A Phosphorus-31 Nuclear Magnetic Resonance Investigation of Intracellular Environment in Human Normal and Sickle Cell Blood

By Yiu-Fai Lam, Allison K.-L. C. Lin, and Chien Ho

Intracellular pH and 2,3-diphosphoglycerate concentration in sickle cell anemia and normal human blood samples were measured by means of phosphorus-31 nuclear magnetic resonance spectroscopy. To monitor the concentrations of various internal phosphorylated metabolites of intact red blood cells, heparinized blood samples were used and were incubated at 37°C with 5.6% CO₂, 25% O₂, and 69.4% N₂. The 31P chemical shifts of phosphorylated compounds, such as 2,3-diphosphoglycerate, adenosine 5'-triphosphate, and inorganic phosphate, depend on pH, and by using an appropriate calibration curve, the intracellular pH of intact erythrocytes can be obtained. The intracellular pH values in fresh sickle cell blood and normal blood were found to be 7.14 and 7.29, respectively. However, the whole-blood pH, as measured by a standard pH meter, was found to be 7.54 for both types of blood. The initial concentration of 2,3-diphosphoglycerate in sickle cell blood was about 30% higher, but it was depleted much faster during incubation than that in normal blood. The difference in intracellular pH between these two types of blood samples remained constant during incubation, even after depletion of 2,3-diphosphoglycerate. These results suggest that there are differences in intracellular environment between normal and sickle cell blood. Thus, 31P nuclear magnetic resonance spectroscopy provides a fast, direct, continuous, and noninvasive way to monitor the intracellular environment of intact erythrocytes.

Recent biochemical and biophysical studies of sickle cell hemoglobin (Hb S) have provided valuable insight into the molecular basis of the sickling phenomenon in sickle cell anemia patients (SS patients). Concentrations of hydrogen ion, hemoglobin, and 2,3-diphosphoglycerate (2,3-DPG), ionic strength, temperature, and degree of oxygenation are some of the factors that can affect the polymerization of Hb S molecules. A recent review of Hb S and sickle cell anemia has been provided by Bunn et al. In spite of considerable effort devoted to sickle cell anemia, the sequence of events that initiates vaso-occlusive crises in SS patients is not well understood. It is known that the frequency and severity of painful crises vary considerably among patients and even in a given individual. SS erythrocytes are known to have elevated red cell levels of 2,3-DPG. However, the
P_{50} value (the partial pressure at 50% oxygen saturation) of SS blood is higher than would be predicted from red cell 2,3-DPG, and there is no significant correlation between P_{50} and red cell 2,3-DPG. Since pH and 2,3-DPG concentration affect not only the oxygen dissociation of hemoglobin but also the polymerization of Hb S, their respective values under various conditions inside SS erythrocytes are of great importance to an eventual understanding of the pathophysiology of this disease. There is controversy over the acid–base status of blood from SS patients. Greenberg and Kass, as well as Barreras and Diggs, reported that venous blood samples taken from SS patients during painful crises were more acidic than those of normal (AA) individuals. Other studies of similar nature have shown no such differences between normal and SS blood samples.

There are two techniques commonly used to measure intracellular pH of red blood cells. The first technique (known as the freeze-thaw method) is to measure the pH of the lysate after hemolyzing the red blood cells. As has been pointed out, not only can the lysing procedure introduce errors in pH measurements, but also the response of a glass electrode in a concentrated protein solution such as a lysate can vary from sample to sample depending on the content of cell water as well as the liquid junction potential. The second method is based on the distribution of an added weak organic acid across the red cell membrane. This method can give an incorrect pH value if there are alterations in the red cell membrane affecting the permeability of the added organic acid. It has been found that the second method usually gives a higher pH value than the freeze-thaw method. In view of these limitations, a new technique is needed to measure intracellular pH.

Recently, {superscript}31P nuclear magnetic resonance (NMR) spectroscopy has been used successfully to investigate the intracellular environment of intact erythrocytes. Moon and Richards were the first to apply this method to measure intracellular pH using rabbit red cells in the carbonmonoxy form. This technique can be used to determine the concentrations of various phosphorylated metabolites, such as 2,3-DPG, adenosine 5'-triphosphate (ATP), sugar phosphate, and inorganic phosphate (P_i), and to measure the intracellular pH inside intact red blood cells. In a NMR spectrum, there are two readily measurable parameters: the position of the resonance (known as the chemical shift) and the area under the resonance. The chemical shift is a sensitive measure of the nature of a chemical group. The area under each resonance is directly proportional to the concentration of that group. A discussion of the principles of NMR has been provided by Pople et al. Thus, by measuring the resonance areas of phosphorylated metabolites as a function of time, their rates of metabolism can be measured. The {superscript}31P chemical shifts of various phosphates are very sensitive to pH, so that by constructing an appropriate calibration curve, the intracellular pH of intact erythrocytes can be determined.

In this study we have applied {superscript}31P NMR spectroscopy to make direct comparisons of normal and sickle cell blood samples. We have found that intracellular pH of fresh sickle cell blood was approximately 0.15 pH unit more acidic than normal blood and that the rates of decay of both 2,3-DPG and ATP are higher in SS blood than in normal blood.

MATERIALS AND METHODS

Fresh whole blood samples were drawn from donors provided by the Sickle Cell Society of Southwestern Pennsylvania, Inc., the Falk Clinic, and the central Blood Bank. All donors were from 21
to 31 yr of age. They were in good health when their blood samples were drawn, and they had taken no medication within 24 hr prior to the drawing of the blood samples. None of the SS donors had had any painful crises or blood transfusions within a 6-mo period. A blood sample from an individual with autoimmune hemolytic anemia (AIHA) was kindly provided by Dr. C. H. Srodes of Pittsburgh. Venous blood was drawn into vacuum tubes (Venoject KT-200-KA, Kimble-Tenumo, Ellston, Md.) containing heparin at 143 international units per 10 ml of whole blood. Samples were kept on ice and transferred to the laboratory for immediate use.

Normal adult hemoglobin (Hb A) was isolated using the standard procedures employed in our laboratory. Stock Hb A solutions were stored in the carbonmonoxy form at 4°C and converted to the oxy form just prior to the NMR studies. Hemoglobin concentrations in the purified solution and in whole blood were determined by Drabkin's method.

2,3-DPG (pentacyclohexyl ammonium salt) was purchased from Calbiochem. Adenosine 5'-triphosphate (disodium salt) was obtained from Sigma. Both compounds were further purified through a cation-exchange resin (AG 50W-X8, Bio-Rad). The concentrations of these two phosphates were determined by standard enzymatic methods. The reagents used were obtained from Sigma for the ATP analysis (using ATP diagnostic kit 366-UV) and from Calbiochem for the 2,3-DPG analysis (using 2,3-DPG Stat-Pack L3062). Other chemicals were of reagent grade and were used without further purification.

**Time-Dependent Study of Intact Whole Blood Cells**

To standardize the gaseous composition of samples from different donors, each freshly obtained whole blood sample was first transferred into a tonometer (Instrumental Laboratory, model 237) and incubated at 37°C for 30 min with a physiologic gas mixture consisting of 5.6% CO2, 25% O2, and 69.4% N2 (purchased from Life-O-Gen, Cambridge, Md.) at a flow rate of 375 ml/min. Zero time for the time-dependent study was defined as the beginning of the incubation. To monitor the intracellular pH and 2,3-DPG content by a NMR method, an aliquot of the incubated sample was immediately transferred into a NMR tube under the same gas mixture and sealed. NMR data were collected continuously for 6-7 hr at 37°C. Other hematologic data (hemoglobin concentration, external blood pH, hematocrit) were also determined with the remainder of the incubated sample. In addition, the 2,3-DPG content and the intracellular pH of the sample were determined by enzymatic assay and the freeze-thaw method, respectively. In the freeze-thaw method, whole blood was centrifuged at 5000 rpm at 37°C in an air-tight plastic syringe (Plastipak, Becton-Dickinson, Rutherford, N.J.). The plasma was removed, and the packed cells were lysed by repeated freezing and thawing. The pH of the lysates was measured immediately with a Radiometer microelectrode unit (model 5021a) attached to a Beckman pH meter (model 3500). The pH meter was calibrated with two standard buffers (1:4 Sorensen and 1:1 phosphate buffers) and cross-checked with a third one (1:3.5 phosphate buffer).

**Calibration of 31P Chemical Shifts in Terms of pH**

To study the pH dependence of the chemical shifts of 2,3-DPG and inorganic phosphate, simultaneous NMR spectra and pH measurements of hemolysates at different pH values were taken. The chemical shifts of these phosphates in lysates did not change as rapidly with time as those in intact red cells. No change in pH value was observed throughout the NMR experiment (30 min). In addition, these chemical shifts were found to be the same before and after lysing the red cells, using either normal or sickle cell blood.

To obtain lysates at different pH, titration with acid or base was carried out gradually on the red cells before lysing. The red cells used were either from fresh blood or from stored blood from the Blood Bank. In the case of fresh blood, the adjustment of pH was easily carried out in the plasma with 0.15-N NaOH or 0.15-N HCl in saline, and then the red cells were recombined with the plasma for 10-15 min before lysing. In the case of stored blood, the red cells were incubated for 3 hr at 4°C with an IPP solution (5-mM inosine, 10-mM pyruvate, and 100-mM NaH2PO4 at pH 7.6). This procedure was used to increase the 2,3-DPG content in the stored blood back to the level of that in normal fresh red cells. The treated cells were then incubated for 4-6 hr with Tris-HCl buffer (5.6-mM glucose, 90-mM NaCl, 5-mM KCl, and 50-mM Tris-HCl buffer) at different pH values. After appropriate pH adjustments, the red cells were separated from the incubation medium (the plasma or the buffer) by centrifugation. These pH-adjusted red cells were lysed by the freeze-thaw method previously described and then used immediately for NMR and pH measurements.
In studying the pH dependence of the ATP chemical shifts, the ATP concentration in lysates was found to be very small (0.4 mM) as compared with that in red cells (1.7 mM), apparently as a result of the lysing process.2 The ATP signals were too small to be observed readily in 30 min. However, the ATP content and pH values of lysates changed gradually over long hours of data averaging (±3 hr). Therefore, purified oxy-Hb A solutions with stable pH and ATP content were used: 16%-18% Hb A, 2.4-mM 2,3-DPG, 1.8-mM ATP, 2.1-mM KH2PO4, 4-mM MgCl2, and 90-mM KCl. Fifty-millimolar Tris-HCl or bis-Tris buffer was used to adjust the pH of the solutions.

NMR Measurements

31P NMR spectra at 36.43 MHz were taken on a Bruker HFX-90 spectrometer modified for pulse Fourier transform operation and equipped with a 2H lock as well as a quadrature phase detector ( Nicolet model TT-1025). A typical spectrum required the accumulation of 880–1760 scans (30–60 min). The resolution used was 0.5 Hz per data point. The probe temperature was maintained at 37 ± 0.5°C. The deuterium signal from D2O inside a coaxial capillary in the NMR samples served as the lock for the field to frequency stabilization.

Samples for NMR measurements were sealed into standard 10-mm NMR tubes (10-mm ID and 150-mm length) under an appropriate gas mixture. Each NMR tube was spun along its long axis throughout the measurements for better signal-to-noise ratio and resolution. When a small volume of whole blood (2 ml) was used, the spinning motion caused packing of red cells and a decrease in the signal-to-noise ratio. Hence, 4–5 ml of whole blood had to be used for optimal results.

The 31P chemical shifts are reported with respect to 85% H3PO4, the usual 31P chemical shift reference. The actual primary standard used was a methylene diphosphonic acid (MDP) solution. This standard solution was sealed inside a capillary and placed coaxially into a NMR sample tube. The composition used was 0.05-M MDP, 0.005-M EDTA, and 0.5-M Tris-HCl in D2O at pH 8.9 and 37°C. The 31P signal from this solution was –607.2 Hz or –16.67 parts per million (ppm) downfield from that of 85% H3PO4. The signal areas of the external reference and the 3-P of 2,3-DPG (3-DPG) were integrated digitally in the transformed spectra. The range of integration was consistently around ±1.5 times the corresponding line width at half-height. The 3-DPG areas were first expressed as a percentage of that of the reference. To calibrate the areas to 2,3-DPG concentrations, the following procedure was used, as it is the most appropriate method for the rapidly decreasing 2,3-DPG signals in the blood samples and can eliminate possible complications due to the nuclear Overhauser effect on different nuclei when proton noise decoupling is used. In this method, enzymatic assays and NMR measurements were first carried out simultaneously on hemolysates of different 2,3-DPG contents (1–8 mM). A concentration-to-area conversion factor for the 2,3-DPG signal was obtained: F = (2,3-DPG enzymatic concentration) x (31P NMR area of the external reference)/(31P NMR area of 3-DPG). With this factor and with the same NMR instrumental settings, the areas of 3-DPG from the blood cells were converted into actual concentrations. The results obtained by this method were in agreement with the independent enzymatic assays with blood cells to be presented in the Results section.

RESULTS

Figure 1 shows a typical 31P NMR spectrum of normal human blood at 37°C. The 31P resonances observed in SS blood (results not shown) were qualitatively the same as in normal blood. The low-field region (Fig. 1A) consisted of several large signals easily detected with only 30 min of signal averaging. These signals arose from the 31P resonances of the 3-P and 2-P of 2,3-DPG (labeled 3-DPG and 2-DPG, respectively), and the extracellular and intracellular inorganic phosphates (labeled as P1out and P1in, respectively). The assignment of the 31P resonances as shown in Fig. 1 was based on published results and on our own work. The assignment of the signals from P1in and P1out was carried out by adding a small amount of inorganic phosphate to whole blood. The P1out signal was found to increase in intensity accordingly. Because of the packing of red cells in a spinning NMR tube, the concentration of P1out was expected to be low in the 31P NMR spectra. In addition, this signal was located between two strong signals, 2-DPG and P1in. Therefore, the
A. Typical 36.43-MHz $^{31}$P NMR spectrum (with proton decoupled) of human normal blood incubated at 37°C with a physiologic gas mixture of 5.6% $\text{CO}_2$, 25% $\text{O}_2$, and 69.4% $\text{N}_2$. A: Low-field region of the spectrum obtained from 880 scans (0.5 hr). B: High-field region of the spectrum obtained from 3520 scans (2 hr). The $^{31}$P signals in this region are much weaker than those in A.

Variation of Intracellular Phosphates as a Function of Time

The $^{31}$P NMR spectra of fresh blood samples drawn from normal individuals, SS donors, and an individual with AIHA were monitored as a function of time. Representative $^{31}$P NMR spectra of 2,3-DPG, $P_i^{\text{in}}$, and ATP from AA and SS blood are shown in Figs. 2 and 3. In Fig. 2, decreases are observed in the signal areas from 2-DPG and 3-DPG, with a corresponding increase in the area of the $P_i^{\text{in}}$ resonance as a function of incubation time. Since the blood cells studied were still intact, they were glycolytically active and were metabolizing 2,3-DPG into inorganic phosphate. Thus, the observed $^{31}$P signals responded as first reported by Henderson et al. in normal adult blood. In the intact blood samples studied, the 2,3-DPG signals in the SS blood and in the AIHA blood diminished much faster than those in...
normal blood. In the earlier phase of incubation (≤2.5 hr), neither P'_i nor P'' signals could be detected in any of the samples studied, probably because of the very low concentration (≤1 mM) of inorganic phosphate in the freshly drawn blood. As shown in Fig. 3, the β- and γ-ATP signals in the SS blood samples also appeared to diminish faster than those in normal blood samples. However, the changes were not so drastic as those shown in Fig. 2. In fact, because of the metal
ion effect on the $\beta$-ATP signals,\(^{31,33,34}\) as well as the possible presence of other signals around the $\alpha$-ATP doublets,\(^{30}\) the $\gamma$-ATP signals were better representatives of the ATP content inside the red cells. However, the ATP signals from AIHA blood were extremely small, even compared with those shown in Fig. 3. Therefore, no comparison can be made in this case.

The signal from 3-DPG was intense and was well resolved from other $^{31}$P resonances in a blood sample (Figs. 1 and 2) and could be used as a convenient and direct measurement of the internal 2,3-DPG concentration of intact erythrocytes. In Fig. 4, the 2,3-DPG concentrations predicted from the NMR method (via the $^{31}$P resonance of the 3-DPG signal area) are plotted as a function of time for the AA, SS, and AIHA blood samples. During the first 2–3 hr, there is an increase in the 2,3-DPG concentration; thereafter, 2,3-DPG concentration decreases as a function of time. In some of these samples, enzymatic assays of the 2,3-DPG concentrations were also carried out simultaneously. The results are included in Fig. 4. Table 1 summarizes the intracellular 2,3-DPG concentration, hemoglobin concentration, and hematocrit for fresh human blood. These results show that the $^{31}$P NMR method and the enzymatic method give essentially the same concentration for intracellular 2,3-DPG.

**Intracellular pH**

Figure 5 shows that there are differences in the $^{31}$P chemical shifts of $P_\text{II}$, 2-DPG, and 3-DPG of 2,3-DPG among AA, SS, and AIHA blood samples incubated with a physiologic gas mixture at 37°C. The initial values of the $^{31}$P chemical shifts obtained at the end of the first hour after the beginning of incubation are shown in Table 2. No inorganic phosphate signal was detected at this stage. To correlate the $^{31}$P chemical shifts of these phosphorylated metabolites to the intracellular pH of intact erythrocytes, appropriate calibration curves were constructed.

The pH calibration curves were constructed with lysates from AA blood samples under a variety of conditions, as shown in Fig. 6. In the figure, symbols $\times$ indicate measurements carried out with lysates incubated with the physiologic gas mixture, whereas the $\circ$, $\Delta$, and $\Delta$ symbols indicate results from lysates incubated with air. The mean values of the phosphates in these lysates were as follows: [2,3-DPG] = 5.1 mM; [P] = 4.6 mM; [ATP] = 0.85 mM. The [Hb A] was 5.0 ± 0.5 mM. The lysates in the $\circ$ series were so prepared that they had variable contents of 2,3-DPG and P, to ±30% from their mean values, respectively. The $\Delta$ and $\Delta$ series indicate

![Fig. 4. Variation of 2,3-diphosphoglycerate concentration in three types of human blood as a function of time at 37°C. The left-hand-side coordinate is the relative $^{31}$P resonance area ratio of the $^{31}$P signal of the 3-P atom of 2,3-DPG (labeled 3-DPG) and the reference compound, methylene diphosphonic acid (MDP). The symbols $\circ$ and $\square$ are results from the $^{31}$P signal area of 3-DPG in normal blood (4 donors); symbols $*$ are results from SS blood (3 donors); symbols $\Delta$ are those from AIHA blood (1 donor). The right-hand-side coordinate with the symbol $\times$ is the concentration of 2,3-DPG determined by an enzymatic assay.](image-url)
results from lysates with additional amounts of ATP (1.7 mmoles added per milliliter of lysate) and Mg$^{2+}$ ion (2.2 mmoles added per milliliter of lysate), respectively. The preceding design was intended to check whether or not the pH dependence of the calibration curves was affected by possible interactions among 2,3-DPG, ATP, P$_i$, Mg$^{2+}$, and Hb A$^+$. These results clearly show that the pH is the most important factor in determining the $^3$P chemical shifts of P$_i$, 2-DPG, and 3-DPG in the lysates. Thus, by using one of these $^3$P chemical shifts, we can determine the intracellular pH of intact erythrocytes from the corresponding calibration curve.

Table 3 summarizes the intracellular pH values of fresh blood samples (i.e., within the first hour after the blood samples were incubated with the physiologic gas mixture) for normal individuals, SS donors, and the AIHA sample. The corresponding pH values obtained by the freeze-thaw method and the extracellular
Table 2. $^{31}$P Chemical Shifts of Intracellular Signals From Freshly Drawn Human Blood*

<table>
<thead>
<tr>
<th>Signal</th>
<th>Chemical Shift (ppm from 85% H$_3$PO$_4$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA Blood</td>
</tr>
<tr>
<td>3-P of 2,3-DPG</td>
<td>$-3.30 \pm 0.04$</td>
</tr>
<tr>
<td>2-P of 2,3-DPG</td>
<td>$-2.42 \pm 0.08$</td>
</tr>
<tr>
<td>$\gamma$-P of ATP</td>
<td>$+5.70 \pm 0.14$</td>
</tr>
<tr>
<td>$\alpha$-P of ATP</td>
<td>$+10.82 \pm 0.03$</td>
</tr>
<tr>
<td>$\beta$-P of ATP</td>
<td>$+19.93 \pm 0.14$</td>
</tr>
</tbody>
</table>

*The $^{31}$P NMR data are averaged measurements between 0.5 and 1.0 hr of incubation. The 0–0.5-hr incubation was carried out in a tonometer before the NMR measurement, as described in the text.

The $^{31}$P NMR data are averaged measurements between 0.5 and 1.0 hr of incubation. The 0–0.5-hr incubation was carried out in a tonometer before the NMR measurement, as described in the text. (or whole-blood) pH values are also given in Table 3. Based on the $^{31}$P NMR method, there is a definite difference in intracellular pH between normal individuals and SS patients, with the SS samples being 0.15 pH unit more acidic than the normal. The intracellular pH of the AIHA blood is perhaps slightly more acidic than that of SS patients, but since only one such blood sample was available, it is difficult to make a firm conclusion at this stage. Within the accuracy of pH measurements, there is no significant difference in intracellular pH between AA and SS donors as determined by the freeze-thaw technique. The extracellular blood pH is the same for the three types of blood examined.

Figure 7 shows the variation of intracellular pH values (determined from the $^{31}$P signals of P, 2-DPG, and 3-DPG) as a function of time for AA and SS blood samples at 37°C. The intracellular pH value decreases as a function of time for all three types of blood examined. However, at longer periods of incubation, even though the rate of decay of 2,3-DPG in SS blood is faster than that in AA blood, approximately the same pH difference existed between these two types of blood. Of special significance is the observation that at the sixth hour of incubation the intracellular pH of the SS blood is still about 0.12 pH unit lower than that of the AA blood. At that time the intracellular concentration of 2,3-DPG is approximately the same in the two types of blood (Fig. 4).

Over the pH range from 6.8 to 7.8 we have observed a much smaller pH variation in the chemical shifts of ATP in the red cells, lysates, and the model solutions. The $\alpha$- and $\beta$-ATP phosphate groups, being diester in structure, are not expected to have a large pH dependence over this pH range. The $\gamma$-ATP phosphate, which has a pK value of approximately 6.5, showed only about a 10-Hz change from pH 6.8 to 7.8, as compared with approximately 20 Hz for 3-DPG (Fig. 6). Furthermore,
Table 3. Intracellular pH Values of Freshly Drawn Human Blood at 37°C

<table>
<thead>
<tr>
<th>Blood Type</th>
<th>Intracellular pH</th>
<th>Freeze-Thaw Method pH (pH meter)</th>
<th>Extra-Cellular pH (pH meter)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3-P of 2,3-DPG</td>
<td>2-P of 2,3-DPG</td>
<td>3-P of 2.3-DPG</td>
</tr>
<tr>
<td>Normal blood</td>
<td>7.30 ± 0.06</td>
<td>7.27 ± 0.08</td>
<td>7.29 ± 0.08</td>
</tr>
<tr>
<td>(5 donors)</td>
<td></td>
<td></td>
<td>7.53 ± 0.08</td>
</tr>
<tr>
<td>SS blood</td>
<td>7.15 ± 0.03</td>
<td>7.13 ± 0.04</td>
<td>7.22 ± 0.04</td>
</tr>
<tr>
<td>(5 donors)</td>
<td></td>
<td></td>
<td>7.54 ± 0.16</td>
</tr>
<tr>
<td>AIHA blood</td>
<td>7.08 ± 0.03</td>
<td>7.07 ± 0.03</td>
<td>7.22 ± 0.02</td>
</tr>
<tr>
<td>(1 donor)</td>
<td></td>
<td></td>
<td>7.53 ± 0.02</td>
</tr>
</tbody>
</table>

the long hours of signal accumulation that are required to detect these weak signals could induce large uncertainty in the physiologic status of the intact cells under investigation. Hence, ATP is not a convenient or suitable monitor for intracellular pH in red cells.

DISCUSSION

There are two important conclusions that can be reached concerning the intracellular environment of intact human erythrocytes based on this 31P NMR investigation. First, the rate of metabolism of organic phosphates in SS blood is higher than that in normal adult blood. Second, the intracellular pH of fresh SS blood is approximately 0.15 pH unit lower than that of normal adult blood.

The faster rate of decay of phosphorylated metabolites (such as 2,3-DPG) in SS blood as compared with that in AA blood may be related to a higher glycolytic activity in the former. This suggestion is supported by the following observations. It is known that young red cells (reticulocytes) have higher glycolytic activity than matured ones. It is also known that both SS blood and AIHA blood have considerably larger populations of reticulocytes than AA blood. The results summarized in Fig. 4 clearly show that the rate of 2,3-DPG depletion in SS blood is much like that in AIHA blood, i.e., it is faster than that in AA blood. This supports the idea that the rate of turnover of phosphorylated metabolites is related to a larger population of young cells present in the SS blood that have a higher rate of glycolysis. A recent study using all populations of red cells also indicated that SS blood has higher hexokinase activity and larger concentrations of glycolytic intermediates (such as glucose-6-phosphate, fructose-6-phosphate, etc.) than normal red cells. In addition to the age factor, consumption of 2,3-DPG can also be activated by inorganic phosphate, whose concentration is increasing and is
higher in SS blood than in AA blood during incubation. Nevertheless, it should be noted that the change in 2,3-DPG concentration in red cells is complex\textsuperscript{44,45} and that there are quantitative differences among these three types of red blood cells (Fig. 4). Further investigation is needed for a better understanding of the factors that affect the rates of both formation and consumption of 2,3-DPG in human blood.

The second major difference among the three types of blood studied by \textsuperscript{31}P NMR spectroscopy is in the intracellular pH. Table 3 summarizes both extracellular and intracellular pH values of AA, SS, and AIHA blood samples as determined by the \textsuperscript{31}P NMR and pH electrode methods. With the conventional pH electrode method there is no difference in extracellular (or whole-blood) pH for the blood samples studied. By using the conventional freeze-thaw method, we have found that the intracellular pH values of AA, SS, and AIHA blood samples at 37°C are 7.29 ± 0.14, 7.22 ± 0.14, and 7.22 ± 0.02, respectively. Thus, within the limitations of this method, the difference in intracellular pH between SS and AA blood is at best marginal. However, by means of \textsuperscript{31}P NMR spectroscopy, we have found a definite difference in intracellular pH among these three types of blood: 7.29 ± 0.08 for AA blood, 7.14 ± 0.04 for SS blood, and 7.08 ± 0.03 for AIHA blood. These are the values of intracellular pH for fresh blood samples (i.e., within 1 hr after blood was drawn). With the exception of the result for AIHA blood (only one donor was available), the data for SS blood and AA blood are based on an average of five donors for each type. These values were also the average from the results based on the \textsuperscript{31}P resonances of 2-DPG and 3-DPG of 2,3-DPG. They give essentially the same pH values. Furthermore, with the appearance of the \textsuperscript{31}P signal due to inorganic phosphate at the end of 2–2.5 hr of incubation at 37°C, we can also determine the intracellular pH values using the P\textsubscript{o} resonance. Again, they yield essentially the same values as those based on 2-DPG and 3-DPG, as shown in Fig. 7. However, at the later phase of incubation (time > 5 hr), deviation between the computed intracellular pH values from the 2,3-DPG and the inorganic phosphate appeared. This is due mostly to the increasing uncertainty in the 2,3-DPG chemical shifts, as these signals are diminishing with time (Fig. 2). On the other hand, the accuracy with the inorganic phosphate signal increases as this compound gradually accumulates within the cell and the signal resolution in the spectra improves. Nevertheless, at any given time throughout the incubation, the same kind of signal (either that of 2,3-DPG or that of P\textsubscript{o}) indicates the same difference in pH values between the AA and the SS blood samples. It is concluded that there is a difference of about 0.15 pH unit between normal and SS blood samples.

In view of the fact that both SS and AIHA blood samples have higher levels of 2,3-DPG, is there a direct relationship between the concentration of 2,3-DPG and intracellular pH? In fact, several investigators have presented data to support this relationship in normal blood.\textsuperscript{46,47} The order of acidity as determined by \textsuperscript{31}P NMR for the blood examined was AIHA > SS > AA. This order coincides with the initial amounts of 2,3-DPG present in these three types of blood (Table 1 and Fig. 4). Our findings support a relationship between 2,3-DPG level and intracellular pH of blood. However, \textsuperscript{31}P NMR results also shows that the intracellular pH of SS blood stays about 0.15 pH unit below that of AA blood over the incubation period studied (Fig. 7), whereas the 2,3-DPG content in SS blood decreases to a level comparable to or less than that of the AA blood (Fig. 4) (e.g., after 5 hr of incubation). As was
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mentioned earlier, the depletion rate of 2,3-DPG is faster in SS blood than in AA blood.

The occurrence of more acidic intracellular pH and higher 2,3-DPG content appears to be an intrinsic property of the SS blood. Unfortunately, higher levels of 2,3-DPG and hydrogen ion are factors that promote the polymerization of Hb S molecules. Bookchin et al.\(^4\) reported that the polymerization process of the SS lysate is sharply dependent on pH over a very narrow range, namely pH 7.15-7.25. It is noteworthy that the intracellular pH of SS blood is at the lower end of this range. Further work is needed to establish a relationship between changes in the intracellular environment and onset of the sickling process in sickle cell anemia.

In summary, these results demonstrate the unique value of \(^3\)P NMR spectroscopy for investigation of the intracellular environment of intact erythrocytes. This technique provides fast, direct, and continuous monitoring of intracellular pH and phosphorylated metabolites without altering the intracellular environment or disrupting the cell membrane. Thus, it is a valuable technique for correlating molecular and cellular studies on sickle cell anemia.

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