pH and Cell Volume Effects on H_2O and Phosphoryl Resonance Splitting in Rapid-Spinning NMR of Red Cells

Timothy J. Larkin, William A. Bubb, and Philip W. Kuchel
School of Molecular and Microbial Biosciences, University of Sydney, New South Wales 2006, Australia

ABSTRACT Two resonances are seen in the ^{1}H-NMR spectrum of water in erythrocyte suspensions spun at the magic angle, a broad signal from water inside the cells and a sharp signal from extracellular water. The splitting is a result of a true chemical shift difference between the two populations, as bulk magnetic susceptibility effects are negated at the magic angle. The pH dependence of this chemical shift difference in erythrocyte suspensions was investigated. Splittings of 16.7 $\pm$ 0.1, 18.9 $\pm$ 0.9, and 21.0 $\pm$ 0.2 Hz were observed at pH 6.0, 7.0, and 8.5, respectively; however, this was accompanied by a change in the mean cell volume. To account for any contribution from the volume change, the osmolality of the pH 6.0 and 8.5 suspensions was adjusted to equalize the cell volume between samples at the three pHs. Under these conditions, the splitting was 18.3 $\pm$ 0.1 and 18.6 $\pm$ 0.1 Hz at pH 6.0 and 8.5, respectively. Thus the observed chemical shift difference between the two water resonances was independent of pH. Therefore the splitting of the water resonance was concluded to be directly proportional to the protein concentration within the cell. Measurements of the magnetic susceptibility difference between the two compartments were also carried out, yielding a value of 2.0 $\pm$ 0.2 $\times$ 10^{-7} (SI units) for erythrocytes in isotonic saline at pH 7.0.

INTRODUCTION

In high-resolution magic angle spinning NMR (MAS-NMR) experiments on erythrocyte suspensions, the signal from ^1H_2O is observed as two separate resonances (1,2). The separate resonances are brought about by the centrifugation of the cells to the inside of the wall of the MAS rotor, creating a two-phase system consisting of two concentric cylinders—the inner one is cell-free supernatant water, and the outer formed of tightly packed cells (3). This spatial separation of cells and supernatant effectively eliminates the exchange of water between the two compartments, giving rise to a broad signal assigned to intracellular water and a sharp signal assigned to the supernatant water. The assignment has been confirmed by the addition of magnetic resonance imaging contrast agents that are unable to cross the erythrocyte membrane and thus broaden and shift only the supernatant resonance (4). The intracellular signal arises from water that is truly intracellular together with a small fraction (~3%) of interstitial water. The water that this signal represents is sometimes described as the pellet water (5). This interstitial water appears not to give a separate ^{1}H-NMR signal but is presumably in rapid exchange with the intracellular fraction. Recently, Bruno et al. (5) have confirmed the insignificance of the exchange on the NMR timescale between the pellet and supernatant waters in MAS experiments on suspensions of red blood cells (RBCs).

For samples oriented at the magic angle, bulk magnetic susceptibility effects are eliminated (6). Chen et al. (3) suggested that the splitting of the water signal in cell suspensions was due to an isotropic susceptibility difference between the cells and supernatant. It has since been shown, however, that by introducing the ''sphere of Lorentz'' correction the splitting is due to a true chemical shift difference between water in the two compartments (2). Aime et al. (7) have demonstrated that the magnitude of the ^1H_2O chemical shift difference is correlated with protein concentration for suspensions of several cell types under MAS conditions and that in solutions of bovine serum albumin (BSA) the chemical shift of water is dependent on protein concentration but not on pH. Their conclusion was that the primary determinant of the magnitude of the water peak separation was the cellular protein concentration, except in the case of erythrocytes where the presence of paramagnetic deoxyhemoglobin leads to an increase in the peak splitting due to a pseudocontact shift (7).

Although the effect of pH on the chemical shift of water in a protein solution has been investigated, we set out to investigate the dependence of the ^1H_2O chemical shift difference on pH in a cellular system, where due to pH buffering by proteins and cellular components a pH gradient across the cell membrane is possible. Erythrocytes are ideal for such a study as they are easily obtainable, they are viable over a large pH range, they have a uniform interior, and the paramagnetic heme can be converted to the stable diamagnetic form in carbonmonoxyhemoglobin to eliminate the pseudocontact shift. In addition, ^{31}P-NMR methods are well established for monitoring their intracellular pH (8), cell volume (9), and membrane potential (10).

MATERIALS AND METHODS

Erythrocyte preparation

Fresh blood was obtained by venipuncture from a single healthy volunteer (T.J.L.), then centrifuged for 10 min at 3000 $\times$ *g* and the buffy coat removed

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Address reprint requests to Philip W. Kuchel, School of Molecular and Microbial Biosciences, Building G08, University of Sydney, NSW 2006, Australia. Tel.: 61-2-9351-3709; Fax: 61-2-9351-4726; E-mail: p.kuchel@mmb.usyd.edu.au.

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by aspiration. The cells were then washed with three volumes of isotonic saline containing 25 mM phosphate, 30 mM hypophosphorous acid (HPA), 30 mM methylphosphonate (MeP), 30 mM dimethyl methylphosphonate (DMMP), 10 mM triethylphosphate (TEP), 2.5 mM 3-(trimethylsilyl)-1-propane-sulfonic acid, and 10 mM glucose, constituted in 15% (v/v) D_2O, with a pH of 6.0, 7.0, or 8.5 (unadjusted glass pH electrode readings). Carbon monoxide was then bubbled through the suspension for 15 min to create diamagnetic carbonmonoxyhemoglobin. The cells were then incubated at 37°C for 1 h to allow for equilibration of MeP between intracellular and extracellular compartments, followed by a further two washes. The cells were suspended to a final hematocrit (Ht) between 77% and 92%, measured using a capillary centrifuge (Clements, North Ryde, NSW, Australia). Red cell counts of the final erythrocyte suspensions and hemoglobin concentrations of fresh blood were measured with a Sysmex KX-21 hematology analyzer (Sysmex, Kobe, Japan).

Hemolysates

Lysate samples for pH titrations of inorganic phosphate (Pi) and MeP were prepared from high Ht RBC suspensions (>98%) washed in buffers with pH between 4 and 9, with three freeze/thaw cycles in liquid nitrogen. Samples from the supernatant from the last wash of these samples were used for calibration of the supernatant pH.

NMR

Erythrocyte samples for variable angle spinning NMR experiments were placed in a 300-μL sealing cell (Doty Scientific, Columbia, SC) whose cap was modified to incorporate a coaxial threaded hole and nylon grub screw to allow for the removal of air bubbles, which interfere with spectral resolution for spectra acquired at angles other than the magic angle. NMR spectra were recorded on a Bruker DRX-400 widebore spectrometer with a variable angle spinning XC7 probe (Doty Scientific). ^{1}H spectra can be acquired at all angles with this probe, but the field frequency lock and X-nucleus channels are not effective when the stator is perpendicular and parallel to $\mathbf{B_0}$, respectively. The samples were spun at 2 kHz for ~30 s to centrifuge the cells to the inside of the wall of the sealing cell and then subsequently at 250 Hz. All experiments were performed at 37°C, with sample temperature calibrated using dry neat ethylene glycol in a sealing cell. For experiments on samples of lysates and supernatants, the 30-s spin at 2 kHz was omitted. The chemical shifts of all relevant peaks were identified using the automatic peak picking routine in Topspin 1.3 (Bruker Biospin, Karlsruhe, Germany) after automatic baseline correction of the regions of interest.

RESULTS

Hemoglobin concentration

The average hemoglobin concentration in the freshly taken blood samples was 155 ± 5 gL^{-1}.

Chemical shift differences

Representative ^{1}H and ^{31}P spectra of the compounds studied are shown in Figs. 1 and 2, respectively. Observed chemical shift

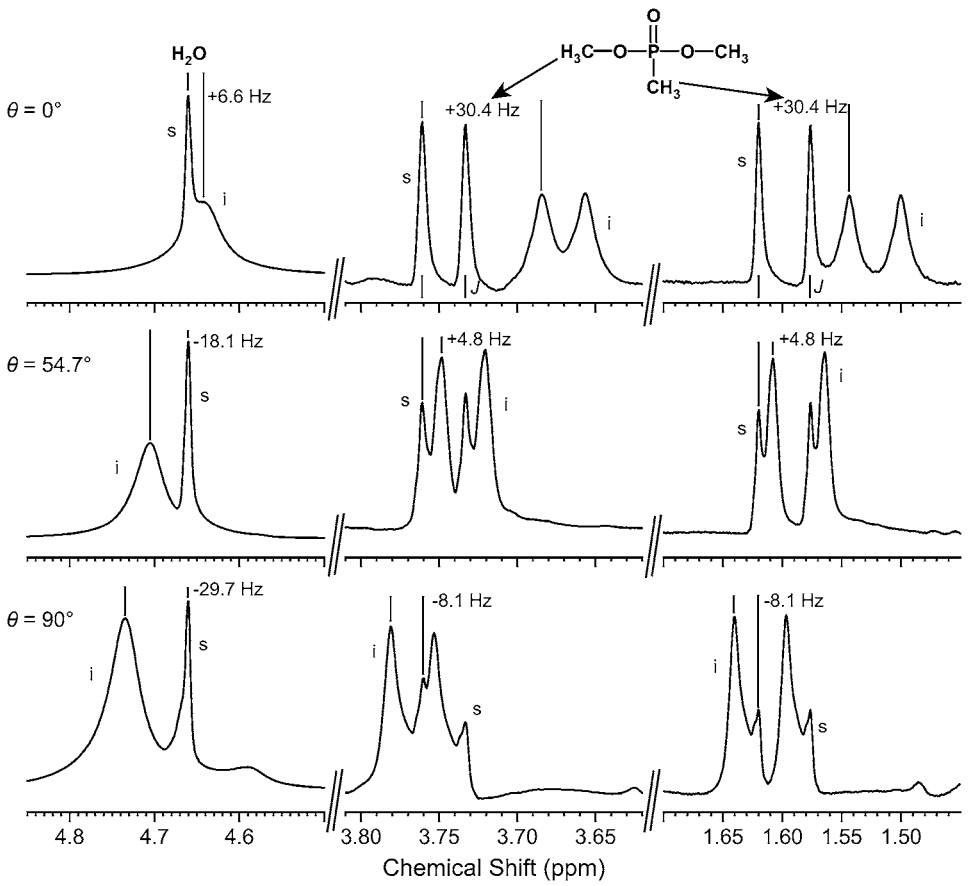

FIGURE 1 400.13 MHz ^{1}H VAS spectra of a suspension of erythrocytes that had been washed in a pH 7.0 medium (Ht = 83%). Similar spectra for pH 6.0 and 8.5 were also obtained (not shown). Spectra in each row were obtained at the same spinning angle: top row $\theta = 0°$ (rotor aligned with main magnetic field); middle row $\theta = 54.7°$ (rotor at the magic angle); and bottom row $\theta = 90°$ (rotor perpendicular to main magnetic field). From left to right the spectral regions contain the resonances of water protons, DMMP ester methyl protons, and DMMP phosphonyl methyl protons, respectively. Different vertical scaling was used for the water and DMMP regions of the spectrum. Each spectrum represents the sum of 64 transients, with presaturation of the water peak for the DMMP spectra. No line broadening was used before Fourier transformation of the free induction decays, and the broad hemoglobin background was removed using automatic baseline correction. Intracellular (i) and supernatant (s) resonances are marked together with their separation, shown as (supernatant frequency—intracellular frequency). ^{1}H/^{31}P scalar couplings (J) in DMMP were 11 Hz for the ester methyl and 17 Hz for the phosphonyl methyl. Spectra were referenced to the supernatant water resonance at 4.66 ppm. Spin rate was 250 Hz. The chemical shift differences shown are those from the particular experiment depicted and are not average values (see Tables 1 and 2 for averages).

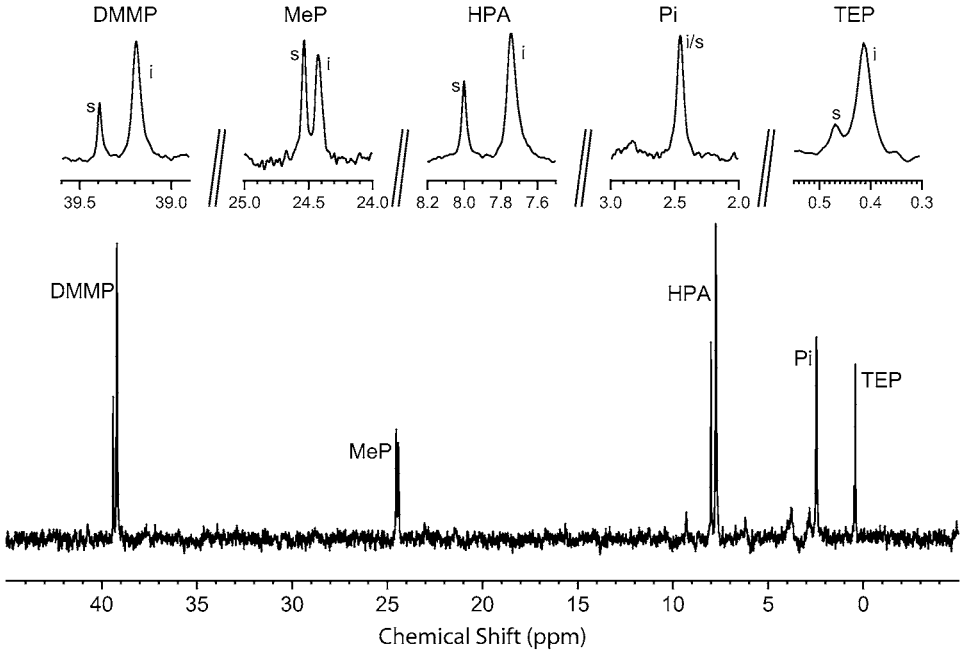

FIGURE 2 161.98 MHz ^{31}P-NMR spectrum of the erythrocyte suspension (pH 7.0) whose ^{1}H spectra are shown in Fig. 1. The sample was spun at 250 Hz at the magic angle. The spectrum was acquired for 256 transients with 3-Hz exponential line broadening applied before Fourier transformation of the free induction decay. The top row shows expansions of the relevant peaks in the full spectrum, with resonances from intracellular (i) and supernatant (s) populations marked. For Pi the intracellular and supernatant resonances were overlapped. The spectrum was referenced to the weighted average chemical shift of TEP at 0.44 ppm.

differences between intracellular and supernatant resonances in ^{1}H and ^{31}P spectra at the magic angle are reported in Table 1, and chemical shift differences from ^{1}H spectra acquired with the stator aligned with and perpendicular to $\mathbf{B_0}$ in Table 2.

TEP normally appears as a single resonance in spectra of RBC suspensions due to its fast exchange across the red cell membrane (11). When spun at the magic angle, physical separation of the two environments significantly reduces exchange, and distinct intracellular and supernatant resonances are seen. For referencing ^{31}P spectra, the weighted average (weighted according to the peak integral) chemical shift of TEP was used.

The relationships between the pH of the suspension-buffer and the peak separation at the magic angle for H_2O (^{1}H), DMMP (^{31}P), and HPA (^{31}P) are shown in Fig. 3. The linear relationship between the peak separation and the buffer pH is noted. Similar linear relationships were seen for the ^{1}H resonances of DMMP and the ^{31}P resonance of TEP for samples spun at the magic angle. The ^{1}H resonances of H_2O and DMMP, when the sample was spun aligned with and perpendicular to $\mathbf{B_0}$, showed a similar linear dependence of the peak separation on the pH of the suspension. No correlation of the variation of peak separation with buffer pH was observed for MeP or Pi.

Cell volume

Mean erythrocyte volume in fL was calculated using the Ht: vol = Ht/cell count. The values for each washing buffer are given in Table 3. For cells in isotonic saline the cell volume varied linearly with the pH of the washing buffer.

pH measurements

Measurement of intra- and extracellular pH using the ^{31}P chemical shifts of MeP and Pi have been performed on

TABLE 1 Chemical shift differences between intracellular and extracellular resonances in erythrocyte suspensions of different pH when spun at the magic angle

pH	H_2O (^{1}H)	DMMP (^{1}H)	DMMP (^{31}P)	TEP (^{31}P)	HPA (^{31}P)	MeP (^{31}P)
6.0	16.7 ± 0.1	−3.2 ± 0.4	−29 ± 1	−7.8 ± 0.7	−45 ± 1	−28 ± 3
7.0	18.9 ± 0.9	−5.0 ± 0.2	−34 ± 2	−9.2 ± 0.3	−44 ± 2	−17 ± 3
8.5	21.0 ± 0.2	−7.1 ± 0.4	−42 ± 1	−11.6 ± 0.6	−43 ± 2	69 ± 3
6.0*	18.3 ± 0.1	−4.2 ± 0.6	−34 ± 1	−8.4 ± 0.3	−50 ± 1	−31 ± 2
8.5*	18.6 ± 0.1	−6.1 ± 0.2	−37 ± 1	−10.4 ± 0.3	−36 ± 1	89 ± 2

Separations were measured from the supernatant resonance to the intracellular resonance and are quoted as the mean ± 1 SD.

*Indicates cells of specified pH washed in osmolality-adjusted buffer to give cells of the same volume as those at pH 7.0 in isotonic buffer.

TABLE 2 Chemical shift differences between intracellular and extracellular resonances in ^{1}H spectra of erythrocyte suspensions of different pH, when aligned with and perpendicular to B_0, respectively

	Vertical		Horizontal	
PH	H_2O	DMMP	H_2O	DMMP
6.0	−7.7 ± 0.6	−27 ± 3	27.3 ± 0.9	8.1 ± 0.8
7.0	−7.6 ± 1.4	−31 ± 1	31.7 ± 2.7	8.2 ± 0.2
8.5	−7.3 ± 1.4	−37 ± 2	36.0 ± 1.0	7.3 ± 0.5
6.0*	−8.0 ± 1.5	−31 ± 1	31.6 ± 0.3	8.0 ± 0.5
8.5*	−7.8 ± 1.5	−34 ± 1	31.9 ± 0.3	7.1 ± 0.5

Values are mean ± 1 SD.

*Indicates cells of specified pH washed in osmolality-adjusted buffer to give cells of the same volume as those at pH 7.0 in isotonic buffer.

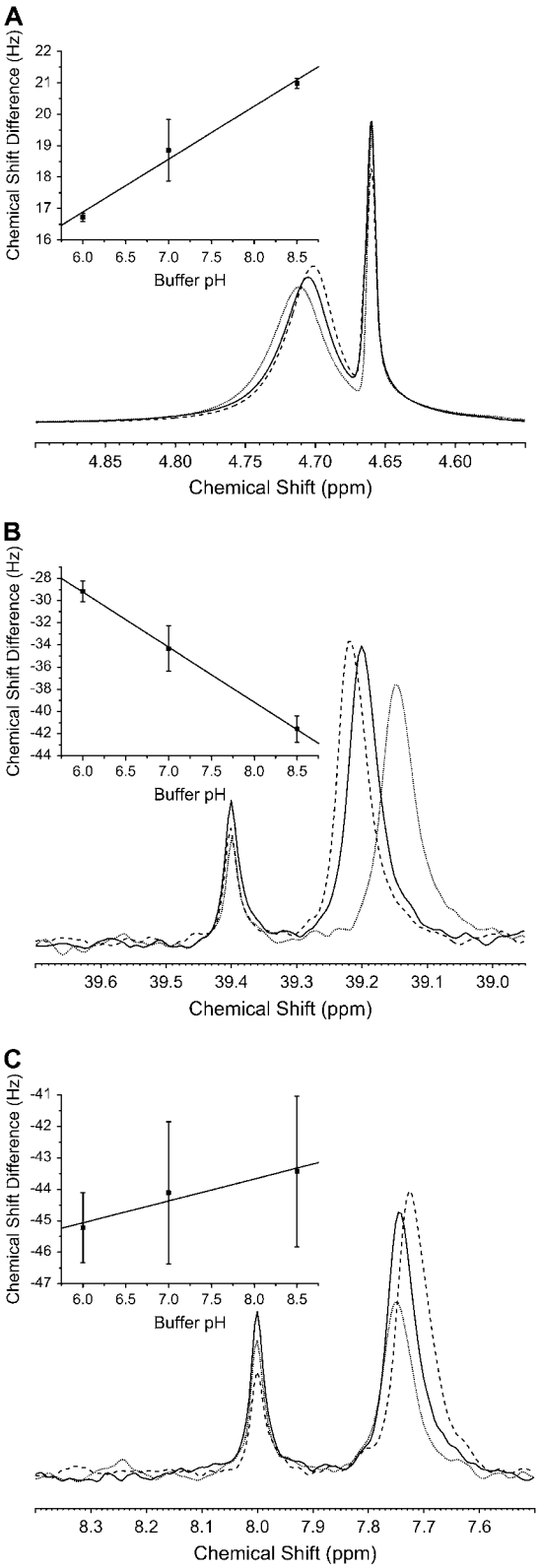

FIGURE 3 (*A*) ^{1}H spectra of ^1H_2O, (*B*) ^{31}P spectra of DMMP, and (*C*) ^{31}P spectra of HPA from erythrocytes at pH 6.0 (*dashed line*), 7.0 (*solid line*), and 8.5 (*dotted line*) acquired at the magic angle. All spectra were referenced to the respective supernatant resonance and were acquired as described in Figs. 1 and 2. Changes in the relative areas of intracellular and supernatant peaks reflect the change in cell volume with pH. Insets show the variation of the chemical shift difference with the pH of the buffer used to wash the cells, with the lines showing linear fits to the data: $y = 1.7x + 6.8$, $y = -5.0x + 0.5$ and $y = 0.7x - 49.2$, for H_2O, DMMP, and HPA, respectively, where y denotes chemical shift difference and x denotes pH. The error bars on each plot show ± 1 SD.

erythrocytes in normal high resolution NMR probes (8). This method uses a modified Henderson-Hasselbalch equation,

$$\mathrm{pH} = \mathrm{pK_a} + \log_{10}(\delta - \delta_a/\delta_b - \delta), \quad (1)$$

where δ is the observed chemical shift of a species and δ_a and δ_b are the chemical shifts (in ppm) of the acid and base forms of it, respectively. Due to the elimination of the contribution to peak separation of bulk magnetic susceptibility under MAS conditions, pH calibration curves for MeP and Pi in lysates and supernatant samples were constructed (Fig. 4). Values of pK_a, δ_a, and δ_b (Table 4) were determined by using nonlinear least squares regression of Eq. 1 onto the data in a Mathematica program (Wolfram, Champaign, IL). The intracellular and supernatant pH values for intact RBCs are given in Table 3.

Membrane potential

The potential (φ) across the erythrocyte membrane was calculated using the relative areas (integrals) of the intracellular and extracellular resonances of DMMP and HPA and a modified Nernst equation,

$$\varphi = \frac{RT}{N_0 F}\ln\left(\frac{\mathrm{HPAi/DMMPi}}{\mathrm{HPAs/DMMPs}}\right), \quad (2)$$

where R is the gas constant, T is the absolute temperature, N_0 is Avagadro's number, F is the Faraday constant, HPAi and DMMPi are the integrals of the intracellular resonances, and HPAs and DMMPs are the integrals of the extracellular resonances. Integrals were measured using the peak deconvolution routine in Topspin 1.3 with pure Lorenzian peaks for supernatant resonances and 7:3 mixed Lorenzian and Gaussian peaks for intracellular resonances.

Magnetic susceptibility

The magnetic susceptibility difference between the intracellular and supernatant compartments ($\Delta\chi$) was determined using the relationship derived by Philp et al. (2),

$$\Delta\chi = 2(\Delta\nu_0 - \Delta\nu_{90})/\nu_r, \quad (3)$$

where $\Delta\nu_0$ and $\Delta\nu_{90}$ are the chemical shift difference (in Hz) between intracellular and extracellular resonances when the sample is parallel to and perpendicular to $\mathbf{B_0}$, respectively, and ν_r is the spectrometer reference frequency. Using this relationship, values of $\Delta\chi$ of $-1.7 \pm 0.2 \times 10^{-7}$, $-2.0 \pm 0.2 \times 10^{-7}$, and $-2.2 \pm 0.1 \times 10^{-7}$ (SI units) were obtained

TABLE 3 Cellular parameters for erythrocytes suspended at different pH values

pH	Cell volume (fL)	Membrane potential (mV)	^{31}P Intracellular pH	^{31}P Last Wash Supernatant pH	^{31}P Whole cell supernatant pH
6.0	94.5 ± 1.2	−2.3 ± 2.8	6.5 ± 0.2	6.1 ± 0.1	6.2 ± 0.1
7.0	88.1 ± 0.6	−9.8 ± 0.8	7.2 ± 0.1	7.0 ± 0.1	7.0 ± 0.1
8.5	77.8 ± 2.6	−21.0 ± 2.3	8.1 ± 0.2	8.3 ± 0.1	7.8 ± 0.1
6.0*	86.3 ± 1.1	−4.3 ± 1.8	6.7 ± 0.1	6.1 ± 0.1	6.7 ± 0.1
8.5*	85.5 ± 0.7	−25.7 ± 1.6	7.9 ± 0.1	8.3 ± 0.1	7.9 ± 0.1

Values are mean ± 1 SD. pH values from ^{31}P spectra were calculated from the shifts of MeP and Pi, together with the chemical shift difference between the two (MeP-Pi). Intracellular pH measurements are from ^{31}P spectra of whole cells.

*Indicates cells of specified pH washed in osmolality-adjusted buffer to give cells of the same volume as those at pH 7.0 in isotonic buffer.

using the ^{1}H resonances of H_2O and DMMP for erythrocytes in isotonic media at pH 6.0, 7.0, and 8.5, respectively. For the cell-volume-adjusted erythrocytes the magnetic susceptibility differences were $-1.9 \pm 0.1 \times 10^{-7}$ and $-2.0 \pm 0.1 \times 10^{-7}$ (SI units) for the pH 6.0 and 8.5 samples, respectively.

DISCUSSION

Kuchel et al. (12) have shown that under MAS conditions there is no correlation between Ht and the magnitude of the separation between intracellular and extracellular resonances of DMMP in ^{31}P spectra. This was confirmed in this study for a range of phosphorus compounds, DMMP, HPA, TEP, and H_2O. All blood samples were taken from a single donor, and there was no significant variation in the hemoglobin concentration in fresh blood samples during the experiments; therefore sample variation should not have contributed to variation in the splitting.

DMMP, HPA, and H_2O displayed a linear variation of the peak separation with the suspension pH, whereas MeP and Pi did not. DMMP and TEP are neutral, so any change in peak separation does not depend on any change in the charge state of the molecules themselves. Therefore it should follow that the changes in peak separation for H_2O and HPA, which is charged, are due to factors other than or in addition to pH. In the case of DMMP the separation between extracellular and intracellular resonances is known to be due to differences in the extent of hydrogen bonding of the phosphoryl oxygen to water inside erythrocytes due to the high concentration of intracellular protein, predominantly hemoglobin (13). Indeed the magnitude of the separation can be used to monitor changes in erythrocyte volume (9,12).

MeP and Pi did not show a linear relationship between suspension pH and chemical shift difference between intracellular and supernatant resonances, and in some cases the intracellular and supernatant resonances overlapped (e.g., Pi resonance in Fig. 3). The separation between resonances for these molecules is dependent on the pH difference across the cell membrane and on the values of pK_a and δ_a and δ_b in the supernatant and intracellular environments.

Philp et al. (2) reported a magnetic susceptibility difference between the intracellular and supernatant compartments for erythrocyte suspensions, from variable angle spinning (VAS) data of -2.2×10^{-7} (SI units). Similar values of magnetic susceptibility were obtained in these experiments; however, the difference was observed to vary with the pH of the sample. As bulk magnetic susceptibility effects are eliminated at the magic angle, the change in magnetic susceptibility with pH will not contribute to the observed change in chemical shift with pH. At angles other than the magic angle, the susceptibility difference may in part account for the change in splitting, together with the observed change in cell volume.

The erythrocyte volume was measured directly at each pH (Table 4), and the relationship between cell volume and separation between supernatant and intracellular resonances for H_2O (^{1}H), DMMP, TEP, and HPA (^{31}P) for suspensions spun at the magic angle is shown in Fig. 5. It is apparent from Fig. 5 that for each of the molecules a linear relationship exists between cell volume and the magnitude of the peak

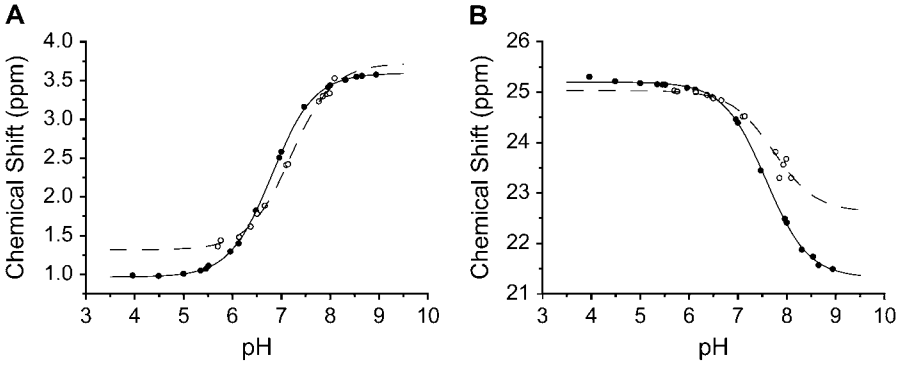

FIGURE 4 pH calibration curves for (*A*) Pi and (*B*) MeP under MAS conditions (spin rate = 250 Hz). Solid and open circles represent data from erythrocyte supernatant and lysate samples, respectively, and the solid and dashed lines are fitted curves derived from nonlinear regression for supernatant and lysates, respectively.

TABLE 4 Parameters for the determination of intracellular pH from chemical shifts in ^{31}P-NMR spectra

Species	pK_a	δ_a (ppm)	δ_b (ppm)
Pi	7.19 ± 0.03	1.32 ± 0.03	3.72 ± 0.04
MeP	7.72 ± 0.05	25.03 ± 0.02	22.6 ± 0.1

Errors are ± SE from nonlinear least squares analysis. δ_a denotes the extreme chemical shift of the acid form, and δ_b denotes the extreme chemical shift of the base form of the molecule.

separation. As the cell volume was increased, so the hemoglobin concentration decreased, and the size of the splitting for H_2O, DMMP, and TEP all decreased. For HPA, however, the opposite was true and the splitting increased with increasing cell volume; this is related to the change in membrane potential with pH (10).

To confirm that the change in cell volume was responsible for the observed changes in peak separation and magnetic susceptibility, RBCs were washed with pH 6.0 and 8.5 buffers with osmolality adjusted to give the same cell volume as cells at pH 7.0 in isotonic saline. Under these conditions the chemical shift differences for H_2O and DMMP in ^{1}H spectra were the same as for cells at pH 7.0, within experimental error, for samples spun at the magic angle and with the stator parallel and perpendicular to the main field. Similarly, in ^{31}P spectra of volume-adjusted RBC samples spun at the magic angle, the DMMP and TEP splittings were the same as those for pH 7.0 cells, whereas the splittings for HPA were significantly different. A comparison of the HPA splitting between RBCs in isotonic saline at pH 6.0 and 8.5 and those from the volume-adjusted cells revealed that the splitting was reduced at pH 6.0 (hypertonic buffer) and increased at pH 8.5 (hypotonic buffer). HPA is not a titratable species at the pHs covered in performing the pH calibrations for supernatant and lysate samples (data not shown), and it exists in the ionized base form, hypophosphite ($H_2PO_2^-$); therefore it carries a single negative charge at these pH values ($pK_a = 1.1$) (10). The change in magnitude of the peak splitting was concluded to be the result of the change in the membrane potential brought about by the change in ionic strength required to alter the volume of the cells.

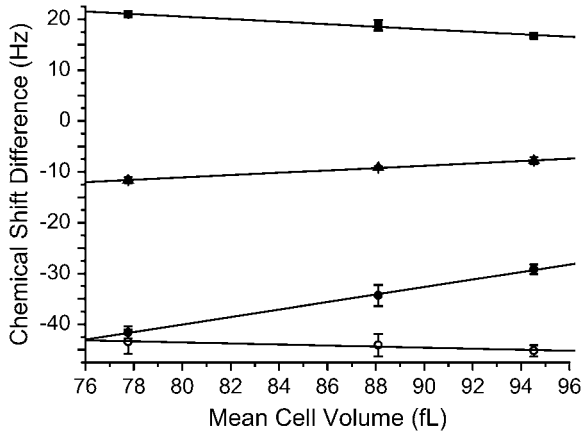

FIGURE 5 The relationship between the mean RBC volume and the magnitude of the chemical shift difference between intracellular and supernatant resonances, for H_2O (*solid squares*), DMMP (*solid circles*), TEP (*solid triangles*), and HPA (*open circles*). The lines shown are linear fits to the data for each compound: $y = -0.2x + 40.5$, $y = 0.7x - 99.0$, $y = -0.1x - 35.3$, and $y = 0.2x - 29.6$, for H_2O, DMMP, HPA, and TEP, respectively, where y denotes chemical shift difference and x denotes the mean cell volume. The error bars indicate ± 1 SD.

The magnetic susceptibility difference in the volume-adjusted erythrocytes at pH 6.0 and 8.5 was the same as that measured for cells at pH 7.0, within experimental error. Therefore the most likely source of the variation in magnetic susceptibility with pH is the change in cell volume, as the overall susceptibility difference is the additive combination of the molar magnetic susceptibilities of the individual components in the two compartments (Wiedemann's additivity law (14)). The change in the concentration of solutes and protein and in the relative amount of water in the cell with the pH-induced volume change is sufficient to account for the change in the overall magnetic susceptibility difference between the intracellular and supernatant compartments.

Aime et al. (7) have shown that the magnitude of the separation between intracellular and extracellular water peaks for several cell types is related to the cellular protein concentration. Additionally, for solutions of BSA the size of the chemical shift change was independent of pH but dependent on the protein concentration. This confirms the earlier work on erythrocytes under static conditions (13). In these experiments a variation of the splitting with pH was observed but was found to be accompanied by a change in cell volume. By washing the cells in specially composed hypo- or hypertonic media at different pH values, the difference in splitting was eliminated, within experimental error. Therefore we conclude that in erythrocytes the cellular protein concentration is the overwhelming determinant of the magnitude of the peak splitting through changes in the extent of hydrogen bonding of water to itself or other probe molecules.

The supernatant pH values for cells washed at pH 8.5 (Table 3) vary depending on the method of measurement, with those calculated from ^{31}P-NMR spectra of whole cell suspensions being significantly less than those from ^{31}P-NMR spectra of the final wash supernatant. This difference is surprising considering that in erythrocyte suspensions the supernatant and cells exist in separate compartments in the rotor. The supernatant resonance in a cell suspension should therefore give a pH value similar to that for a supernatant only sample. The source of this discrepancy is not clear, but the higher pH from the last wash supernatant is considered to be more reliable in this case, as the buffering by hemoglobin inside the cell should give an intracellular pH lower than that of the supernatant in an erythrocyte suspension at pH 8.5.

We conclude that the hydrogen-bonding ''split-peak'' effect that occurs with phosphoryl compounds in ^{31}P-NMR spectra of erythrocytes under MAS conditions also occurs with water in ^{1}H-NMR spectra. We show here that the

variation in the peak splitting with pH is due to the change in cell volume with pH.

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