Simultaneous Determination of Low Free Mg$^{2+}$ and pH in Human Sickle Cells using $^{31}$P NMR Spectroscopy

The concentrations of free magnesium, [Mg$^{2+}$]$_{\text{free}}$, and [ATP] are important in the dehydration of red blood cells from patients with sickle cell anemia, but they are not easily measured. Consequently, we have developed a rapid, noninvasive NMR spectroscopic method using the phosphorus chemical shifts of ATP and 2,3-diphosphoglycerate (DPG) to determine [Mg$^{2+}$]$_{\text{free}}$ and pH, simultaneously in fully oxygenated whole blood. The method employs theoretical equations expressing the observed chemical shift as a function of pH, K$^+$, and [Mg$^{2+}$]$_{\text{free}}$ over a pH range of 5.75–8.5 and [Mg$^{2+}$]$_{\text{free}}$ range 0–5 mM. The equations were adjusted to allow for the binding of hemoglobin to ATP and DPG, which required knowledge of the intracellular concentrations of ATP, DPG, K$^+$, and hemoglobin. Normal oxygenated whole blood ($n = 33$) had a pH of 7.20 ± 0.02, a [Mg$^{2+}$]$_{\text{free}}$ of 0.41 ± 0.03 mM, and [DPG] of 7.69 ± 0.47 mM. Under the same conditions, whole sickle blood ($n = 9$) had normal [ATP] but significantly lower pH, (7.10 ± 0.03) and [Mg$^{2+}$]$_{\text{free}}$ (0.32 ± 0.05 mM) than normal red cells, whereas [DPG] (10.8 ± 1.2 mM) was significantly higher. Because total magnesium was normal in sickle cells, the lower [Mg$^{2+}$]$_{\text{free}}$ could be attributed to increased [DPG] and therefore greater magnesium binding capacity of sickle cells.

Magnesium is the second most abundant intracellular cation after potassium and is important in the regulation of more than 300 enzymes (1–3) and ion transport across cell membranes (4, 5). Some diseases, such as hypertension, pre-eclampsia, and sickle cell anemia exhibit pathologies linked to low magnesium levels (6–8). The genetic defect in sickle cell anemia results in the synthesis of an abnormal $\beta$ hemoglobin (Hb) subunit, which polymerizes upon deoxygenation. This polymerization produces the characteristic sickle deformation in red blood cells and leads to microcirculatory occlusion and consequent tissue ischemia. Sickling depends strongly on the intracellular Hb concentration (9) and is enhanced by dehydration of erythrocytes, a process involving potassium and sodium transport across cell membranes, which is in turn thought to be regulated by cytosolic free magnesium, [Mg$^{2+}$]$_{\text{free}}^\ast$ via the KCl cotransporter (KCC1) (5, 10). Thus dehydration of sickle cells, and thereby the process of sickling, may be controlled by changes in the magnesium content (8, 11).

To assess its role in sickle cell anemia, an accurate and reproducible method was required for the measurement of [Mg$^{2+}$]$_{\text{free}}$. Although several methods have been developed to determine [Mg$^{2+}$]$_{\text{free}}$ in red blood cells, such as null point titration with metallochromic dyes (12), magnesium-sensitive electrodes (13, 14), use of divalent cation ionophores (15), and $^{31}$P NMR spectroscopy (16, 17), the reported values vary 3-fold, from 0.2 to 0.6 mM (18–20). Of these methods, the one noninvasive technique that has been used extensively is that of $^{31}$P NMR spectroscopy (16–19, 21–25). However, there are a number of potential errors associated with using the observed NMR signal to determine [Mg$^{2+}$]$_{\text{free}}$, including uncertainty in the binding constant between ATP and magnesium (26), the effect of intracellular ionic content on this interaction (27), and the effect of metabolite-Hb interactions (23). Gupta and co-workers (2), the first to use this method, addressed the first two problems by determining an apparent MgATP binding constant, $K_{\text{app}}$ or MgATP, from the difference between the end points of the chemical shifts of the magnesium bound and unbound $a$ and $\beta$ phosphorus nuclei of ATP, $\delta_{\text{app}}$ or MgATP and $\delta_{\text{app}}$-ATP. This decreased the number of equilibria that had to be considered and overcame the need to rely on adjusted published values of $K_{\text{app}}$, which varied 100-fold (28). However, as with any simplification, problems are associated with this approach, which include the possible difference in ionic strength between solutions and in vivo conditions, the accuracy of the chemical shift end points required to determine $K_{\text{app}}$ (29), and the assumption that $\delta_{\text{app}}$-ATP did not vary.

Because the chemical shifts of $^{31}$P NMR phosphorus peaks depend on many different factors (30), accurate analysis should take all factors into account, which has become possible with the increased capacity of computation. Previous analyses using phosphorus chemical shifts have been used to obtain either pH (24) or [Mg$^{2+}$]$_{\text{free}}$ (16) in red blood cells. Only one approach used the $^{31}$P resonances of ATP to determine both pH and [Mg$^{2+}$]$_{\text{free}}$ (31), but this averaged all ATP species with an apparent binding constant and made no allowance for the effect of Hb and K$^+$ binding upon the observed chemical shift. It is possible that $a$- and $\beta$-ATP, phosphate group at the $a$ position of ATP; DPG, 2,3-diphosphoglycerate; $\delta_{\text{app}}$-MgATP and $\delta_{\text{app}}$-ATP, chemical shift difference between $a$- and $\beta$-phosphorous nuclei of ATP when bound to magnesium and unbound, respectively; $\delta_{\text{obs}}$, observed chemical shift; $K_{\text{app}}$ or MgATP binding constant; $K_{\text{app}}$ or MgATP, apparent dissociation constant for MgATP; LW, half-peak line width; MgT, total concentration of magnesium; 2P- and 3P-DPG, phosphate group on the second and third carbon atom of DPG, respectively.

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metabolite relaxation times become so large upon binding to HB that such species do not contribute to the observed NMR peak, making them “NMR-invisible,” and hence they could be omitted from analysis. Therefore, the effect of HB on metabolite NMR “visibility” was investigated here. In addition, standard titration curves of ATP and DPG phosphorus chemical shifts were expressed in equations as functions of pH and \([\text{Mg}^{2+}]_{\text{free}}\). Adjusting these equations for HB and \(K^+\) binding to ATP and DPG improved the method for measuring pH and \([\text{Mg}^{2+}]_{\text{free}}\) in red blood cells. This newly developed NMR-based analysis allowed the simultaneous determination, for the first time, of the intracellular pH and \([\text{Mg}^{2+}]_{\text{free}}\) in normal and sickle blood. Preliminary reports of this work have been published in abstract form (32, 33).

### EXPERIMENTAL PROCEDURES

**Preparation of Standard Solutions for Titration Curves**—Solutions were prepared as described previously (34) and contained 5 mM each \(K_\text{ATP}\cdot2H_2O\), \(Na_\text{DPG}\cdot3.5H_2O\) (Sigma), and \(P_i\) (British Drug House, Poole), at 310.15 K and an ionic strength of 0.25. The pH of the solutions ranged from 5.75 to 8.50, and the total magnesium varied from 0 to 15.9 mM. Ionic strength was adjusted to 0.25 by adding KCl, and the pH was adjusted using 1 N KOH or HCl. In total, 130 samples were prepared, with 13 pH values, at 10 \([\text{Mg}^{2+}]_{\text{free}}\) concentrations. Using the relevant equilibrium constants (Table I) for each particular ion species, the amount of total MgCl\(_2\), \([\text{Mg}^{2+}]_{\text{free}}\), added to achieve a desired \([\text{Mg}^{2+}]_{\text{free}}\), was calculated using the Equation 1,

\[
[\text{Mg}^+] = [\text{Mg}^{2+}]_{\text{free}} \left[1 + \frac{[\text{Mg}^{2+}]_{\text{free}}}{[\text{Mg}^+]}ight] \quad \text{(Eq. 1)}
\]

where \([\text{Mg}^+]\) is a ratio of the ionized ligand, \(A^{2-}\), to the total ligand, \(A^3\),

\[
[\text{Mg}^+] = \frac{[\text{Mg}^{2+}]_{\text{free}}}{[\text{Mg}^{2+}]_{\text{free}} + [\text{Mg}^{2+}]} \quad \text{(Eq. 2)}
\]

For the calibration solutions containing the three magnesium binding ligands, ATP, DPG, and \(P_i\), the \([\text{Mg}^+]\) required for each solution was calculated from Equation 3.

\[
\begin{align*}
[\text{Mg}^+] &= \frac{[\text{Mg}^{2+}]_{\text{free}}}{1 + \frac{[\text{P}_i] \times [\text{Mg}^{2+}]_{\text{free}}}{[\text{Mg}^{2+}]_{\text{free}}} + \frac{[\text{ATP}] \times \left(\frac{[\text{K}_{\text{ATP}}]}{[\text{K}_{\text{ATP}}] - [\text{K}_{\text{ATP}}]} + [\text{H}^+]\right)}{[\text{ATP}]} + \frac{[\text{DPG}] \times \left(\frac{[\text{K}_{\text{DPG}}]}{[\text{K}_{\text{DPG}}]} + [\text{H}^+]\right)}{[\text{DPG}]} + \frac{[\text{H}_2\text{PO}_4^-] \times [\text{K}_{\text{HDPG}}]}{[\text{H}_2\text{PO}_4^-]}}
\end{align*}
\]

\[
[\text{Mg}^+] = \frac{[\text{Mg}^{2+}]_{\text{free}}}{1 + \frac{[\text{P}_i] \times [\text{Mg}^{2+}]_{\text{free}}}{[\text{Mg}^{2+}]_{\text{free}}} + \frac{[\text{ATP}] \times \left(\frac{[\text{K}_{\text{ATP}}]}{[\text{K}_{\text{ATP}}] - [\text{K}_{\text{ATP}}]} + [\text{H}^+]\right)}{[\text{ATP}]} + \frac{[\text{DPG}] \times \left(\frac{[\text{K}_{\text{DPG}}]}{[\text{K}_{\text{DPG}}]} + [\text{H}^+]\right)}{[\text{DPG}]} + \frac{[\text{H}_2\text{PO}_4^-] \times [\text{K}_{\text{HDPG}}]}{[\text{H}_2\text{PO}_4^-]}}}
\]  

The solution of Equation 3 and the simultaneous equations involved in providing the correct ionic strength and pH (see Appendix I) were achieved using Mathematica™ (Wolfram Research, Champaign, IL). Using this method, MgCl\(_2\) was added to give solutions containing \([\text{Mg}^{2+}]_{\text{free}}\) of 0.05, 0.10, 0.25, 0.50, 0.75, 1.00, 1.25, 2.50, and 5.00 mM. 31P NMR Spectroscopic Analysis of Standard Solutions—31P NMR spectra of the solutions were acquired using a 9.4-tesla, Oxford Instruments wide bore superconducting magnet interfaced with a Varian Nova spectrometer operating at a phosphorus frequency of 161.9 MHz. A 10-mm probe was used, with the sample temperature set to 310.15 K. The homogeneity of the magnetic field was optimized by shimming on the 1H free induction decay to give 1H spectral line widths of 9 ± 2 Hz. For each sample, a 90° pulse (18.5 µs) was used with an interpulse delay of 18 s, a spectral width of 8 KHz, and no proton decoupling. The delay time was based on preliminary experiments in which T1 values were determined for sample solutions by running a preinstalled macro on the Varian spectrometer, using a standard inversion recovery method. Each spectrum consisted of 128 summed transients. Up to 256 transients were acquired for samples in which the \(\beta\)-phosphorus of ATP was especially broad.

A capillary containing ~50 µl of 0.5 M phenylphosphonic acid was used as an external chemical shift reference, standardized relative to phosphocreatine at 0.00 ppm. Prior to Fourier transformation, the signal/noise ratio was increased by multiplying the 31P NMR free induction decays by an exponential function sufficient to generate a line broadening of 1 Hz, and the time domain signals were zero filled once. The NMR1 program (Tripos, St. Louis, MO) was used to fit peak areas, line widths, and chemical shifts of spectral resonances.

### Theoretical Fitting of the Observed Chemical Shifts—The chemical shifts for the 3-phosphorus of DPG and \(\beta\)-phosphorus of ATP peaks were plotted against pH and \([\text{Mg}^{2+}]_{\text{free}}\) generating a three-dimensional surface. This was then fitted to an equation using the nonlinear fit algorithm in Mathematica™ with the main parameters being the chemical shifts of the \(H^+, K^+\), and \([\text{Mg}^{2+}]_{\text{free}}\) bound forms of \(\beta\)-ATP and 3P-DPG, and the binding constants of each species to \(H^+, K^+\), and \([\text{Mg}^{2+}]_{\text{free}}\).

The equation used for fitting was based on the principle that the observed chemical shift (\(\delta_{\text{obs}}\)) could be described by combining the chemical shifts of all of the relevant forms of a substance present in the equilibrium and weighting their contribution to the observed chemical shift according to the ratio of their population to that of the total substance present (35).

\[
\delta_{\text{obs}} = \frac{[X_{\text{species}1}]_{\text{Total}}}{[X_{\text{species}2}]_{\text{Total}}} \times \delta_{\text{species}1} + \frac{[X_{\text{species}2}]_{\text{Total}}}{[X_{\text{species}2}]_{\text{Total}}} \times \delta_{\text{species}2} + \ldots \quad \text{(Eq. 4)}
\]

Hence for ATP, assuming all ATP to consist of several species,

\[
\begin{align*}
[ATP^+] &= [ATP^+] + [HATP^+] + [H_2ATP^+] + [MgATP^+] \\
&+ [MgHATP^+] + [KATP^+] + [KHATP^+]
\end{align*}
\]

the theoretical chemical shift can be described by Equation 6.

\[
\begin{align*}
[ATP^+] \times \delta_{\text{ATP}} + [HATP^+] \times \delta_{\text{HATP}} + [H_2ATP^+] \times \delta_{\text{H_2ATP}} + [MgATP^+] \times \delta_{\text{MgATP}} + [MgHATP^+] \times \delta_{\text{MgHATP}} + [KATP^+] \times \delta_{\text{KATP}} + [KHATP^+] \times \delta_{\text{KHATP}}
\end{align*}
\]

Using the equilibrium constants (assuming activity coefficients of unity), the concentrations of all forms can be expressed relative to the concentration of the completely ionized form (f values). Taking these and substituting in the above equation, the observed chemical shift is represented by Equation 7.

\[
\begin{align*}
\delta_{\text{obs}} &= \frac{[X_{\text{species}1}]_{\text{Total}}}{[X_{\text{species}2}]_{\text{Total}}} \times \delta_{\text{species}1} + \frac{[X_{\text{species}2}]_{\text{Total}}}{[X_{\text{species}2}]_{\text{Total}}} \times \delta_{\text{species}2} + \ldots
\end{align*}
\]

Similarly for DPG, the following equation describing the observed chemical shift for DPG was formulated, assuming the forms present were \(\text{DPG}^+, \text{HDPG}^+, \text{H}_2\text{DPG}^+, \text{MgDPG}^+, \text{MgHDPG}^+, \text{KDPG}^+, \) and \(\text{KHDPG}^+\).
with $F_i$, the value of the ith data point, and $f_i$ is the value obtained from the fit.

Model of the Red Blood Cell—The chemical shifts of the intermediate species were calculated from the above fitting equations and were used in new multiple equilibria equations, which included the binding of Hb to ATP$^+$, MgATP$^-$, and DPG$^-$. To achieve this, it was assumed that the chemical shifts of each species were not affected by binding to Hb (2, 14). Binding constants for Hb were taken from Berger et al. (36), where $K_{HbATP}$ is 360 M$^{-1}$, $K_{HbMgATP}$ is 39 M$^{-1}$, and $K_{HbDPG}$ is 250 M$^{-1}$.

Therefore,

$$\delta_{\text{MgATP}} = \left( \left[ \frac{[\text{MgATP}]+[\text{HbMgATP}]}{[\text{ATP}]_\text{tot}} \right] \times \delta_{\text{MgATP}} \right) + \left( \left[ \frac{[\text{ATP}_\text{tot}]+[\text{HbATP}]}{[\text{ATP}]_\text{tot}} \right] \times \delta_{\text{ATP}} \right) + \left( \left[ \frac{[\text{HATP}]}{[\text{ATP}]_\text{tot}} \right] \times \delta_{\text{HATP}} \right) + \left( \left[ \frac{[\text{MgHATP}]}{[\text{ATP}]_\text{tot}} \right] \times \delta_{\text{MgHATP}} \right)$$

(Eq. 10)

and

$$\delta_{\text{DPG}} = \left( \left[ \frac{[\text{MgDPG}]}{[\text{DPG}]_\text{tot}} \right] \times \delta_{\text{MgDPG}} \right) + \left( \left[ \frac{[\text{DPG}_\text{tot}]+[\text{HbDPG}]}{[\text{DPG}]_\text{tot}} \right] \times \delta_{\text{DPG}} \right) + \left( \left[ \frac{[\text{HDPG}]}{[\text{DPG}]_\text{tot}} \right] \times \delta_{\text{HDPG}} \right) + \left( \left[ \frac{[\text{MgHDPG}]}{[\text{DPG}]_\text{tot}} \right] \times \delta_{\text{MgHDPG}} \right)$$

(Eq. 11)

The above equations are simplified from the Mathematica® program presented in Appendix II. After determination of chemical shifts of the $\beta$-phosphate peak of ATP ($\delta_p$) and the phosphate on the 3 carbon of DPG ($\delta_{3p}$) from acquired spectra of red blood cells, these equations were solved simultaneously to calculate pH and [Mg$^{2+}$]$_{free}$ in red blood cells using the Solve algorithm in Mathematica (Appendix II). A requirement of this method is that the total amounts of ATP, DPG, Hb, and K$^+$ must be entered in the algorithm before a solution can be found. ATP and DPG concentrations were assayed as described below, and Hb and K$^+$ concentrations (mM/liter cell water) were assumed to be 7 mM (2, 37) and 160 mM (38), respectively. Erythrocyte total magnesium was calculated by summing the concentrations of all magnesium-containing species, determined from the solution of this algorithm, for comparison with the total magnesium determined using a standard colorimetric assay (see later).

Preparation of Whole Human Blood Samples—This study was approved by the Ethics Committee, Medical School, University of Birmingham. Blood from volunteers (8–10 ml) or from consenting homozygous sickle cell disease (HbSS) patients (5 ml) was collected by venipuncture into lithium-heparanized syringes. To simulate in vivo fully oxygenated blood, whole blood samples were prepared for analysis by equilibration with a mixture of O$_2$ and CO$_2$ (95%/5%), adjusted to give a final P$_{O2}$ of 40 mmHg and P$_{CO2}$ between 200 and 400 mmHg, determined using a blood gas analyzer (Radiometer ABL 330). The P$_{O2}$, P$_{CO2}$, and pH$_{tot}$ were measured before and after NMR analysis, at which time the P$_{O2}$ remained > 150 mm Hg, to ensure complete oxygenation at all times during analysis. To minimize cell degradation, time from phlebotomy to analysis was kept to a minimum (1–3 h), during which blood samples were stored on ice.

Separation of Sickle Cells by Density—Sickle cells are heterogeneous with respect to total magnesium, DPG content, and pH (39, 40), which vary with cell density as the cell sickles, therefore we separated sickle cells into three fractions using a discontinuous arabinogalactose density gradient before analysis, thereby allowing comparison with unfractio-

layered on the Stractan gradient. After ultracentrifugation, three layers were harvested, light (d < 1.090 g/ml), medium (1.090 g/ml < d < 1.099 g/ml), and dense (d > 1.099 g/ml), with the dense layer containing most of the irreversibly sickled cells. The density-fractionated red cells were washed three times at 4 °C, using the above buffer, before being resuspended in pooled, heparinized plasma from the same two patients, to give final hematocrits of 35–50%. Normal ATP concentrations (see later) and microscopic observation at high magnification showed that no agglutination occurred in the pooled, unmatched blood samples, possibly because of the high heparin concentrations (43). The “whole blood” samples were prepared for NMR analyses as described above. The fully oxygenated whole blood (~ 4.5 ml), prepared as above, was sealed in a 10-mm NMR sample tube together with an external reference capillary containing 50 µl of 0.3 m phenylphosphonic acid and brought to 37 °C before 31P NMR spectroscopic analysis in a 10-mm probe. The probe temperature was set to 310.15, the samples were not spun, and no proton decoupling was used. The homogeneity of the magnetic field was optimized by shimming on the $^1$H free induction decay to a line width of 30 ± 2 Hz (normal cells) or 23 ± 2 Hz (sickle cells). A 45° pulse was used with an interpulse delay of 0.52 s to give peaks of a high signal/noise ratio (25). The spectral width was 8 kHz, with an acquisition time of 0.272 s (4,500 data points), and 2048 transients were summed. The samples were at 37 °C for a maximum of 38 min. Prior to Fourier transformation, the signal/noise ratio was increased by multiplying the 31P NMR free induction decays by an exponential function sufficient to generate a line broadening of 7 Hz. The time domain signals were zero filled once, giving a digital resolution of

$^a$V. L. Lew, personal communication.
1.1 Hz/point. The NMR1 program was used to fit peak areas, line widths, and chemical shifts of spectral resonances.

**Chemical Assays**—Immediately after the NMR measurement, the blood was poured from the NMR tube and samples taken for light microscopy to count the number of sickled cells, and for determination of hematocrit and ATP and DPG concentrations using diagnostic kits (Sigma). Total magnesium concentrations in plasma and trichloroacetic acid extracts of whole blood were determined in duplicate using diagnostic kits (Sigma). The total erythrocyte magnesium concentrations were then calculated by subtracting the plasma total magnesium from the whole blood measurement. Concentrations are presented per liter of cell water, assuming a 95% packing efficiency and 70% cellular water content for normal cells, 66% for whole sickle blood, and 65 and 62% for medium and dense sickle cells, respectively (37). The hematocrits were determined after centrifugation at 13,000 × g for 10 min in a microhematocrit centrifuge using a Hawksley microhematocrit reader, with all measurements made in triplicate.

**Effect of Hb on ATP and DPG NMR Visibility**—To alleviate concerns that metabolites bound to Hb would be NMR-invisible, solutions were prepared containing 2 mM K$_2$ATP:2H$_2$O, 7 mM Na$_5$DPG:3.5H$_2$O (Sigma), 190 mM KCl, 3 mM MgCl$_2$:6H$_2$O with and without 6 mM human Hb (Sigma), and the pH was adjusted to 7.2 at 37 °C using 1 M KOH or HCl. Typical $^{31}$P NMR spectra of the solutions acquired as described above. Immediately after the NMR measurement, ATP and DPG concentrations were determined as described above.

**Statistics**—Data are presented as means ± S.D. Significance was tested using analysis of variance, with repeated measures where appropriate, with $p < 0.05$ considered significant.

**RESULTS**

Typical $^{31}$P NMR spectra of the titration solutions acquired between a pH 6 of 8 and [Mg$^{2+}$]$_{\text{free}}$ between 0 and 5 mM are shown in Fig. 1. The ATP, DPG, and P$_i$ peaks increased in chemical shift as pH increased, reflecting a decrease in electronic shielding upon proton association. The maximal chemical shift changes are given in Table II. Increasing [Mg$^{2+}$]$_{\text{free}}$ altered the δ of all ATP peaks (β (3.26 ppm) and γ (3.72 ppm) more than α (0.46 ppm)) but led to only slight changes in those of P$_i$ (0.03 ppm) and 3P- and 2P-DPG (0.34 and 0.76 ppm, respectively). The chemical shifts of the 2P-DPG and P$_i$ peaks were more sensitive to changes in pH than to changes in magnesium (3.15 and 2.35 ppm, respectively), whereas those of the β- and γ-phosphate peaks of ATP were equally sensitive to both pH and [Mg$^{2+}$]$_{\text{free}}$. At the physiological pH of 7.2 and [Mg$^{2+}$]$_{\text{free}}$ of 0.3 mM, the β-ATP triplet could not be fully resolved because of line broadening caused by chemical exchange, but the peak could still be used for chemical shift determination. The P$_i$ peak overlapped with the 2P-DPG doublet, thus neither could be used for pH or [Mg$^{2+}$]$_{\text{free}}$ determination in red blood cells. Instead, it was necessary to use the 3P-DPG peak.

**Three-dimensional Curves from the Chemical Shifts of β-Phosphate of ATP and 3-Phosphate of DPG**—Using the ob-
served β-ATP peak chemical shifts ($\delta_{\beta}$), a three-dimensional surface curve was generated as a plot of pH and [Mg$^{2+}$]$\text{free}$ with the section relevant to physiological conditions expanded (Fig. 2a). Equation 7 was fitted to these data and plotted in Fig. 2b. Using the observed 3P-DPG peak chemical shifts ($\delta_{3P}$), a three-dimensional surface curve was generated as a plot of pH and [Mg$^{2+}$]$\text{free}$ (Fig. 3a). Equation 8 was fitted to these data and plotted in Fig. 3b. The final values of the fitting parameters of chemical shifts and explicit equilibrium constants are given in Table III.

Effect of Hb on ATP and DPG NMR Visibility—The line widths of DPG and ATP peaks each increased by 60% on addition of Hb (α-LW from 28 to 45 Hz, and β-LW from 50 to 80 Hz). Despite this, when the normalized peak integral was divided by the assayed total metabolite concentration, the values changed by less than 3% on addition of Hb. This indicated that ATP and DPG NMR visibility was not significantly impaired by binding to Hb, in that the Hb-bound component of the observed chemical shift was not so broad as to be lost in the noise of the spectrum.

Fig. 4 shows a typical $^{31}$P NMR spectrum of normal red blood cells. The DPG peaks were relatively sharp (LW $\approx$ 20 Hz), whereas the line width of the β phosphate of the ATP peak was significantly greater than the line widths of the other ATP peaks (β-LW $\approx$ 65 Hz, α-LW $\approx$ 35 Hz). Using the 3P-DPG and β-ATP phosphate peak chemical shifts, the intracellular pH and [Mg$^{2+}$]$\text{free}$ were determined simultaneously using the values of ATP and DPG determined by enzymatic analysis.

Analysis of Normal and Sickle Red Blood Cells—Fractionation of the 8–10 ml of packed sickle cells gave fraction volumes in the approximate ratio 3.5:2 for light:medium:dense fractions. When resuspended in their plasma, the hematocrits were in the range of 35–50%. Morphological microscopic examination, as estimated by criteria described previously (42), revealed that the dense fraction contained between 50 and 70% irreversibly sickled cells, the medium contained $\approx$5%, and the light had fewer than 1%.

The determined levels of DPG, ATP, pH$\text{i}$, [Mg$^{2+}$]$\text{free}$, and Mg$\text{T}$, both predicted using the model and assayed, are presented in Table IV for normal and sickle whole blood and for each of the separated sickle cell fractions, light, medium, and dense. DPG was elevated significantly in all three sickle cell fractions, by a maximum of 2.4 mm in the medium density fraction, compared with normal blood cell DPG of 7.7 mm. However, the DPG concentration of the dense cells was significantly lower than that of the other sickle cells, but not as low as the normal cells. ATP was normal in all of the sickle cell samples. The PCO$\text{2}$ of whole blood and cell fractions was set to the same for all the analyses, which gave normal blood a measured pH$\text{ex}$ of 7.39 $\pm$ 0.03. However, at a PCO$\text{2}$ of 40 mm Hg, the pH$\text{ex}$ of whole sickle blood was 0.07 pH unit more acidic than normal blood. After fractionation, the pH$\text{ex}$ of sickle cells was 0.23 pH unit more acidic than whole control blood. The PO2 never dropped below 150 mm Hg and usually was around 300 mm Hg. In parallel with their more acidic pH$\text{ex}$, the pH$\text{i}$ of all sickle cell samples was 0.10–0.13 pH unit more acidic than normal cells. [Mg$^{2+}$]$\text{free}$ was decreased significantly by $\approx$0.09 m in the medium and the dense cells, and in the whole sickle blood compared with normal cells. There was no change in Mg$\text{T}$, either predicted from the model or measured by assay.

| Phosphate group | pH 5.75–8.5 | [Mg$^{2+}$]$\text{free}$ 0–5 mM | ATP 0.05 mM | 5.75 pH | to 8.5 pH |
|----------------|-------------|-------------------------------|------------|---------|---------|
| α-ATP          | 0.37        | 0.46                          | 0.59       |         |         |
| β-ATP          | 2.54        | 3.26                          | 3.82       |         |         |
| γ-ATP          | 4.16        | 3.72                          | 4.66       |         |         |
| 3P-DPG         | 2.79        | 0.34                          | 2.79       |         |         |
| 2P-DPG         | 3.15        | 0.76                          | 3.15       |         |         |
| P$_i$          | 2.35        | 0.03                          | 2.35       |         |         |

**TABLE II**

Maximum chemical shift changes of all phosphate groups at different pH and/or [Mg$^{2+}$]$\text{free}$

**DISCUSSION**

Less than 0.5% of body magnesium resides in blood plasma, and yet plasma total magnesium is commonly used as a marker of body magnesium status because of the absence of an easy reproducible method for measuring [Mg$^{2+}$]$\text{free}$. Because intracellular [Mg$^{2+}$]$\text{free}$ is important in regulating biochemical reactions, it may serve as a better marker of status, and, of all tissues, red blood cells are the most readily available for study in humans. The best method by which to measure [Mg$^{2+}$]$\text{free}$ is frequently discussed, but the effect of pH on [Mg$^{2+}$]$\text{free}$ measurements is often not taken fully into account (27). Consequently, we have developed a method to quantify both [Mg$^{2+}$]$\text{free}$ and pH in red blood cells under a wide range of conditions.

31P NMR studies (44) on the effects of Mg$^{2+}$ on ATP have shown that Mg$^{2+}$ induces the greatest chemical shift on the β-phosphorus and the least on the α-phosphorus resonance of ATP. This has been interpreted as indicating that Mg$^{2+}$ binds mainly to the β- and γ-phosphate groups (45, 46), possibly via a cyclic six-membered transition state that is energetically favored (Fig. 5) and has little interaction with the α residue. Thus, previous studies have used the changing difference of the chemical shift of the β from the α resonance of ATP, $\delta_{3P}$, to determine intracellular [Mg$^{2+}$]$\text{free}$ in many tissue types (16, 47, 48) and thereby use the α-ATP peak as an internal chemical shift standard. However, it was seen (Fig. 1) that the α-phosphate chemical shift was not only variable over the ranges of pH and magnesium studied, but also α-phosphorus and β-phosphorus changed as pH and magnesium were varied. Therefore, in this study, all chemical shifts were measured relative to an external capillary standard.

**Use of Explicit Equilibrium Constants**—One important new aspect of this study was the use of explicit equilibrium constants as opposed to apparent constants, which allows the ready adaptation of the technique to a much wider range of conditions, either in red blood cells or other tissues. However, it
also necessitates that a greater number of constants be accurate. This necessity was overcome in the original method of Gupta et al. (16) by measuring an apparent dissociation constant for MgATP, $K_{app/MgATP}$, under in vitro conditions thought appropriate to the in vivo situation. However, this had three major problems. First, the two conditions would not have the same ionic strength, despite possessing same concentration of $K^+$. This is because of the presence of other charged species, such as DPG, in the in vivo environment, and therefore the measured $K_{app/MgATP}$ would also differ between in vitro and in vivo conditions. For example, the ionic strengths would have changed from 0.16 to 0.19 M as ATP and magnesium concentrations were varied in the in vitro solutions. The addition of 7 mM DPG would have increased the ionic strength to 0.24 M in vivo. Assuming that $K_{app/MgATP}$ was measured at 0.17 M ionic strength to be 38 mM, it would correspond to 60 mM at this in vivo ionic strength (adjusted using standard thermodynamic equations (3)). Because the calculated $[Mg^{2+}]_{free}$ is directly proportional to $K_{app/MgATP}$, this would have the effect of also increasing $[Mg^{2+}]_{free}$ by a factor of 1.5. Second, an accurate assessment of $K_{app/MgATP}$ from 31P NMR requires an accurate knowledge of the chemical shift end points $δ_{ATP}$ and $δ_{MgATP}$. Although $δ_{ATP}$ may be relatively straightforward to determine, $δ_{MgATP}$ is more difficult because of the presence of the MgATP species, which would affect the overall observed chemical shift.

\[ \begin{array}{l}
\text{Table III} \\
\text{Parameters used to produce the best fit of the theoretical chemical shift to that of the observed, including interactions with potassium} \\
\text{Equilibrium constants are explicit binding constants at 37 °C and } I = 0.25. \text{ Chemical shifts are presented as ppm relative to phosphocreatine at 0 ppm. Errors are presented as standard errors.} \\
\hline
\text{Parameter} & \beta\text{-ATP} & 3P\text{-DPG} \\
\hline
K_{AX} & 2.00 ± 0.06 \times 10^{-7} \text{ M} & 7.80 ± 0.07 \times 10^{-5} \text{ M} \\
K_{ATP} & 1.91 \times 10^{-4} \text{ M} & 4.46 \pm 0.05 \times 10^{-3} \text{ M}^{-1} \\
K_{MgATP} & 7.50 \times 0.2 \times 10^4 \text{ M}^{-1} & 2.40 \pm 0.06 \times 10^{-3} \text{ M}^{-1} \\
K_{MgATP} & 5.50 \pm 0.64 \times 10^3 \text{ M}^{-1} & 3.78 \pm 0.01 \times 10^{-4} \text{ M}^{-1} \\
K_{MgATP} & 1.20 \pm 0.25 \times 10^{-4} \text{ M}^{-1} & 1.30 \pm 0.02 \times 10^{-4} \text{ M}^{-1} \\
K_{MgATP} & 4.00 \pm 3.18 \times 10^{-4} \text{ M}^{-1} & 1.08 \pm 0.02 \times 10^{-4} \text{ M}^{-1} \\
\delta_{ATP} & 19.10 ± 0.15 & 6.90 ± 0.023 \\
\delta_{ATP} & 20.65 ± 0.13 & 5.69 ± 0.024 \\
\delta_{ATP} & 21.50 \pm 0.02 & 3.13 ± 0.015 \\
\delta_{ATP} & 15.69 \pm 0.02 & 6.35 ± 0.009 \\
\delta_{ATP} & 17.40 ± 0.11 & 3.79 ± 0.05 \\
\delta_{ATT} & 18.36 ± 0.07 & 6.74 ± 0.01 \\
\delta_{ATT} & 19.60 ± 0.02 & 4.74 ± 0.011 \\
\end{array} \]

*Errors not calculable for region studied.*
...effect on $K_b$ would have to be determined experimentally. Under a range of conditions, including a pH change, a new apparent binding constant, including the effect of oxygenated erythrocytes (2), is determined to be 7.23 ± 0.01 and 0.41 ± 0.04 mM, respectively, using Berger's constants (36), but 7.25 ± 0.01 and 0.21 ± 0.03 mM using those of Gupta et al. (2). The analysis presented under "Results" used Berger's constants because errors may be associated with the methodology (23) used by Gupta (16).

Effect of $K^+$ and $Hb$—For a complete description of the erythrocytes, the effects of both $K^+$ and $Hb$ should be taken into account. However, once $K^+$ is specified in the magnesium-ATP equilibrium, it also needs to be specified in the ATP-Hb equilibrium. Both Berger (36) and Gupta (2) measured apparent $Hb$ binding constants using a physiological $[K^+]_r$. This is independent of every other, and therefore the addition of $Hb$ cannot, per se, alter the magnesium binding ATP equilibrium, the observed chemical shift is not independent because it intrinsically reflects a weighted average of all species present. Therefore the effect of $Hb$ binding on the observed chemical shifts of both DPG and ATP must be taken into account. This can be achieved, to a first approximation, by assuming that chemical shifts did not change when metabolite species bound to $Hb$. This is true within a 5% error in oxygenated erythrocytes (2, 23). Simultaneous equations were then formulated which included the effect that $Hb$ binding had on the equilibria for ATP and DPG and hence on the overall observed chemical shifts (Equations 10 and 11 and Appendix II). These equations also required the binding constants for $Hb$ binding to DPG, ATP, and MgATP. There are two generally accepted sets of constants (2), which largely agree except on the value of MgATP binding to $Hb$. This affected the positions of the equilibria to a different extent as illustrated in Fig. 6, a and b).

Effect of $Hb$ on Metabolite NMR Visibility—If the metabolite relaxation times were sufficiently long when bound to $Hb$, the $Hb$-bound component of the overall observed chemical shift would be broad enough to be lost in spectral noise. Thus, $Hb$-bound metabolites would be NMR-invisible and could be omitted from the analysis. However, we found no less in metabolite visibility on binding to $Hb$, so the effect of oxygenated $Hb$ binding must be included in the analysis of $[Mg^{2+}]_{free}$. Furthermore, when the concentration of ATP in red blood cells was estimated from the relative peak integrals of ATP and DPG in the observed spectra using the assayed concentration of DPG, there was less than a 10% variation between this value and that of the spectrophotometrically measured ATP concentration. This confirms the finding that DPG and ATP remain NMR-visible when bound to $Hb$.

Effect of $Hb$ on Metabolite NMR Visibility—In the original $[^3P]NMR$ method, the effect that metabolite-$Hb$ interactions had on the equilibria was considered (16). However, by 1983 (48), this complexity in the method was lost, perhaps because, according to their equilibrium constants, $Hb$ bound MgATP and ATP equally strongly, which would allow the $Hb$ effect to be ignored (23). Also, this added complexity, combined with the lack of computational power at the time, perhaps made solving the necessary multiple equilibria too difficult to be achieved routinely. A disregard of the effect of $Hb$ has existed ever since, until 1997 when the effect of metabolite-$Hb$ interactions was reevaluated (23).

The standard titration solutions did not include $Hb$ because the extent of DPG and ATP binding to $Hb$ was uncertain, and its effect would complicate the analysis of the important interactions among $H^+$, Mg$^{2+}$, $K^+$, ATP$^{4-}$, and DPG$^{5-}$. However, after the observed experimental chemical shifts were modeled by equations describing the effect of all intermediates as functions of $H^+$ and Mg$^{2+}$, these equations were altered to allow for $Hb$ binding (Equations 10 and 11). Although each equilibrium is independent of every other, and therefore the addition of $Hb$ cannot, per se, alter the magnesium binding ATP equilibrium, the observed chemical shift is not independent because it intrinsically reflects a weighted average of all species present. Therefore the effect of $Hb$ binding on the observed chemical shifts of both DPG and ATP must be taken into account. This can be achieved, to a first approximation, by assuming that chemical shifts did not change when metabolite species bound to $Hb$. This is true within a 5% error in oxygenated erythrocytes (2, 23). Simultaneous equations were then formulated which included the effect that $Hb$ binding had on the equilibria for ATP and DPG and hence on the overall observed chemical shifts (Equations 10 and 11 and Appendix II). These equations also required the binding constants for $Hb$ binding to DPG, ATP, and MgATP. There are two generally accepted sets of constants (2), which largely agree except on the value of MgATP binding to $Hb$. This affected the positions of the equilibria to a different extent as illustrated in Fig. 6, a and b).
49918

**pH and [Mg\(^{2+}\)]\(_{\text{free}}\) in Sickle Cells**

**TABLE IV**

| Assumed water content | Whole normal (n = 33) | Fractionated sickle cells (n = 3) | Whole sickle (n = 9) |
|-----------------------|-----------------------|----------------------------------|---------------------|
|                       | Light (<1.099 g/ml)   | Medium (1.099 - 1.099 g/ml)     | Dense (>1.099 g/ml) |
| [DPG]                 | 70%                   | 65%                              | 66%                 |
| [ATP]                 | 7.7 ± 0.5             | 10.0 ± 0.9\(^a\)                | 9.7 ± 0.2\(^a\)     |
| pH\(_i\)              | 7.32 ± 0.13           | 7.12 ± 0.02                      | 7.10 ± 0.03\(^a\)   |
| [Mg\(^{2+}\)]\(_{\text{free}}\) | 0.41 ± 0.03          | 0.37 ± 0.03                      | 0.33 ± 0.02\(^a\)   |
| Model Mg\(_{T}\)      | 3.10 ± 0.31           | 3.30 ± 0.07                      | 3.29 ± 0.20          |
| Assay Mg\(_{T}\)      | 3.50 ± 0.39           | 3.69 ± 0.17                      | 3.31 ± 0.29          |

\(^a\) p < 0.05 vs. whole normal blood.

\(^a\) ND, not determined.

Following this procedure, using end points determined here and assuming \(K_{\text{app}} = 38 \mu M\) (51), \([\text{Mg}^{2+}]_{\text{free}}\) was calculated to be 0.24 ± 0.02 and 0.16 ± 0.02 mM for normal and sickle cells, respectively, significantly different from the values of 0.41 ± 0.03 and 0.32 ± 0.05 mM found in this work (Table V). Also, a comparison was made with values calculated using the original technique (16), where \(pH_i\) is, again, assumed to be 7.2, but Hb interactions are included. This also leads to significantly different values.

**Use of Explicit Constants for the Generation of in Vitro Solutions**—Solutions are often required in experimental practice to mimic the conditions found in vivo. These frequently require an accurate \([\text{Mg}^{2+}]_{\text{free}}\) in ATP-containing solutions, but all too often this is calculated using equilibrium constants that are inappropriate for the conditions used. This is partly because of constants being quoted as apparent ones, thus making it difficult to adjust them over a range of conditions. However, in this work, explicit constants have been determined which readily allow the generation of a specific \([\text{Mg}^{2+}]_{\text{free}}\) in an ATP-containing solution over any range of pH, potassium, ATP, DPG, Hb, temperature, and ionic strength. Furthermore, this technique can be used in other cell types if a replacement peak chemical shift is used instead of DPG, ideally Fe^3+.

**Normal versus Sickle Red Blood Cells**—We went on to compare normal red cells with those from sickle patients. We found that well oxygenated sickle cells had significantly lower \([\text{Mg}^{2+}]_{\text{free}}\) and \(pH_i\) than normal red cells but unchanged [ATP]. Our results agree well with the seminal study by Ortiz and co-workers (40), conducted using the null point (A23187) method. Thus, values for [DPG], \([\text{Mg}^{2+}]_{\text{free}}\), and Mg\(_T\) in normal red cells were very similar in the two studies. In both studies, oxygenated sickle cells had high [DPG], low \([\text{Mg}^{2+}]_{\text{free}}\), and unchanged Mg\(_T\). A reduction in \([\text{Mg}^{2+}]_{\text{free}}\) in sickle cells, compared with normal cells, could result from an increased buffering capacity for magnesium because of high [DPG], concomitant with unchanged [ATP]. We also found similar results in our oxygenated dense (density > 1.099 g/ml) sickle cell fraction. By contrast, in their dense fraction (density > 1.118 g/ml), Mg\(^{2+}\) to Hb, which, it has been suggested, can account for the remaining 10% (49). Because Mg\(^{2+}\) does not compete with ATP and DPG in binding Hb, this factor did not significantly change the analysis for \([\text{Mg}^{2+}]_{\text{free}}\).

**Comparisons with Previous Methods**—In the most common analysis of NMR spectra to yield \([\text{Mg}^{2+}]_{\text{free}}\) (16, 48, 50), \(\Delta_{\text{ATP}}\) and \(\delta_{\text{MgATP}}\) end points and an apparent dissociation constant are determined from in vitro solutions. It is then assumed that \(pH_i\) is 7.2.

\([\text{Mg}^{2+}]_{\text{free}} = K_{\text{app}} \times (\delta_{\text{ATP}} - \delta_{\text{Hb}})/(\delta_{\text{ATP}} - \delta_{\text{MgATP}})\) (Eq. 10)

Fig. 5. Possible structure of MgATP\(^{2-}\), with Mg\(^{2+}\) bound in an energetically favored six-member cyclic transition state.

Fig. 6. Effects of allowing Hb binding to ATP, MgATP, and DPG on the theoretical chemical shift \(\beta\)-ATP (a) and 3P-DPG (b), with no Hb (line), with 2 mM ATP, 5 mM DPG, and 7 mM Hb and using equilibrium constants from either Gupta et al. (2) (longer dashes) or Berger et al. (36) (shorter dashes).

partly verified by summing all MgX species to find the model prediction for erythrocyte total magnesium. This was calculated to be 3.1 mM, which is ~90% of that found experimentally. However, the model does not include the low affinity binding of Mg\(^{2+}\) to Hb, which, it has been suggested, can account for the remaining 10% (49). Because Mg\(^{2+}\) does not compete with ATP and DPG in binding Hb, this factor did not significantly change the analysis for \([\text{Mg}^{2+}]_{\text{free}}\).
Ortiz et al. (40) reported an increased [Mg\(^{2+}\)]\(_{\text{i,free}}\) and decreased Mg\(_{\text{p}}\). This elevation in [Mg\(^{2+}\)]\(_{\text{i,free}}\) was markedly exacerbated on deoxygenation, reversing the usual inward magnesium concentration gradient and providing a mechanism for the magnesium depletion that they observed in this fraction. The difference in oxygenated dense cells between the two studies could be attributed to the difference in the densities chosen for cell fractionation and/or a difference in magnesium buffering. Although the blood samples were gassed with 95% O\(_2\) to produce a PO\(_2\) > 150 mm Hg, it is possible that the dense sickle cells were not fully oxygenated, causing sickle Hb (Hb S) to polymerize (52). To our knowledge, there are no constants available for binding of metabolites to Hb polymer, but polymerization would alter [Mg\(^{2+}\)]\(_{\text{i,free}}\) estimated using our method and may explain the low [Mg\(^{2+}\)]\(_{\text{i,free}}\) in the dense cell fraction.

We found that the pH\(_{\text{i}}\) of whole sickle blood was 0.1 pH unit more acidic than that of normal red cells, with a pH\(_{\text{i}}\) of 7.10 in agreement with previous NMR studies of buffer-washed red cells (24). However, at the same PO\(_2\), sickle blood pH\(_{\text{ex}}\) was also significantly more acidic, by 0.07 pH unit, than normal. Low [Mg\(^{2+}\)]\(_{\text{i,free}}\) and low pH will both activate KCC1 (53), which may partially explain the high activity of this transporter in sickle cells. Cell shrinkage, perhaps via overactivity of KCC1, remains the principal hazard in irreversible sickling. For a patient with sickle cell anemia in a steady state, the percentage of red blood cells that are irreversibly sickled is about 15%. With a hematocrit of 20%, this corresponds to 3% of total blood volume. Therefore, although it is important to fractionate cells and analyze the dense cell fraction to understand the mechanisms underlying cell sickling, it is clinically important to analyze whole sickle blood, using considerably smaller volumes, to assess the in vitro state of the majority of red blood cells in an effort to prevent sickling.

In summary, we have modeled the observed chemical shift of \(\beta\)-ATP and 3P-DPG over a wide range of pH and [Mg\(^{2+}\)]\(_{\text{i,free}}\) using appropriate theoretical equations. These equations included contributions to the overall chemical shift from all possible intermediates present in solution and represented the multiple equilibria formed by the interactions among H\(^+\), Mg\(^{2+}\), K\(^+\), ATP\(^{4-}\), and DPG\(^{5-}\). An adjustment for Hb binding was made to the equations to allow a new, more complete, determination of intracellular pH and [Mg\(^{2+}\)]\(_{\text{i,free}}\) using \(^{31}P\) NMR spectroscopic analysis of red blood cells. Thus, pH\(_{\text{i}}\) and [Mg\(^{2+}\)]\(_{\text{i,free}}\) were determined in whole, oxygenated normal and sickle blood, with pH\(_{\text{ex}}\), Mg\(_{\text{p}}\), ATP, and DPG concentrations. The pH\(_{\text{i}}\), pH\(_{\text{ex}}\), and [Mg\(^{2+}\)]\(_{\text{i,free}}\) were significantly lower in sickle cells, whereas [DPG] was significantly higher, with no changes in [ATP].
pH and $[\text{Mg}^{2+}]_{\text{free}}$ in Sickle Cells

49920

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