2,3-Diphosphoglycerate and Intracellular pH as Interdependent Determinants of the Physiologic Solubility of Deoxyhemoglobin S

By William N. Poillon and Bak C. Kim

We have established that 2,3-diphosphoglycerate (2,3-DPG) content and intracellular pH exert separate, but interdependent, effects on the equilibrium solubility (c_{sat}) of deoxyhemoglobin S (deoxy-Hb S) that act in concert to modulate intracellular polymer formation. In a nonphysiologic c_{sat} assay system, a steep dependence of c_{sat} on pH in the physiologic range 7.0 to 7.6 was shown for both stripped (Hb) and DPG-saturated deoxy-Hb S (Hb-DPG). The solubility-pH profile for Hb under near-physiologic buffer conditions also showed that c_{sat} increased steeply in the same pH range (6.8 to 7.6). The effect of 2,3-DPG on c_{sat} under near-physiologic conditions was evaluated separately. At pH 7.20, the pH of the human red blood cell, c_{sat} values for Hb and Hb-DPG were 19.56 ± 0.14 and 17.95 ± 0.45 g/dL, respectively, indicating that the solubility of Hb-DPG is lower than that of Hb by 8.2% ± 2.3%. Thus, binding of 2,3-DPG in the β-cleft promotes the polymerization of deoxy-Hb S, the ultimate determinant of cell sickling. Furthermore, because of the abnormal Bohr effect of sickle blood (approximately double that of normal blood), the intracellular pH of deoxygenated sickle erythrocytes should be ~0.28 pH unit higher than that of oxygenated cells (ie, 7.41 v 7.13). At the higher pH, the corresponding c_{sat} for Hb-DPG is 20.22 g/dL, which is the best estimate of the intrinsic solubility of T-state Hb S under conditions that approximate closely those of pH, temperature, ionic strength, and 2,3-DPG saturation in the fully desaturated sickle erythrocyte.

© 1990 by The American Society of Hematology.
units of whole blood from hospitalized sickle cell anemia patients who required partial exchange transfusions. Preparation of hemolysates and purification of Hb S by ion-exchange chromatography on columns of DEAE-Sephalogel (Pharmacia Fine Chemicals, Piscataway, NJ) are described elsewhere.17 This procedure effectively removes all endogenous RBC organophosphates.

2,3-DPG. This organic phosphodiester was obtained (Sigma) as the pentasodium salt. Stock solutions (20 to 100 mmol/L) were prepared in the appropriate buffer and adjusted to neutral pH with 1N NaOH.

Solubility measurements. The method for determining equilibrium solubility, or saturation concentration \( c_{sat} \), of deoxy-Hb S by ultracentrifugation and its modification to a microscale are described elsewhere.18,19 Samples of Hb S were dialyzed against a Bistris ([bis(2-hydroxyethyl)amino]tris(hydroxymethyl)-methane) buffer whose ionic strength approximated that of the cytoplasm of the RBC (0.05 mol/L Bistris, 0.1 mol/L KCl, and 0.02 mol/L NaCl) and whose osmolality was in the range of normal serum (280 to 290 mOsm/kg). Although it has no analogue in the RBC, we chose to use Bistris for buffering capacity because its pK\(_b\) (6.5 to 8.5) is close to physiologic. In addition, the use of sodium dithionite to ensure rigorous deoxygenation makes it virtually impossible to perform \( c_{sat} \) assays in CO\(_2\)-bicarbonate buffer, a more stringently physiologic medium. The Bistris-based buffer may then be considered quasi-physiologic, at least. Use of dithionite was necessary because it rapidly deoxygenates oxy-Hb S and reduces any residual met-Hb.20 However, in an aerobic atmosphere, dithionite may generate activated O\(_2\) products that could be harmful to the Hb molecule. Accordingly, the following precautions were taken to ensure anaerobicity during manipulation: the amount of solid dithionite needed for a 1 mol/L solution was placed in a 5-mL volumetric flask, evacuated through a rubber septum, and flushed with nitrogen. A portion of distilled water (10 to 15 mL) was deaerated by evacuation for 1 hour on a water aspirator, then purged with nitrogen. Exactly 5 mL of degassed water was transferred anaerobically by Hamilton syringe to the dithionite, which quickly dissolved. An aliquot of oxygen-free dithionite (10 \( \mu \)L) was transferred to the centrifuge tube containing Hb S and effector (total volume: 250 \( \mu \)L), mixed, and overlaid with mineral oil. To ensure that dithionite itself does not influence the solubility, an experiment was performed in which Hb S was deoxygenated by nitrogen inside a glove bag; a small amount of dithionite (0.75 mol/mol heme) was then used to reduce residual met-Hb (less than 9%); \( c_{sat} \) was measured and compared with values obtained in which deoxygenation was achieved with the usual amount of dithionite (3.0 mol/mol heme). Overall, two kinds of experiments were performed at 37°C: (1) Stripped deoxy-Hb S was subjected to different pHs above 7 by initially dialyzing against Bistris buffer of variably higher pH (8.2 to 8.8) at 25°C. Subsequent addition of various amounts of sodium dithionite, encompassing a molar excess (based on heme) from 2 to 4, resulted in pH values for deoxy-Hb S in the range desired (7.0 to 7.6) at 37°C. Below pH 7.5, where the buffering capacity of Bistris is stronger, a lower initial pH (6.6 to 7.2) at 25°C for the dialysis buffer was sufficient to achieve the desired pH range (6.2 to 6.8) at 37°C, again with a variable molar excess of dithionite. In four rotors, 24 replicates were measured that encompassed the pH range of interest (6.2 to 7.6). (2) The solubility of deoxy-Hb S was measured, in the absence or presence of a saturating amount of 2,3-DPG, at a fixed (norminal) pH of 7.20 at 37°C. Four rotors, each containing triplicate assays for Hb and Hb-DPG, were used. To compensate for the proton uptake evoked by 2,3-DPG binding in the \( \beta \)-cleft, a slightly greater molar excess of dithionite was used to deoxygenate Hb S in the presence of 2,3-DPG (3.5-fold) than in its absence (threefold). After equilibration for 1.5 hours in a water bath at 37°C, samples were placed in an SW 50.1 rotor and allowed to equilibrate in the chamber of a preparative ultracentrifuge (Beckman L55) at 37°C for an additional 0.5 hour, after which they were centrifuged for 1 hour at 242,000g. After separation of the two component phases of the gel, the pH of the supernatant was measured through mineral oil with an Ingold combination microelectrode and a Radiometer pH meter (model PHM61), while the equilibration temperature was maintained, as described previously.18,19 The concentration of Hb S in the supernatant (ie, \( c_{sat} \)) was measured spectrophotometrically as cyanmethemoglobin by using the millimolar extinction coefficient of 11.0 cm\(^{-1}\) (per heme) at 540 nm17 and 16,100 for the molecular weight of Hb monomer.

RESULTS

ATP and 2,3-DPG content of normal and sickle erythrocytes. Intracellular concentrations of ATP and 2,3-DPG in whole blood samples from 19 normal individuals and 38 sickle cell anemia patients are compared in Table 1. Mean values of both organophosphates are elevated in sickle erythrocytes, ATP by 22% and 2,3-DPG by 27%, as others have shown.6,22,23 However, in our patient population, the 2,3-DPG concentration ranges from 3.5 to 9.5 mmol/L, a nearly threefold variation. Comparable values for normal controls span a much narrower range, from 4.0 to 6.0 mmol/L. Thus, a greater heterogeneity of 2,3-DPG levels occurs in sickle erythrocytes compared with normal erythrocytes.

Effects of pH and 2,3-DPG on the physiologic solubility of deoxy-Hb S. To facilitate comparison of the effect of pH on both stripped (Hb) and DPG-saturated (Hb-DPG) deoxy-Hb S under near-physiologic conditions, the effect of pH on both species under less physiologic conditions is also of interest. Results of such an investigation appear in an earlier publication.12 Because of their importance to the present work, a modified version of the solubility profiles (\( c_{sat} \) vs pH) from that study is included here as Fig 1. Under the nonphysiologic conditions outlined in the legend to Fig 1, the solubility-pH profile for Hb-DPG was displaced downward from that for Hb throughout the pH range examined (6.5 to 7.6) by a decrement that was somewhat smaller (\(-1.8 \text{ g/dL}\)) above pH 7.0 than below it (\(-3.0 \text{ g/dL}\)). The weaker effect in the physiologic pH range (7.0 to 7.6) parallels the diminished strength of binding for 2,3-DPG at the elevated pH. (The strength of 2,3-DPG binding to deoxy-Hb A is nearly 10 times less at pH 7.8 than at pH 7.0.)13

The solubility-pH profile for stripped deoxy-Hb S under near-physiologic conditions over the pH range 6.2-7.6 is shown in Fig 2. Although \( c_{sat} \) is invariant at pH 6.2 to 6.8, it increases steeply between pH 6.8 and 7.6. This behavior is

Table 1. Organophosphate Concentrations in Erythrocytes From Normal Subjects and Patients With Sickle Cell Anemia

<table>
<thead>
<tr>
<th>Subject</th>
<th>Concentration (( \mu \text{mol/mL} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATP</td>
</tr>
<tr>
<td>Normal</td>
<td>19</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>1.12 ± 0.22</td>
</tr>
<tr>
<td>Range</td>
<td>0.82-1.56</td>
</tr>
<tr>
<td>Sickle cell anemia</td>
<td>38</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>1.37 ± 0.22</td>
</tr>
<tr>
<td>Range</td>
<td>0.95-1.82</td>
</tr>
</tbody>
</table>

From www.bloodjournal.org by guest on September 24, 2017. For personal use only.
temperature displacement of the profile for Hb-DPG in the nonphysiologic buffers and, to a much lesser extent, from the difference in ionic composition between the two buffer systems may be ascribed solely to their ionic strength, and temperature that approximate those of the normal erythrocyte, the solubility of DPG-saturated deoxy-Hb S is lower than that of stripped deoxy-Hb S by 8.2% ± 2.3%. Because it generates more intracellular poly-

virtually identical to that shown in Fig 1 for both Hb and Hb-DPG under nonphysiologic conditions.

The data of Figs 1 and 2 were subjected to linear regression analyses over the pH range in which $c_{sat}$ increases steeply (7.0 to 7.6). The slopes of these linear solubility-pH profiles ranged from 10.3 g/dL per pH unit for Hb-DPG in the nonphysiologic system to 10.8 g/dL per pH unit for stripped deoxy-Hb S in the physiologic system. The nearly identical slopes for stripped deoxy-Hb S, regardless of the ionic composition of the buffer, as well as the downward displacement of the profile for Hb-DPG in the nonphysiologic case (Fig 1), indicate that the effects of pH and 2,3-DPG on T-state Hb S are separate phenomena, whose combined influences are additive.

The mean values of solubility for Hb and Hb-DPG in the pH region of $c_{sat}$ invariance under nonphysiologic buffer conditions (pH 6.5 to 7.0; Fig 1), as well as the value for stripped Hb under near-physiologic buffer conditions (pH 6.2 to 6.8; Fig 2), have been compiled in Table 2. For the nonphysiologic case, the $c_{sat}$ value for Hb-DPG (15.24 ± 0.47 g/dL) is 17% lower than that for Hb (18.27 ± 0.21 g/dL). For the near-physiologic buffer system, in the pH range 6.2 to 6.8, the $c_{sat}$ value for Hb is 16.10 ± 0.27 g/dL. It is noteworthy that $c_{sat}$ for Hb in the nonphysiologic buffer system is 14% higher than this. The disparity results primarily from the difference in ionic composition between the two buffers and, to a much lesser extent, from the difference in temperature (30°C v 37°C). Therefore, to a first approximation, the differences in solubility for stripped deoxy-HbSs in the two buffer systems may be ascribed solely to their ionic compositions (Table 2). Furthermore, because the inorganic components of the physiologic buffer (Na+, K+, and Cl-) are relatively weak salting-in ions, the higher solubility in the nonphysiologic buffer may be ascribed to the more potent salting-in properties of the Bistris cation (unpublished results).

The exquisite sensitivity of the deoxy-Hb S polymer to its ionic environment is further demonstrated by the data in Table 3. Because the $c_{sat}$ experiments aimed at establishing the solubility of Hb-DPG under near-physiologic buffer conditions were performed near a single pH (7.20), it was not possible to obtain a solubility-pH profile for this species, as was done in the nonphysiologic buffer system (Fig 1). Instead, multiple $c_{sat}$ assays (11 in all) were performed for both Hb and Hb-DPG at the nominal pH of 7.20. Table 3 presents the mean values of $c_{sat}$ and pH obtained for both Hb and Hb-DPG at 37°C under these conditions. The $c_{sat}$ values of 19.34 ± 0.14 and 17.95 ± 0.45 g/dL obtained for these two species were measured at a mean pH of 7.18 ± 0.02 and 7.20 ± 0.01, respectively. Thus, the solubility of DPG-saturated deoxy-Hb S is 7.2% lower than that of Hb. Final pH values for the two species were close, but not identical. By using the slope of the solubility-pH profile in the physiologic pH range shown in Fig 2 (10.8 g/dL per pH unit), it is possible to obtain the $c_{sat}$ value of Hb at pH 7.20 (19.56 g/dL) for comparison with that of Hb-DPG at the same pH (17.95 g/dL; Table 3). Thus, under conditions of pH, ionic strength, and temperature that approximate those of the normal erythrocyte, the solubility of DPG-saturated deoxy-Hb S is lower than that of stripped deoxy-Hb S by 8.2% ± 2.3%. Because it generates more intracellular poly-

![Fig 1. Effect of 2,3-DPG on the equilibrium solubility ($c_{sat}$) of deoxy-Hb S as a function of pH under nonphysiologic conditions. Two buffer systems (0.1 mol/L Bistris, triangles; 0.1 mol/L Tris, circles) were used to encompass the pH range 6.5 to 7.6 at 30°C. At any particular pH, $c_{sat}$ values were determined for paired samples: stripped Hb S and Hb S plus a saturating amount of 2,3-DPG (DPG/Hb molar ratio = 1.5). Plots of $c_{sat}$ versus pH are given for deoxy-Hb S in the absence (filled symbols) and presence (open symbols) of 2,3-DPG.](image1)

![Fig 2. Effect of pH on the equilibrium solubility ($c_{sat}$) of stripped deoxy-Hb S under near-physiologic conditions. Samples of Hb S were dialyzed against an isotonic buffer of varying pH containing 0.06 mol/L Bistris, 0.1 mol/L KCl, and 0.02 mol/L NaCl; deoxygenation with a variable molar ratio of dithionite/hemoglobin was used to achieve the desired final pH. Four rotors (six replicates in each) were used to encompass the pH range 6.2 to 7.6 at 37°C. The symbols (C, A, Θ, I) denote the four individual rotors used to generate the 24 data points. The slope of this solubility-pH profile in the pH range 6.8 to 7.6 (10.8 g/dL per pH unit) can be used to estimate the effect of intracellular acidosis or alkalosis on the solubility of deoxy-Hb S.](image2)
mer, a solubility decrease of this magnitude would enhance the tendency of SS RBCs to sickle and should exacerbate the clinical manifestations of sickle cell disease.

**DISCUSSION**

*Molecular bases for the effects of pH and 2,3-DPG on solubility.* The steep pH dependence of c_sat above pH 7 under either nonphysiologic (Fig 1) or near-physiologic (Fig 2) buffer conditions reflects the rapid change in net charge on the deoxy-Hb S molecule due to the progressive titration of histidine side chains. Of the 10 histidine residues in the a-chain and nine in the b-chain of Hb S, a total of 10 participate in intermolecular contacts within the deoxy-Hb S polymer. For six of these (2 b, 20 b, 77 b, 116 b, 72 a, and 89a), only one of the two symmetrically related residues makes an intermolecular contact, whereas for the other two histidine residues (45 a and 117 b) both participate in intermolecular contacts. Therefore, it appears that some or all of the imidazolium groups of these individual histidines begin to deprotonate above pH 7, thereby destabilizing the polymer.

The molecular basis for the effect of 2,3-DPG on the solubility of deoxy-Hb S is an alteration of the deoxy (T) quaternary structure that occurs when the effector binds to the b-cleft. The alteration of T-state Hb S induced by 2,3-DPG binding, in which the A-helices of the b-chain move closer together by 0.2 nm, facilitates polymerization by altering the spatial disposition of the two Val 68 residues in such a way that the crucial intermolecular contact of 1 Val 68 b, with 2 Phe 85 b, and 2 Leu 88 b, is strengthened, thereby stabilizing the polymer.

**Intrinsic solubility of deoxy-Hb S.** The values of 19.6 ± 0.1 and 18.0 ± 0.5 g/dL reported in Table 3 for stripped and DPG-saturated deoxy-Hb S, respectively, pertain to the nominal intracellular pH (7.20) and temperature (37°C) of the normal human RBC.

Estimates by others of the solubility of stripped deoxy-Hb S have been made, for the most part, in a buffer composed of 0.15 mol/L KPO₄ at the nominal pH of 7.20 and 35°C to 37°C. In five different studies performed in this buffer, the c_sat values obtained fell into two sets: 16.0 to 16.2 and 17.2 to 17.3 g/dL. The higher values may be attributed to an effective pH somewhat greater than that for the lower values. (There is no way to evaluate this point from the experimental details provided.) Both sets of values determined in the nonphysiologic phosphate-based buffer are considerably lower (by 18% and 12%, respectively) than the value of 19.6 g/dL observed for stripped deoxy-Hb S in our more nearly physiologic Bistris-based buffer (Table 3). This discrepancy arises primarily from differences in the composition of the two buffers. In the phosphate buffer, ionic strength (approximately 0.40 before the addition of dithionite [0.05 mol/L]) was, with one exception, provided solely by orthophosphate at a concentration (0.15 mol/L) that considerably exceeds the ionic strength of the normal erythrocyte (μ = 0.17; reference 35). In addition, in only one case was a provision made for the inclusion of chloride, the permeant anion whose transmembrane migration maintains the osmotic equilibrium of the human RBC. Furthermore, orthophosphate behaves as a salting-out anion in the c_sat assay system, by contrast, Bistris acts as a salting-in cation. Accordingly, one would expect that solubilities determined in phosphate buffer would be significantly lower than those obtained in Bistris buffer.

Our near-physiologic Bistris buffer adheres more closely to

Table 2. Solubility of Deoxy-Hb S in the pH Region of c_sat Invariance for Nonphysiologic and Near-Physiologic Buffer Conditions

<table>
<thead>
<tr>
<th>Buffer (mol/L)</th>
<th>pH Range</th>
<th>Species T (°C)</th>
<th>c_sat (g/dL)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 Bistris or 0.1 Tris (nonphysiologic)</td>
<td>6.5-7.0</td>
<td>Hb</td>
<td>18.27 ± 0.21†</td>
</tr>
<tr>
<td>0.1 Bistris or 0.1 Tris (nonphysiologic)</td>
<td>6.5-7.0</td>
<td>Hb-DPG†</td>
<td>15.24 ± 0.47</td>
</tr>
<tr>
<td>0.05 Bistris, 0.10 KCl, 0.02 NaCl (near-physiologic)</td>
<td>6.2-6.8</td>
<td>Hb§</td>
<td>16.10 ± 0.27†</td>
</tr>
</tbody>
</table>

*Mean ± SD.
†Data presented in Fig 1.
‡The c_sat versus pH data of Fig 1 were also fit to a single quadratic in which the pH of minimal solubility corresponds to the isoelectric pH (pI). For Hb, values of 6.81 and 18.01 g/dL were obtained for pl and the minimal solubility, whereas for Hb-DPG, the corresponding values were 6.62 and 15.16 g/dL.
§Data presented in Fig 2.
¶For experiments in which oxy-Hb S was deoxygenated with N₂ and residual met-Hb (<9%) was reduced with 1/4 as much dithionite (0.75 mol/mol heme): 16.7 ± 0.3 g/dL (n = 8) with the suboptimal amount versus 16.4 ± 0.7 g/dL (n = 12) with the optimal amount. Therefore, dithionite has no appreciable effect on the solubility of deoxy-Hb S and may be considered an inert constituent of the c_sat assay system.

†Because it functions as a reducing agent in the c_sat assay and is converted, first to SO₄⁻, then to SO₄³⁻, the dithionite dianion contributes little to the ionic strength of the medium (see Table 2, footnote ‡); the sodium cation contributes an additional 0.025 to the ionic strength. Moreover, in a study in which c_sat was measured by analytical ultracentrifugation, it was noted that runs done without dithionite were no different from those done with it.
Table 3. Solubility of Deoxy-Hb S, in Presence or Absence of 2,3-DPG, Under Near-Physiologic Conditions

<table>
<thead>
<tr>
<th>Species</th>
<th>n</th>
<th>pH (37°C)</th>
<th>c_{sat} (g/dL)</th>
<th>ΔpHi [unit]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb</td>
<td>11</td>
<td>7.18 ± 0.02</td>
<td>19.34 ± 0.14</td>
<td>—</td>
</tr>
<tr>
<td>Hb-DPG</td>
<td>11</td>
<td>7.20 ± 0.01</td>
<td>17.95 ± 0.45</td>
<td>-7.2</td>
</tr>
</tbody>
</table>

Buffer conditions: 0.05 mol/L Bistris, 0.1 mol/L KCl, 0.02 mol/L NaCl, pH 7.20 (nominal), osmolality 280 mOsm/kg; pO₂ = 0 mmHg.

*Mean ± SD.
†Because the measured pH did not correspond exactly to that for Hb-DPG, c_{sat} was normalized to the measured pH 7.20 by use of the value of 10.8 g/dL per pH unit for the slope of the linear plot of c_{sat} versus pH (in the pH range 6.8 to 7.0) presented in Fig 2.
‡DPG/Hb molar ratio = 1.5; c_{sat} was invariant over the range 1.0 to 2.0. This value was chosen because the 2,3-DPG content of sickle erythrocytes is elevated by 30% to 50%.

Because pH, and 2,3-DPG are separate determinants of the solubility of deoxy-Hb S, one must deduce the individual contribution of each variable before assigning a value of c_{sat}, the intrinsic solubility of deoxy-Hb S under intracellular conditions. This may be done as follows: Whereas the measured pH of 7.18 in Table 3 for the DPG-saturated species pertains to the nominal intracellular pH (7.20) of the normal erythrocyte, it has long been known that the arterial hypoxia of sickle cell anemia induces an elevation of intraerythrocyte 2,3-DPG levels which, in turn, evokes an acidification of the RBC interior due to adjustment of the Donnan equilibrium.

The best estimates of pH, for AA and SS RBCs are those obtained by 31P-NMR spectroscopy. Two sets of values have been reported for pH, of AA versus SS RBCs: 7.29 ± 0.08 versus 7.14 ± 0.04 and 7.24 ± 0.07 versus 7.13 ± 0.04, respectively. Thus, fully deoxygenated sickle erythrocytes are more acidic by 0.11 to 0.15 pH unit than normal erythrocytes. The binding of 2,3-DPG to Hb A inside normal erythrocytes has also been measured by 31P-NMR spectroscopy. The results showed that 2,3-DPG bound nearly stoichiometrically to T-state Hb near pH 7, but only weakly to R-state Hb. Similar results were obtained with stripped Hb A in vitro.

A recent investigation of the interaction between 2,3-DPG and Hb A by 1H- and 31P NMR spectroscopy shows that the binding site of 2,3-DPG to HbCO (R-state) contains at least some of the same amino acid residues responsible for binding to the deoxy form. To a first approximation, then, 2,3-DPG binds to intracellular Hb S only when the cell is deoxygenated and its Hb is in the deoxy (T) quaternary structure.

Moreover, because of the alkaline Bohr effect, there is an uptake of protons on deoxygenation of AA RBCs that produces an increase in pH, which the magnitude of the pH increment has been established by a 31P-NMR spectroscopy study which showed that when normal erythrocytes were deoxygenated, pH, increased by 0.14 unit. Thus, the corresponding increase in pH, when sickle erythrocytes are deoxygenated would be from 7.13 to 7.27.

However, this analysis does not take into account the linkage between oxygenation and polymerization. A study of functional abnormalities of sickle cell anemia blood has shown that there is a normal decrease in O₂ affinity in the pH region of pathophysiologic interest (7.0 to 7.2). Thus, the Bohr effect for sickle blood was more than double that of normal; the Bohr coefficients (B_i) were -0.99 and -0.42, respectively. This abnormally high value of B_i for sickle blood could result from an effect of H⁺ on polymerization that depends on O₂ binding. That is, the additional uptake of protons on deoxygenation of SS RBCs should be approximately double that which occurs when AA RBCs are deoxygenated (ie, four, rather than two, protons per Hb molecule). Therefore, the increment in pH, that companions deoxygenation should be roughly twice as large for sickle as for normal blood (ie, a pH increment of 0.28, rather than 0.14 unit). Thus, on deoxygenation, the pH_i of SS RBCs should change from 7.13 to about 7.41. The solubilities corresponding to these two extremes of pH_i as well as that at the intermediate value, are given in Table 4. The solubility at pH 7.41 is 20.2 g/dL and may be considered the best approximation to the intrinsic solubility (c_{sat}) of DPG-saturated deoxy-Hb S under conditions that conform closely, with one exception, to those of the deoxygenated sickle erythrocyte.
PHYSIOLOGIC SOLUBILITY OF DEOXYHEMOGLOBIN S

where \( c, \) is the total Hb concentration, taken as 34 g/dL for a homogeneous RBC population; \( c, \) is the concentration of Hb in the polymer phase, taken as 70 g/dL; and \( c_{\text{sat}} \) is the intrinsic solubility of deoxy-Hb S under conditions in which the 2,3-DPG binding site is saturated and pH, varies according to the uptake of Bohr protons on deoxygenation.

Furthermore, it has been shown\(^4\) that sickling of SS RBCs at half \( O_2 \) saturation was partially inhibited by varying \( pCO_2 \) from 10 to 80 mm Hg, particularly in the higher pH ranges. This is to be expected since carbamate (RNHCOO\(^-\)) formation, in competition with 2,3-DPG binding, becomes greater at higher pH.\(^4\) Moreover, because carbamate formation inhibits polymerization and 2,3-DPG binding promotes it, any interaction between these two physiologic ligands at alkaline pH would raise the value of \( c_{\text{sat}} \) cited above. On balance then, this value of the intrinsic solubility (20.2 g/dL) may represent an underestimation.

Intracellular polymer content depends on the magnitude of the intrinsic solubility of deoxy-Hb S. A reliable estimate of the intrinsic solubility \( c_{\text{sat}} \) of deoxy-Hb S is crucial for calculating the mole fraction of polymer \( f_p \) inside the fully desaturated sickle erythrocyte. Our value of \( c_{\text{sat}} \) (20.2 g/dL) is considerably higher (by 26% and 17%) than either of the two earlier values (16.0 and 17.2 g/dL, respectively) used to estimate \( f_p \) from the conservation of mass equation:\(^30,69\) The theoretical calculations used in the past\(^52,50-52\) to quantitate polymer content under various intracellular conditions need to be revised accordingly. For example, when this is done for the simplest case (homozygous SS disease), the value of \( f_p \), calculated on the basis of our higher value of \( c_{\text{sat}} \), is 0.57 (compared with 0.69 and 0.66, respectively, calculated with the earlier, lower values). Thus, in the correlation between \( f_p \) and disease severity proposed for various sickling syndromes,\(^25\) \( f_p \) will be correspondingly lower when our higher value of \( c_{\text{sat}} \) is used, as will the threshold oxygen saturation at which polymer disappears. Therefore, less polymer may be present in sickle erythrocytes at very high oxygen saturation levels than has been postulated\(^63\) on the basis of equilibrium solubility considerations.

Interdependence between 2,3-DPG concentration and intracellular pH as determinants of solubility. Although other studies have focused on the separate effect of either pH or 2,3-DPG on \( c_{\text{sat}} \), none before this has considered the interdependence between these two intracellular variables. The data presented in Table 3 clearly show that because of the strong dependence of \( c_{\text{sat}} \) on pH in the physiologic range, no disparity in pH between Hb and Hb-DPG can be tolerated if only the effect of 2,3-DPG on \( c_{\text{sat}} \) is to be measured. Lack of appreciation for the interplay between these two variables has undoubtedly contributed to the conflicting results reported over the years\(^11\) with regard to the effect of 2,3-DPG on the solubility of deoxy-Hb S. Our results, in which the effects of pH and 2,3-DPG were carefully dissected, show conclusively that intracellular pH and the allosteric effector 2,3-DPG must both be considered modulators of the solubility of deoxy-Hb S inside the sickle erythrocyte.

Furthermore, it has been demonstrated\(^37\) that the induction of supranormal levels of 2,3-DPG inside normal erythrocytes causes a corresponding decrease in pH, (ie, because 2,3-DPG is an impermeant polyanion, a compensatory chloride/proton shift occurs to maintain the Donnan equilibrium). Therefore, because of the heterogeneity in 2,3-DPG content of sickle erythrocytes (Table 1), a corresponding heterogeneity in pH, values should also exist. Using the empirical correlation derived by Duhm\(^17\) (ie, an increase in intracellular 2,3-DPG by 0.5 mmol/L lowers pH, by 0.01 unit), one can estimate the pH, corresponding to the concentration of 2,3-DPG for any particular sickle blood. This is done in Table 5 for the range of 2,3-DPG levels found in our patient population (Table 1). Estimated pH, values for their deoxygenated RBCs range from 7.48 (at the low end) to 7.34 (at the high end of the 2,3-DPG concentration scale). The corresponding solubilities and mole fractions of polymer at each pH, are also collected in Table 5 (columns 4 and 5). Values of \( c_{\text{sat}} \) encompass a range from 21.0 g/dL for the lowest 2,3-DPG concentration to 19.5 g/dL for the highest, while the respective values of \( f_p \) range from 0.55 to 0.59. Thus, this reciprocal relationship between pH, and intraerythrocytic 2,3-DPG concentration dictates that the amount of intracellular polymer will vary both within a particular patient's RBC population and among subpopulations of

<table>
<thead>
<tr>
<th>pH,</th>
<th>( c_{\text{sat}} ) (g/dL)(^*)</th>
<th>( f_p )</th>
<th>Uptake Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.13</td>
<td>17.19</td>
<td>0.66</td>
<td>None</td>
</tr>
<tr>
<td>7.27</td>
<td>18.71</td>
<td>0.61</td>
<td>R = T transition</td>
</tr>
<tr>
<td>7.41</td>
<td>20.22</td>
<td>0.57</td>
<td>R = T transition</td>
</tr>
</tbody>
</table>

*Estimated by use of the reference value 17.95 g/dL at pH 7.20 (see Table 2) and the slope of the \( c_{\text{sat}} \) versus pH plot from Fig 2 (10.8 g/dL per pH unit).

†Mole fraction of polymer was calculated for each solubility by use of the conservation of mass relationship:

\[
f_p = \frac{c_p (c_0 - c_{\text{sat}})}{c_0 (c_p - c_{\text{sat}})}
\]

where \( c_0 \) is the total Hb concentration, taken as 34 g/dL for a homogeneous RBC population; \( c_p \) is the concentration of Hb in the polymer phase, taken as 70 g/dL; and \( c_{\text{sat}} \) is the intrinsic solubility of deoxy-Hb S under conditions in which the 2,3-DPG binding site is saturated and pH, varies according to the uptake of Bohr protons on deoxygenation.
Table 5. Interrelationship Between 2,3-DPG Content and Intracellular pH of Sickle Erythrocytes

<table>
<thead>
<tr>
<th>2,3-DPG (μmol/mL)</th>
<th>pH* (estimated)†</th>
<th>c0.5 [g/dL]‡</th>
<th>f§</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5</td>
<td>0.73</td>
<td>7.48</td>
<td>20.98</td>
</tr>
<tr>
<td>4.8</td>
<td>1.00</td>
<td>7.44</td>
<td>20.54</td>
</tr>
<tr>
<td>6.1</td>
<td>1.27</td>
<td>7.41</td>
<td>20.22</td>
</tr>
<tr>
<td>7.0</td>
<td>1.46</td>
<td>7.39</td>
<td>20.00</td>
</tr>
<tr>
<td>8.0</td>
<td>1.67</td>
<td>7.37</td>
<td>19.79</td>
</tr>
<tr>
<td>9.5</td>
<td>1.98</td>
<td>7.34</td>
<td>19.46</td>
</tr>
</tbody>
</table>

*Range of 2,3-DPG content of sickle erythrocytes in our patient population, expressed relative to that of normal erythrocytes; mean values for each class are (in mmol/L): normal, 4.82 ± 0.55; sickle, 6.10 ± 1.00 (see Table 1).
†Based on a pH of 7.41 for unfractionated, completely deoxygenated sickle erythrocytes (see text). Other pH values were estimated by use of the empirical relationship established for the induction of elevated 2,3-DPG levels in normal RBCs: an increment of 0.5 mmol/L in 2,3-DPG concentration produces a decrement of 0.01 unit in pH.
‡Based on an average intrinsic solubility of 20.22 g/dL for deoxy-Hb S at pH of 7.41 inside the fully desaturated sickle erythrocyte. Other solubility values were calculated by use of the pH values in column 3 and the slope of the c0.5 versus pH profile from Fig 2 (10.8 g/dL per pH unit).
§Mole fraction of polymer was calculated for each pH, and concentration of 2,3-DPG according to the conservation of mass relationship given in Table 4, footnote †.

RBCs from various patients according to the 2,3-DPG content of those erythrocytes. Moreover, one can predict that those patients with relatively high 2,3-DPG levels in their RBCs should have lower pH values than patients whose 2,3-DPG levels are relatively low. All else being equal, one would also expect there to be more sickling in the relatively acidotic RBCs as well.§ In any case, because the desolubilizing effect of 2,3-DPG on solubility is manifest only until its binding site in the β-cleft is saturated, any further effect on c0.5 beyond this point must be mediated by Donnan-induced pH changes.57

Among those factors influencing intracellular polymerization, which include oxygen saturation, non-Hb S composition, and Hb concentration, the interdependent variables of 2,3-DPG and pH, should also be included now. Because the amount of polymer produced during partial deoxygenation in the microcirculation is the ultimate determinant of sickling,§ a treatment modality based on the sustained reduction of intraerythrocytic 2,3-DPG should be therapeutically beneficial.

ACKNOWLEDGMENT

We are grateful to Drs John Kark and Richard Labotta for careful reading of the manuscript, and to Dr Oswaldo Castro for providing blood samples for the study.

§That lowering pH, increases the propensity of deoxy-Hb S to polymerize, and also increases cell sickling, is well-documented.34,36 Furthermore, part of this pH effect is independent of O2 binding, indicating that ionization of side chains at regions of intermolecular contact contributes at least as much to the overall polymerization-linked effect as does the Bohr effect.

REFERENCES

PHYSIOLOGIC SOLUBILITY OF DEOXYHEMOGLOBIN S

1035


40. Tehrani AY, Lam YF, Lin AK, Dosch SF, Ho C: Phosphorus-31 nuclear magnetic resonance studies of human red blood cells. Blood Cells 8:245, 1982


43. Russu IM, Wu SS, Bupp KA, Ho NT, Ho C: 1H and 31P nuclear magnetic resonance investigation of the interaction between 2,3-diphosphoglycerate and human normal adult hemoglobin. Biochemistry 29:3785, 1990


From www.bloodjournal.org by guest on September 24, 2017. For personal use only.

54. Lange RD, Minnich V, Moore CV: Effects of oxygen tension and pH on the sickling and mechanical fragility of erythrocytes from patients with sickle cell anemia and sickle cell trait. J Lab Clin Med 37:789, 1951


2,3-Diphosphoglycerate and intracellular pH as interdependent determinants of the physiologic solubility of deoxyhemoglobin S

WN Poillon and BC Kim