rotation. Ordinarily, the nuclear Overhauser enhancement (NOE) of the phosphorus resonance could be used to estimate the dipole-dipole contribution to the relaxation rate constant [27]. However, the NOE of ^{31}P nuclei in a molecule depends upon several factors including pH, the nature of the countercation, the concentration of the species and the ionic strength of the solution [28]. In the present work, T_1 data were used to estimate the dipole-dipole contribution to the relaxation rate constant. If isotropic molecular reorientation occurs then the rate constant for the intra-molecular dipole-dipole mechanism (R_{1DD}, corresponding relaxation time T_{1DD}) is given by [29],

$$R_{1DD} = 1/T_{1DD} = 1/10(\hbar\gamma_H\gamma_P)^2 r_{P-H}^{-6}\chi, \qquad (2)$$

where

$$\chi = \frac{\tau_c}{1+(\omega_H-\omega_P)^2\tau_c^2} + \frac{3\tau_c}{1+\omega_P^2\tau_c^2} + \frac{6\tau_c}{1+(\omega_H+\omega_P)^2\tau_c^2},$$

and γ_H and γ_P are the respective magnetogyric ratios of proton and phosphorus nuclei, r_{P-H} the phosphorus–proton bond length, and ω_H and ω_P the respective proton and phosphorus Larmor frequencies.

Because the coupling of the spin-rotation Hamiltonian to the nuclear angular momentum is proportional to T (compared with the dipole-dipole interaction where it does not increase with increasing angular momentum) the nuclear relaxation rate constant (R_{1SR}, corresponding relaxation time T_{1SR}) is directly proportional to temperature [30,31]. If the motion is isotropic then

$$R_{1SR} = 1/T_{1SR} = 2IkTC^2\tau_{SR}/3\hbar^2, \qquad (3)$$

where I is the moment of inertia of the molecule, k Boltzmann's constant, T the absolute tempera-

ture, C^2 the squared average of the spin rotation tensor, and τ_{SR} the spin-rotation correlation time that describes a molecule's collision-interrupted rotation, and is therefore a quantity from which microdynamical information can be obtained [32]. Eq. 3 can be made more experimentally useful if we note that for a spherical molecule undergoing isotropic Brownian motion

$$\tau_{SR}\tau_c = I/6kT, \tag{4}$$

provided $\tau_{SR} \ll \tau_c$ [30]. So eq. 3 becomes

$$R_{1SR} = I^2C^2/9\hbar^2\tau_c. \tag{5}$$

Now, if we consider eq. 5 and note that $T_{1DD} \propto \tau_c^{-1}$ we have

$$T_{1DD}T_{1SR} = \text{constant}. \tag{6}$$

Thus, at the maximum of a graph of T_1 vs. temperature we have $T_{1DD} = T_{1SR}$ = twice this maximum T_1 value (see eq. 1). Also, the plot of T_1 vs. temperature is non-linear, thus allowing ready 'separation' of the contributions of each of the mechanisms to longitudinal relaxation [27,33]. In other words, the temperature dependence of the spin-rotation relaxation is opposite to that of the dipole-dipole mechanism, therefore, it is possible to determine experimentally the rate constants that relate to each mechanism.

3.2. Rotational correlation time and viscosity

After relating correlation time to longitudinal relaxation time it is necessary to relate correlation time to viscosity and diffusion coefficient.

The correlation time of a molecule in solution depends on many factors including molecular size and symmetry [34]. To be useful as a viscosity probe a molecule must not rotate under perfect 'slip' conditions for in this case the correlation time would reflect no viscosity dependence and the molecule would reorientate inertially [35]. To ascertain whether the molecular reorientation can be described by rotational diffusion, the correlation time is compared with that of a classical free rotor (τ_{fr}),

$$\tau_{fr} = 2\pi/9(I/kT)^{1/2}. \tag{7}$$

If $\tau_c \gg \tau_{fr}$ then the assumption of free rotational diffusion is valid.

The correlation time is related to the solution viscosity by way of the Debye theory for dielectric dispersions in polar liquids [20]:

$$\tau_c = 4\pi\eta r_0^3/3kT, \tag{8}$$

where η is the solution viscosity. The Stokes radius may be estimated experimentally (using the Debye equation) or calculated by considering molecular dimensions. However, both estimates are equal only if the rotating sphere is much larger than the structural inhomogeneity of the surrounding liquid ('stick boundary' condition [36]).

The Debye model assumes that the solvent molecules surrounding the solute molecules can be treated as a continuum. Gierer and Wirtz [37] derived a modification of the Debye theory by allowing for the discontinuous nature of the solvent. However, in the present work the coefficient of microfriction has not been included, since r_0 was determined experimentally.

From a given value of τ_c the viscosity can be calculated by rearranging eq. 8. For this expression to remain valid the molecular size and shape need to be concentration independent. If the probe molecule is rotating under 'extreme narrowing' conditions and the relaxation mechanism is purely dipolar, the fact that the longitudinal relaxation rate is inversely proportional to viscosity can be used to relate T_1 to viscosity [20] by

$$\eta_x = \eta_0 T_0/T_x, \tag{9}$$

where T_0 is the T_1 of the probe molecule in the medium of known viscosity η_0, and η_x is the unknown viscosity in a medium in which the solute relaxation time is T_x. However, as hypophosphite is relaxed by a mixture of relaxation mechanisms the T_1-ratio method cannot be used. With a knowledge of r_0 and η it is possible to calculate the translational diffusion coefficient of the probe molecule using the Stokes-Einstein relationship [38,39],

$$D = kT/6\pi\eta r_0. \tag{10}$$

3.3. Concentration dependence of viscosity

Formulae have been derived relating solute concentration to bulk viscosity [9,39–43], for which

the volume fraction of the solute and solvent is required. The volume fraction for each sample was calculated from the expression,

$$\phi = c\bar{v}, \tag{11}$$

where c is the solute concentration (g/ml) and $\bar{v}$ its partial specific volume (ml/g). Herrmann and Müller [36] reported that in red cells the intracellular viscosity depends solely on the hemoglobin concentration. In the present work the value used for $\bar{v}$ of hemoglobin was 0.7546 ml/g [44] with that for glycerol being 0.4617 mg/g; the latter was derived assuming that the glycerol molecules were spherical with the same packing density as liquid water [45]. This probably results in an underestimate of ϕ but the general conclusions drawn from the analysis (see below) are unchanged [9].

3.4. Computing

Non-linear least-squares analyses were performed using the modified Levenberg-Marquardt-Morrison algorithm [46].

4. Results

4.1. Relaxation mechanisms of the ^{31}P nucleus in hypophosphite

T_1 measurements performed on hypophosphite samples, with and without EDTA, showed no significant difference, thus implying that the contribution of paramagnetic impurities to relaxation was negligible.

Measurements performed on the same reference sample at 310 K gave T_1 values of 6.75 ± 0.47 and 6.39 ± 0.30 s at 80.98 and 161.5 MHz, respectively. These results reveal, noting the difference in field strengths, that at 161.5 MHz $T_{1CSA} = 90$ s, thus contributing less than 10% to the observed relaxation rate constant.

The T_1 values for the 0.5 M hypophosphite solutions in various $^2H_2O/H_2O$ concentrations (^2H_2O (%), T_1 (s); 0, 6.03 ± 0.22; 20, 5.45 ± 0.16; 60, 6.03 ± 0.11; 80, 5.88 ± 0.23; 6.44 ± 0.13) were the same within experimental error. This implied that intermolecular dipolar interactions were insignificant and that only intramolecular dipolar interactions need be considered.

Table 1

Hypophosphite ^{31}P-NMR relaxation times in aqueous solutions at 161.5 MHz

Temperature (K)	T_1 (s) ($\pm$S.D.)
277.6	4.17 ± 0.17
286.1	4.77 ± 0.12
296.1	5.49 ± 0.14
301.2	5.95 ± 0.25
309.8	6.36 ± 0.09
310.0	6.39 ± 0.30
312.0	6.36 ± 0.08
313.4	6.54 ± 0.19
316.8	6.31 ± 0.06
322.9	6.24 ± 0.11
323.0	5.98 ± 0.15
327.0	6.07 ± 0.14
331.7	5.97 ± 0.23
334.0	5.57 ± 0.12
342.2	5.23 ± 0.14
345.1	5.14 ± 0.10
352.3	4.89 ± 0.29

Table 1 contains relaxation data for hypophosphite solutions at 161.5 MHz at various temperatures. The maximum T_1 value (6.5 s) occurred at 313 K, thus according to eq. 6 T_{1DD} (total) = T_{1SR} = 13.0 s. If we use a value of $r_{P-H} = 1.42$ Å [19] in eq. 4 and $T_{1DD} = 26.0$ s (since there are two contributing protons), we obtain a value for τ_c of 3.39×10^{-12} s. It was assumed that hypophosphite undergoes isotropic reorientation (which is probably justified in view of the compact geometry of the molecule) and inserted this value for τ_c, $T_1 = 13.0$ s and $I = 7.851 \times 10^{-46}$ kg m^2 (calculated using a formula given by Hirschfelder [47]) into eq. 5 to calculate an initial estimate of C^2 of 1.073×10^9 s^{-2}.

To obtain a better estimate of C^2, T_1 vs. temperature data were fitted by eq. 1, combining terms for T_{1DD} (eq. 2) and T_{1SR} (eq. 5). In order to achieve this fitting a relationship was needed to relate τ_c and T. This was obtained by substituting the Arrhenius equation for viscosity,

$$\eta = a \exp(\Delta E_{vis}/RT), \tag{12}$$

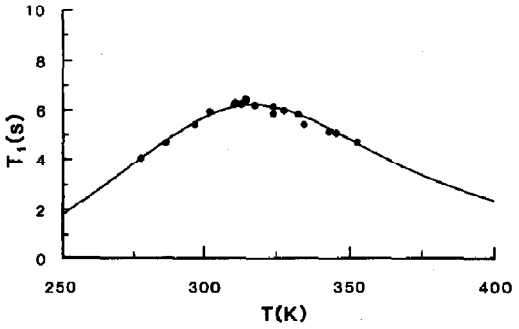

Fig. 1. ^{31}P-NMR spin-lattice relaxation times of hypophosphite in a reference solution (see section 2.2) as a function of temperature at 161.5 MHz. The solid line is a theoretical curve obtained using eqs. 1, 2, 5 and 13, to describe hypophosphite relaxation, and the experimentally derived constants in the expression (see text).

into eq. 8, where a is a constant and ΔE_{vis} the activation energy for viscous flow [48], to give,

$$\tau_c = 4\pi r_0^3 a \exp(\Delta E_{vis}/RT)/3kT. \quad (13)$$

Because hypophosphite is a nearly-spherical molecule it is very likely that the temperature dependence of its viscosity obeys an Arrhenius relationship [49]. By regressing eq. 12 onto viscosity vs. temperature data for H_2O [19], initial estimates were obtained for a and ΔE_{vis} of 9.80×10^{-7} and 16.80 kJ/mol, respectively. Accordingly, r_{P-H} was used as an initial estimate of the Stokes radius.

Eq. 13 was substituted into eqs. 2 and 5, then by using eq. 1 we regressed the resulting function onto the data in table 1 to obtain $r_0 = 1.738$ Å, $\Delta E_{vis} = 16.87$ kJ/mol, $a = 1.134 \times 10^{-6}$ and $C^2 = 1.135 \times 10^9$ s^{-2}. Using this value for C^2, rotational correlation times could then be computed from the T_1 values; the theoretical curve is shown in fig. 1.

A spectrum of the solution containing hypophosphite at high pH with ^2H_2O showed the presence of three chemical species: $H_2PO_2^-$, $H^2HPO_2^-$ and $^2H_2PO_2^-$ present in the relative proportions 8 : 5 : 1. The fully protonated species appeared as a characteristic triplet with a phosphorus-proton coupling constant (J_{PH}) of 517 Hz. The mono-deuterated species appeared as a doublet of triplets with a phosphorus-deuterium coupling constant (J_{P^2H}) of 79 Hz and $J_{PH} = 517$ Hz.

Table 2

The ^{31}P-NMR T_1, correlation time, and viscosity and hypophosphite/glycerol solutions; at 310 K

The T_1 measurements were performed at 161.5 MHz.

Glycerol (g/100 g)	T_1 (s)	τ_c (s) ($\times 10^{12}$)	η_N [a] (mPa s)	η_B (mPa s)
0	5.56 ± 0.32	5.79 ± 0.72	1.13	0.70
5	4.67 ± 0.30	7.85 ± 0.77	1.53	0.79
20	4.85 ± 0.28	7.41 ± 0.70	1.44	1.21
35	4.25 ± 0.18	8.96 ± 0.53	1.74	2.02
45	3.96 ± 0.23	9.85 ± 0.77	1.92	3.04
60	2.35 ± 0.06	18.06 ± 0.54	3.52	6.44
82	0.90 ± 0.05	49.68 ± 3.03	9.67	35.00

[a] From correlation time, calculated using $r_0 = 1.738$ Å.

Finally, the bis-deuterated species appeared as a quintet with $J_{P^2H} = 79$ Hz. A T_1 measurement at 310 K on this solution gave a value of 5.52 ± 0.15 s for the central resonance of the triplet arising from $H_2PO_2^-$ and 10.60 ± 1.01 s from the central resonance of the quintet of the $^2H_2PO_2^-$ species.

4.2. NMR-hypophosphite and bulk viscosity of glycerol solutions

T_1, bulk viscosity (η_B) data, as well as those derived from NMR measurements of the viscosity (η_N) of hypophosphite in glycerol solutions are

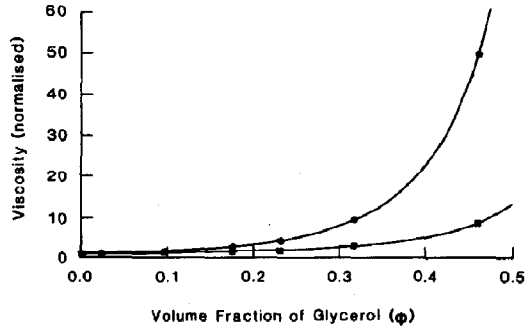

Fig. 2. Bulk viscosity (measured by capillary viscometry) (●) and NMR-derived (from hypophosphite correlation time) viscosity (■) vs. volume fraction of glycerol at 310 K. Viscosity was adjusted by altering the glycerol concentration. The lines drawn were computed from the function $\eta = \exp[\nu\phi/(1 - \kappa\phi)]$ where ν and κ are empirical hydrodynamic interaction parameters [9] determined by non-linear least-squares regression of the equation onto the data. The interaction parameters obtained were: for bulk viscosity, $\nu = 5.074 + 0.013$ and $\kappa = 0.882 \pm 0.003$; for NMR-derived viscosity $\nu = 2.152 \pm 0.219$ and $\kappa = 1.178 \pm 0.103$. Viscosity measurements were normalized so that at $\phi = 0$, $\eta = 1$.

Table 3

The ^{31}P-NMR T_1 of hypophosphite and the resulting calculated viscosity of lysates and whole cells, and the bulk viscosity of lysates Temperature, 310 K; the T_1 measurements were performed at 161.5 MHz.

	Cell volume (fl)		
	65.5	75.8	100.1
[Hemoglobin] (g/ml)	0.41	0.36	0.28
ϕ (hemoglobin)	0.31	0.27	0.21
T_1 (s) (intracellular)	3.00±0.05	3.47±0.09	3.69±0.17
T_1 (s) (lysate)	3.27±0.06	3.42±0.04	3.99±0.05
τ_c ($\times 10^{11}$) (s) (intracellular)	1.37±0.02	1.16±0.03	1.08±0.06
τ_c ($\times 10^{11}$) (s) (lysate)	1.24±0.03	1.18±0.01	0.98±0.02
η_N [a] (intracellular) (mPa s)	2.67	2.26	2.10
η_N [a] (lysate) (mPa s)	2.42	2.30	1.90
η_B (lysate) (mPa s)	132.32 [b]	19.48	5.63

[a] From correlation time, calculated using $r_0 = 1.738$ Å.
[b] From two measurements only.

listed in table 2. All the NMR-derived quantities were calculated using parameter estimates reported in section 4.1. The bulk viscosity and the NMR-derived quantity from τ_c are plotted vs. the volume fraction of glycerol in fig. 2. For increasing solute concentration the increase in NMR-derived viscosities was less than that in bulk viscosity.

4.3. NMR-hypophosphite and bulk viscosity of lysates and intact cells

T_1 and derived quantities for hypophosphite in human erythrocytes at three cell volumes are summarized in table 3. A comparison of η_N for the whole cells and η_B for the corresponding lysates is shown in fig. 3.

Table 4

Diffusion coefficients of hypophosphite derived from viscosity estimates, at 310 K

Sample	τ_c ($\times 10^{12}$) (s)	Diffusion coefficient ($\times 10^9$) (m^2/s)		η_B (mPa s)	η_N (mPa s)
		a	b		c
Glycerol (g/100 g)					
0	5.79	1.16	1.95	0.70	1.13
5	7.85	0.86	1.66	0.79	1.53
20	7.41	0.91	1.08	1.21	1.44
35	8.96	0.75	0.65	2.02	1.74
45	9.85	0.68	0.43	3.04	1.92
60	18.06	0.37	0.20	6.44	3.52
82	49.68	0.14	0.04	35.00	9.67
Cell volume (fl)					
65.5	13.7	0.49	0.01	132.32	2.67
75.8	11.6	0.58	0.07	19.48	2.26
100.1	10.8	0.62	0.23	5.63	2.10

[a] Calculated using η_N computed from correlation time.
[b] Calculated using η_B.
[c] Calculated using $r_0 = 1.738$ Å.

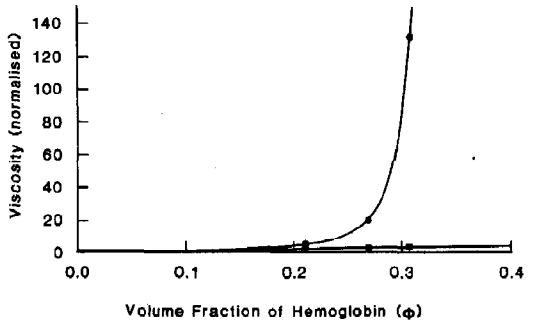

Fig. 3. Bulk viscosity of lysate measured by capillary viscometry (●) and NMR-derived viscosity for whole cells obtained using correlation times (■), vs. volume fraction (ϕ) of hemoglobin at 310 K. The intracellular hemoglobin concentration was altered by changing cell volume. The lines were computed using the equation given in fig. 2 with the interaction parameters obtained by non-linear least-squares regression of the equation onto the data. The calculated values for the interaction parameters were: for bulk viscosity, $\nu - 3.538 \pm 0.056$ and $\kappa = 2.542 \pm 0.011$; for NMR-derived viscosity, $\nu = 4.027 \pm 1.640$ and $\kappa = -0.924 \pm 1.843$. Viscosity measurements were normalized so that at $\phi = 0$, $\eta = 1$.

4.4. Diffusion coefficients of hypophosphite in glycerol, lysate and intact cells as derived from T_1 measurements

From correlation times derived from the T_1 measurements of hypophosphite in glycerol solutions, lysates and intact cells translational diffusion coefficients were calculated using eq. 10; the results are reported in table 4.

5. Discussion

The relaxation mechanism of hypophosphite appears to be a combination of intra-molecular dipole-dipole and spin-rotation interactions, with a small contribution (just detectable at 9.4 T) from chemical shift anisotropy. At temperatures below about 313 K in low-viscosity solutions, the dominant form of relaxation is dipole-dipole, while above this temperature the spin-rotation mechanism dominates. In more viscous media such as glycerol solutions, and red cell cytoplasm, the dipole-dipole mechanism dominates. The molecular reorientation of hypophosphite in solution has been taken to be isotropic in view of its molecular symmetry. Errors in the calculated mean moment of inertia do not affect any final conclusions relating to viscosity and diffusion, since eq. 5 is linear with respect to C^2 and I^2 and the C^2 term compensates for any error in I.

The T_1 measurements of the solution containing hypophosphite in various states of deuteration allowed a check on the proposed relaxation mechanism of hypophosphite. If the CSA contribution is taken to be negligible (< 10%), then at 310 K the relaxation mechanism of hypophosphite is equally intra-molecular dipole-dipole and spin-rotation. Hence, from the T_1 value of 5.52 ± 0.15 s for $H_2PO_2^-$, $R_{1DD} = R_{1SR} = 9.06 \times 10^{-12}$ s^{-1}. However, for the fully deuterated species the dipole-dipole contribution, due to the smaller magnetogyric ratio of the deuteron, should be about 1/36 of that resulting from proton interactions. Hence, for the fully deuterated species we expect $R_{1DD} = 1.51 \times 10^{-2}$ s^{-1}. Thus, the T_1 value for the fully deuterated species should be about 10.74 s; this is in reasonable agreement with our experimental value of 10.6 ± 1.01 s.

The Debye model of molecular reorientation has been used to interpret the motion of hypophosphite. Other, more complex models such as the 'extended J-diffusion' model [50] or the Fokker-Planck-Langevin model [51,52] could have been used, however, analysis of our data with these theories was considered to be outside the scope of the present experimental work. The more complex models require data derived from other forms of spectroscopy. Analysis of the ^{31}P relaxation of larger, more anisotropic molecules has been successfully completed without using these more elaborate models [53]. In any case, since for the range of viscosities we studied in the present work with hypophosphite $(kT/I)^{1/2}\tau_{SR} \ll 1$ and in this limit the extended J-diffusion model and the Fokker-Planck-Langevin model give the relationship stated in eq. 4 [54].

Since for hypophosphite $\tau_{fr} \ll \tau_c$ ($\tau_{fr} \approx 3 \times 10^{-13}$ s) the rotational diffusion assumption is valid. In the quantification of the relaxation mechanism by the regression analysis using eqs. 1, 2, 5 and 13, the Stokes radius was initially set equal to a phosphorus–proton bond length (1.42 Å) but

the regression analysis gave this value to be 1.738 Å. However, when the molecular volume was calculated from the known structure and the Van der Waals radii of the atoms [55,56], the radius of the equivalent sphere was found to be 2.78 Å. Thus, the rotation of hypophosphite has characteristics of both 'inertial limitation' (slipping rotation) and interaction with the solvent (stick boundary condition) [36]. Instead of assuming hypophopshite to be spherical in solution, the slip ellipsoidal model of Hu and Zwanzig [57] could have been used. This would have resulted in a larger experimentally determined value of r_0, more in keeping with the Van der Waals radius, although the estimates of D and η would be unchanged.

Hypophosphite is a valuable ^{31}P-NMR probe of the intracellular milieu of red cells for two reasons; (a) it is rapidly transported into the cells [7,8] and (b) the intra- and extracellular species appear as distinct resonances in the NMR spectrum [7,8]. However, because the hypophosphite molecule is small, thus enabling rapid transport across the red cell membrane, its correlation time does not necessarily agree well with the viscosity predicted by the Debye equation. For the relaxation time to 'report' on macroviscosity would require that the relaxation of the probe molecule would involve whole domains of the liquid and not just the rotation of a single molecule [58]. The cytoplasmic bulk viscosity is determined almost exclusively by hemoglobin [36], but the correlation time of hypophosphite is not proportional to η_B/T since changes in viscosity caused by the motion of the hemoglobin molecules are not directly reflected by changes in the local mobility of hypophosphite [59]. Thus, bulk viscosity (i.e., that measured via the capillary viscometer) is not a good guide to the behaviour of small-solute ^{31}P-NMR relaxation times, because the implicit assumptions of the Debye theory are violated. In that theory the solvent molecules (in the present case, hemoglobin and H_2O) are seen as a continuum, and this can only occur when the solute molecules are large compared with the solvent molecules, which is clearly not the case with hypophosphite.

The theory developed by Gierer and Wirtz [37] takes into account the non-continuous nature of the solvent but does not consider solute-solvent interactions, nor does it allow for a concentration dependence of the solvent radius. (In the present case the solvent radius would be a function of the ratio of the hemoglobin and water concentrations.) The effect of the altered hemoglobin concentration is well demonstrated by comparing the T_1 and bulk viscosity of the most shrunken cells (table 3) to those of the glycerol solutions (table 2). The most viscous glycerol solution ($\eta_B = 35$ mPa s) causes hypophosphite to have a relaxation time of 0.90 s, whereas the lysate from 65.5-fl cells, with almost 4-times the bulk viscosity, results in a hypophosphite relaxation time of 3.27 s. The NMR-derived viscosity does not portray the enormous changes in bulk viscosity caused by much less dramatic changes in cell volume (thereby concentrating the enclosed hemoglobin solution). This behaviour can be quite clearly understood in terms of 'excluded volume effects', which are further complicated by the small changes in hemoglobin solvation, that have been observed with varying solute concentration [36]. Thus, it can be seen that bulk viscosity and NMR-measured viscosity are two distinct entities; the former is largely determined by the macromolecules in the solution while the latter depends on the size of the probe molecule, relative to that of the solvent molecules. The two viscosity values are only able to be related empirically for a particular solute-solvent system [9], and yet the changes in NMR viscosity can be used empirically to monitor changes in cell volume [9].

The cytoplasmic viscosities calculated using the Debye equation from hypophosphite relaxation times were larger (2.10–2.67 mPa s for cells ranging in volume from 65.5 to 100.1 fl), and the corresponding translational diffusion coefficients smaller, than those obtained from similar measurements using ^{13}C-NMR [9]; the difference is attributed to the particular choice of the Stokes radius of the probe molecule. Translational diffusion coefficients calculated from hypophosphite relaxation times were larger than those derived from bulk viscosity measurements, except in the case of the least viscous solutions. The NMR-derived diffusion-coefficient estimates were smaller, however, when the Stokes radius derived from

molecular geometry was used. The NMR-derived diffusion coefficients changed by approx. 20% in red cells ranging in size from 65.5 to 100.1 fl. However, diffusion coefficients estimated from bulk viscosity of lysates of these cells altered by approx. 230%.

In conclusion, hypophosphite is not an ideal probe of bulk (macro-) viscosity because of limited theory relating macro- to microviscosity. However, one reason for studying intracellular viscosity is to understand its effect on enzyme action [60] especially with regard to diffusion control of enzyme rates. In order to detect diffusion control the probe molecule should ideally be similar in size to that of the enzyme substrate of interest. Thus, the rate of diffusion-controlled reactions involving substrate molecules similar in size to hypophosphite could be expected to alter by approx. 20% in a red cell as the volume changes from 66 to 100 fl. This may be the case in the reaction catalysed by the enzyme carbonic anhydrase, since the bicarbonate ion is of similar size to hypophosphite. The transient time of an enzyme system (defined as the time required for diffusion and accumulation of intermediate substrate/products species to levels sufficient to sustain a steady state of enzyme-substrate complex) is a function of the viscosity of the medium [60]. In the case of (small) metabolites this is a function of the (NMR-estimated) microviscosity and not the macroviscosity.

Acknowledgements

The work was supported by a grant to P.W.K. from the Australian NH and MRC. W.S.P. received a Commonwealth Postgraduate Research Award. Mr. W.G. Lowe is thanked for valuable technical assistance and Mr. B.T. Bulliman for assistance with computing. Mr. K. Kirk is thanked for advice and discussions on the use of hypophosphite in red cell suspensions and Dr. R.J. Labotka for pointing out the means for deuterating hypophosphite. Dr. M. Batley and Dr. A.-T. Hoa are thanked for their assistance with the work on the Varian XL-200 spectrometer.

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