Antisickling Effects of 2,3-Diphosphoglycerate Depletion

By W.N. Poilllon, B.C. Kim, R.J. Labotka, C.U. Hicks, and J.A. Kark

Elevation of 2,3-bisphosphoglycerate (2,3-DPG) in sickle erythrocytes (SS RBCs) and concomitant acidification of the cell interior promote polymerization by decreasing the solubility (c∞) of deoxygenated S. The antisickling effect of 2,3-DPG depletion was evaluated after activation of the 2,3-DPG phosphatase activity of bisphosphoglycerate mutase by glycolate-2-phosphate, leading to rapid loss of intracellular 2,3-DPG. To ensure its maximal reduction in a physiologic medium, isosmotic CO2/bicarbonate-buffered saline, pH 7.0, was used. Substitution of K+ for Na+ as the major extracellular cation suppressed K,Cl cotransport, prevented cell shrinkage, and allowed demonstration of the full antisickling effect of 2,3-DPG depletion. The modest effect on solubility per se of removing intraerythrocytic 2,3-DPG (Δc∞ = 1.6 g/dL) was amplified into a much larger antisickling effect by interaction with three other cellular variables affecting solubility and polymer content (intracellular pH, O2 saturation, and mean cell hemoglobin concentration). Acting in concert, these four antisickling effects (three solubilizing, one osmotic) reduced polymer fraction of glycolate-treated SS RBCs by 32% to 63%, with a concomitant decrease in sickling of 46% to 95% at the nominal pO2 of the microcirculation (20 mm Hg). A decrement in sickling of this magnitude should significantly ameliorate the vasoocclusive severity of sickle cell disease. © 1995 by The American Society of Hematology.

As a constituent of the sickle erythrocyte (SS RBC), 2,3-bisphosphoglycerate (2,3-DPG) is not entirely benign. An earlier report showed that 2,3-DPG and intracellular pH (pHi) are separate but interdependent determinants of the intrinsic solubility of deoxygenated S (deoxy-Hb S) in SS RBCs. That is, at pH 7.2, the nominal pH of the human red blood cell, the equilibrium solubility (c∞) of fully deoxygenated, 2,3-DPG-saturated Hb S (Hb-DPG) is 8.2% ± 2.3% lower than that of stripped deoxy-Hb S. This indicates that the binding of 2,3-DPG in the β-cleft promotes gelation by a slight distortion of the T-state. Furthermore, the steep decrease in solubility observed for both Hb and Hb-DPG as the pH was lowered from 7.6 to 6.8 indicates that intracellular acidity is also a determinant of c∞. Thus, the binding of 2,3-DPG to deoxy-Hb S and the cytoplasmic acidification resulting from the variable elevation of 2,3-DPG in SS RBCs compared with normal, act in concert to decrease the solubility of deoxy-Hb S. Sustained reduction of this glycolytic intermediate in SS RBCs should, therefore, provide a definite antisickling effect.

2,3-DPG metabolism occurs at a branch point in the pathway of anaerobic glycolysis, the Rapaport-Luebering shunt, that is erythroid cell-specific. The two enzymatic activities that govern 2,3-DPG metabolism reside in a single protein, 3-phosphoglycerate mutase (BPGM; 3-phospho-D-glycerate 1,2-phosphomutase; EC 5.4.2.4), which, in addition to synthesizing and hydrolyzing glycerate 2,3-bisphosphate, also catalyzes the isomerization of 3-phosphoglycerate to 2-phosphoglycerate (monophosphoglycerate mutase activity). These three activities occur at the same active site of erythrocyte BPGM. Because the unstimulated phosphatase activity of BPGM is low (approximately 1,000-fold lower than its synthase activity), the steady-state concentration of 2,3-DPG is very high in normal human erythrocytes (4 to 6 μmol/mL RBC).

An earlier study of the effect of 2,3-DPG depletion on sickling used the divalent anion metabisulfite S2O52- to stimulate 2,3-DPG phosphatase activity and produce SS RBCs devoid of 2,3-DPG. That study showed a significant inhibition of sickling in 2,3-DPG-depleted cells. It also showed that stimulation of 2,3-DPG to supranormal levels by incubation of SS RBCs with inosine, pyruvate, and orthophosphate markedly potentiated sickling. Thus, the tendency of SS RBCs to sickle is very sensitive to the intracellular concentration of 2,3-DPG.

Glycolate-2-phosphate (2-PG) was shown by Rose to rapidly deplete normal erythrocytes (AA RBCs) of 2,3-DPG during brief incubations in isosmotic medium at physiologic pH and temperature. 2-PG, a minor metabolite of the human erythrocyte, is an extremely potent stimulator of the 2,3-DPG phosphatase activity of BPGM at supranormal concentrations. As a nondiffusible polyanion, it must be generated inside the red cell by the action of pyruvate kinase on glycolate. We sought to establish, therefore, whether exposure of SS RBCs to glycolate would generate sufficient 2-PG to deplete 2,3-DPG in a like manner to AA RBCs and, if so, what effect this would have on sickling. To do this, however, our incubations were performed at slightly lower pH (7.0) in a physiologic medium that contained K+, rather than Na+, as the major extracellular cation. This was necessary to maximize 2,3-DPG depletion without causing cell shrinkage due to activation of K,Cl cotransport in oxygenated sickle erythrocytes. Our results show that the tendency of SS RBCs depleted of 2,3-DPG to sickle is considerably less than that for 2,3-DPG-replete controls. This metabolic intervention approach, therefore, has potential as an antisickling therapy.

MATERIALS AND METHODS

Materials and instrumentation. Glycolic acid, inosine, glucose, and bovine serum albumin were purchased from Sigma Chemical Co, St Louis, MO. All other chemicals were reagent grade. The O2

From the Center for Sickle Cell Disease and the Department of Pediatrics and Child Health, Howard University College of Medicine, Washington, DC; the Division of Hematology/Oncology, Department of Pediatrics, University of Illinois at Chicago College of Medicine, Chicago, IL; and the Department of Hematology, Division of Medicine, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, DC.

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Address reprint requests to W.N. Poillon, PhD, Department of Pediatrics (M/C 8556), University of Illinois at Chicago College of Medicine, 840 S Wood St, Chicago, IL 60612.

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affinities of control and treated cells were measured in a Hemox Analyzer (TCS Medical Products Co, Huntingdon Valley, PA), which was also used to evaluate sickling as a function of P02.

**Cell preparation.** Informed consent was obtained before blood collection from adult sickle cell anemia (SCA) patients in the steady state who were homozygous for Hb S (Hb SS). Blood specimens (approximately 30 mL) were drawn in tubes anticoagulated with heparin and used for metabolite depletion experiments within 24 hours. Hemoglobin phenotypes (SS) were verified by cellulose acetate electrophoresis at pH 8.6 (Helena Labs, Beaumont, TX). Blood was centrifuged at 670g for 10 minutes, followed by aspiration of plasma and buffy coat. Packed RBCs were washed twice in isotonic saline and resuspended [hematocrit (Hct), 10%] in an isosmotic high-K medium (CO2/bicarbonate-buffered saline), in which K+ replaced Na+ as the extracellular cation. This medium mimics closely the cationic composition of the cytoplasm. Control medium consisted of 115 mmol/L KCl, 2 mmol/L CaCl2, 1 mmol/L MgCl2, 2 mmol/L Na3HPO4, 1 mmol/Lucose, 24 mmol/L NaHICO3, and 10 mmol/L glucose, with a final pH of 7.4 (37°C) obtained by bubbling with CO2 (5.6%) for 30 minutes to achieve a PCO of 40 mm Hg. Medium for treated cells contained 30 mmol/L glycine and 5 mmol/L glucose at a final pH of 7.0 (37°C). Osmolality was maintained at 290 mOsm/kg by reducing KCI to achieve a final K+ concentration of 115 mmol/L KCl, 5 mmol/L NaCl, 2 mmol/L CaCl2, 1 mmol/L MgCl2, 2 mmol/L Na3HPO4, 1 mmol/L glucose, and 24 mmol/L NaHICO3, and 10 mmol/L glucose, with a final pH of 7.4 (37°C) obtained by bubbling with CO2 (5.6%) for 30 minutes to achieve a PCO of 40 mm Hg. Medium for treated cells contained 30 mmol/L glycine and 5 mmol/L glucose at a final pH of 7.0 (37°C). Osmolality was maintained at 290 mOsm/kg by reducing KCl to 100 mmol/L.

**General methods.** Mean cell hemoglobin concentration (MCHC) was determined using the spun Hct, and the hemoglobin concentration was measured with Drabkin’s reagent.57 Hemoglobin F was measured by alkali denaturation.58 Red blood cell adenosine triphosphate (ATP) and 2,3-DPG levels were measured using Sigma kits. Oxygen dissociation curves (ODCs) were measured on whole blood (at an Hct of 0.5%) with a Hemox analyzer in an N-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES)-based isosmotic buffer, pH 7.4 at 37°C.9 Red blood cell density profiles were measured by phthalate ester flotation.20,22 Two parameters were extracted from each density profile: (1) Dso, the median density, and (2) the percentage of dense cells (ie, density greater than 1.12 and MCHC greater than 37 g/dL).22 Extent of sickling was assessed with a Leitz Orthoplan microscope equipped with Nomarski interference optics (Leitz Co, Rockleigh, NJ) and an (×100) oil immersion objective.57,74 Two observers, blinded to the identity of glutaraldehyde-fixed samples, evaluated the percentage of reversibly sickled cells (%RSC). The number of cells classified as normal, sickled, or deformed (300 to 500) was chosen to identify a 5% decrease in sickling. Cells were considered reversibly sickled (RSCs) if they had sharp, pointed projections (spicules); other aberrant morphologies were considered deformed.57 In most cases, greater than 80% of the sum of reversibly sickled and deformed cells were RSCs. No correction was made for ISC counts because they were so low (less than 5%). From plots of %RSC versus PO2 and %O2 saturation (%HbO2), two sickling parameters were derived: (1) %RSC at PO2 equal to 20 mm Hg, the predicted PO2 of fully deoxygenated RBCs.44 Simultaneously with the generation of ODCs for control or treated cells, samples at O2 tensions in the range of pathophysiologic interest were fixed in 2% glutaraldehyde in phosphate-buffered saline, pH 7.4, for evaluation of morphologic sickling, as described above.

**Depletion of 2,3-DPG by treatment with glycolate.** Washed erythrocytes were suspended to an Hct of 0% in isosmotic CO2/bicarbonate-buffered saline, pH 7.4 for control cells and 7.0 for treated cells. After incubation for 6 hours at 37°C with shaking and bubbling with CO2 (5.6%), packed, and resuspended to the original Hct. Washed RBCs were stored overnight at 4°C before equilibration at 37°C in the sample chamber of a TCS Hemox Analyzer. Simultaneously with the generation of ODCs for control or treated cells, samples at O2 tensions in the range of pathophysiologic interest were used to monitor intracellular pH and 2,3-DPG levels during incubations at 37°C for 5 hours. Washed erythrocytes (normal or sickle) were suspended in control buffer (isomotic HEPES-buffered saline, pH 7.4) and incubated at 37°C for 1 hour to equilibrate diffusible anions. Packed cells were then resuspended (Hct 40%) in control buffer in the absence or presence of 30 mmol/L glycolate. NMR spectra were obtained at 37°C on a Nicolet NIC 360NB spectrometer (Nicolet Instruments Corp, Madison, WI) operating at 146.16 MHz for 31P, using the protocol of Laboka and Kleps.7 The 31P signal was used to monitor 2,3-DPG.26 Control and glycolate-treated cell suspensions contained methylphosphonate (15 mmol/L) as an intracellular pH reference.27,28

**Quantitation of cellular variables that affect polymerization.** Five variables determine the intracellular polymerization of partially liganded Hb S that underlies sickling: (1) 2,3-DPG concentration, (2) intracellular pH (pHi), (3) hemoglobin composition, (4) O2 saturation, and (5) total hemoglobin concentration (ct). Each of these variables was measured directly for control and glycolate-treated SRS RBCs, except pHi, which was determined indirectly by use of appropriate 2,3-DPG concentrations and the relationship between pHi and 2,3-DPG deduced by Duhm9 for AA RBCs with supranormal levels of 2,3-DPG (ΔpH/Δ2,3-DPG = −0.023 pH unit/mmol/L 2,3-DPG). Thus, at 37°C the solubility of 2,3-DPG–saturated deoxy-Hb S (Hb-DPG) is 8.2% ± 2.3% lower (Δcct = −1.6 g/dL; eq 1a) than that of stripped deoxy-Hb S at pH 7.2.1 Using the slope of the cct versus pH profile for deoxy-Hb S in isomotic Bistris buffer over the pH range of 6.8 to 7.6 (10.8 g/dL per pH unit2; eq 1b), a value of 18.0 g/dL is obtained for cct, the intrinsic solubility of Hb-DPG at pH 7.41, the predicted pH of fully deoxygenated SS RBCs. [This cct value has been corrected for the solubilizing effect of the buffering species (0.05 mol/L Bistris), which amounts to a decrement of 2.2 g/dL; the value given by Poillon and Kiml is higher by this amount.] The sparing effect of Hb F and Hb A2 may be evaluated from the dependence of cct on %Hb (F + A2) (0.334 g/dL per 1% non-S Hb; eq 2). The variation of cct, with O2 saturation was estimated from an empirical equation (eq 3) derived by others.31 The effect of each of these four variables on cct can be summarized as:

\[
\text{2,3-DPG} / \text{pH} = \Delta c_{ct} / \Delta \text{pH} = -1.6 \text{ g/dL (Hb-DPG - Hb)} \quad (1a)
\]

\[
\Delta c_{ct} / \Delta \text{pH} = 10.8 \text{ g/dL/pH unit} \quad (1b)
\]

\[
\text{Non-S hemoglobins:} \quad \Delta c_{ct} / \Delta \text{pH} = 10.8 \text{ g/dL/pH unit} \quad (1b)
\]

\[
\text{Ligand saturation:} \quad c_{ct} = c_{ct}^0 + 9.24y + 9.80y^2 + 23.5y^3 \quad (3)
\]

\[
\text{where } c_{ct}^0 \text{ is the intrinsic solubility of Hb-DPG at pH 7.41, the predicted pH of fully deoxygenated SS RBCs.}
\]

A fifth variable, MCHC (identical to cct, total hemoglobin concentration), influences polymerization by its neideal behavior at high Hb S concentration.

Knowledge of these five variables permits calculation of the mean polymer fraction (fct) for individual cell samples without direct measurement of cct, the equilibrium solubility of intraerythrocytic HbS. This is achieved by substituting the appropriate values of cct, cct, and cct (polymer concentration)32 into the conservation of mass equation37 (see notes to Table 4).
RESULTS

Effect of glycolate on intracellular pH. Using 31P-NMR spectroscopy, we measured the effect of incubation with glycolate in isosmotic HEPES-buffered saline, pH 7.4, on pH, and 2,3-DPG concentration for both AA and SS RBCs. Results are shown in Table 1. In both cases, loss of 2,3-DPG in glycolate-treated cells was substantially greater than in controls (respectively, 74% v 22% for AA and 51% v 9% for SS). Furthermore, for both cell types, pH, was higher for 2,3-DPG-depleted cells than for controls (by 0.09 unit for AA and 0.08 unit for SS cells). These increments in pH, agree well with those calculated using the Duhm factor (see Materials and Methods; 0.08 and 0.07, respectively). This result validates use of this factor to estimate pH, in control and glycolate-treated SS RBCs.

Effect of glycolate on O2 affinity and sickling tendency. Results for a representative experiment on glycolate-induced depletion of 2,3-DPG in isosmotic high-K medium are shown in Table 2. ATP values were relatively unchanged for control and treated cells after 6-hour incubation, whereas 2,3-DPG concentrations increased by 6% for control cells and decreased by 83% for treated cells. MCHC, which reflects cellular hydration status, decreased for both control and treated cells after 6-hour incubation (by 2.1 and 4.3 g/dL, respectively), indicating that cell swelling was twice as large in the glycolate-treated cases.

The effect of 2,3-DPG depletion on %RSC as a function of pO2 at 5 to 51 mm Hg is shown in Fig 1, with the corresponding plot for O2 saturation shown in Fig 2. For control cells, %RSC increased gradually below 60% saturation, to a value of 62% near complete deoxygenation; for treated cells, %RSC was relatively flat throughout the range of O2 tension examined, ranging from 3% at 31 mm Hg to 21% at 5 mm Hg. Thus, near full desaturation, the sickling tendency of 2,3-DPG–depleted SS RBCs was lower than that of 2,3-DPG–replete cells by 66%.

The two sickling parameters that characterize SS RBCs in isosmotic CO2/bicarbonate-buffered saline (derived from Fig 1) are shown in Table 2. Sickling parameter 1 (%RSC at pO2 = 20 mm Hg, the nominal O2 tension of the microcirculation) was 29% lower for treated cells. A reduction of this magnitude reflects the significant decrease of polymer content in SS RBCs devoid of 2,3-DPG. Sickling parameter 2 (%RSC at P50) was 11% lower for treated cells, indicative of inhibition of intracellular gelation independent of changes in O2 affinity. The percentage decrease in sickling (71% and 48%, respectively) shows that the sickling potential of these 2,3-DPG–depleted SS RBCs has been substantially diminished.

<table>
<thead>
<tr>
<th>System</th>
<th>Incubation Time (h)</th>
<th>MCHC (g/dL)</th>
<th>ATP (μmol/mL RBC)</th>
<th>1,3-DPG (μmol/mL RBC)</th>
<th>P50 (mm Hg)</th>
<th>1</th>
<th>2</th>
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<tr>
<td>Control (pH 7.4)</td>
<td>0</td>
<td>36.4</td>
<td>1.10</td>
<td>7.45</td>
<td>27.5</td>
<td>41</td>
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<tr>
<td></td>
<td>6</td>
<td>34.3</td>
<td>1.16</td>
<td>7.92</td>
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<tr>
<td>Treated (pH 7.0)</td>
<td>0</td>
<td>35.0</td>
<td>1.15</td>
<td>6.57</td>
<td>19.5</td>
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<td>6</td>
<td>30.7</td>
<td>1.23</td>
<td>1.34†</td>
<td></td>
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</tbody>
</table>

† These data pertain to patient A in Table 3.
* Derived from ODCs used to generate sickling curves shown in Fig 1.
† These parameters are derived from Fig 1 as (parameter 1) reversibly sickled cells (%RSC at pO2 = 20 mm Hg, the nominal O2 tension of the microvasculature, and (parameter 2) %RSC for O2 tension at half-saturation (P50).
‡ The low 2,3-DPG concentration in treated cells is probably an overestimate, because its detectable limit by the coupled enzymatic oxidation of reduced nicotinamide-adenine dinucleotide (NADH) is being approached.
Red cell density profiles shown in Fig 3 for control and treated cells indicate that at the inflection point (D\textsubscript{50}), glycolate-treated SS RBCs have densities lower than controls by 0.012 unit. Thus, 2,3-DPG-depleted sickle erythrocytes are considerably more hydrated than controls because of adjustment to the osmotic equilibrium by which Cl\textsuperscript{−} and H\textsuperscript{+} are translocated across the cell membrane.

**Osmotic and antisickling effects of 2,3-DPG depletion.** We evaluated in detail the osmotic and antisickling effects of 2,3-DPG depletion on erythrocytes from four Hb SS patients; results are presented in Table 3. The percentage of dense cells in whole blood was much less for patient A (32%) than for patients B through D (range, 8.7% to 21.8%), presumably reflecting the sparing effect on polymerization of the elevated Hb F level for patient A. Composite data on the difference between treated and control cells for six measured (\Delta A, \Delta P\textsubscript{50}, \Delta MCHC, \Delta D\textsubscript{50}, and \Delta sickle parameters 1 and 2) and two calculated (\Delta c\textsubscript{un} and \Delta f\textsubscript{p}) parameters show the following: (1) a reduction in 2,3-DPG concentration that varied from −37 to −6.6 mmol/L, corresponding to a loss of 2,3-DPG of 69% to 83%; (2) a significant increase in O\textsubscript{2} affinity shown by a decrement in P\textsubscript{50} ranging from −8.0 to −10.5 mm Hg; (3) a reduction in cell density with good correlation (r = 0.986) between \Delta D\textsubscript{50} and \Delta MCHC, the two parameters that reflect the cell’s hydration status; (4) an inhibition of sickling at pO\textsubscript{2} = 20 mm Hg, the nominal O\textsubscript{2} tension of the capillary bed (sickling parameter 1), that was greatest for patient B (95%) and least for patient D (46%); (5) an inhibition of sickling at half-saturation (sickling parameter 2) that was greatest for patient B (68%) and least for patient D (11%); (6) an increase in c\textsubscript{un} estimated (see Materials and Methods) to vary from 4.4 to 6.0 g/dL, and (7) a decrease in the calculated value of f\textsubscript{p} that ranged from −0.18 to −0.33, corresponding to decrements of 53%, 63%, 39%, and 32% for sickle erythrocytes from patients A, B, C, and D, respectively.

**Direct and indirect effects of 2,3-DPG depletion on intracellular polymerization.** Table 4 quantitates all intra-erythrocytic variables (2,3-DPG, pH\textsubscript{i}, MCHC, and O\textsubscript{2} saturation) that interact to affect the polymerization of partially liganded Hb S in a 2,3-DPG–dependent fashion. Non-S hemoglobins (Hbs F and A\textsubscript{2}) also inhibit polymerization, as shown in our recent report,\textsuperscript{30} but this sparing effect is independent of the cell’s 2,3-DPG status. [In any case, for the patient from Table 3 (B) chosen for this analysis, the Hb F concentration is so low (3.0%) that its solubilizing effect (<1.0 g/dL\textsuperscript{30}) can be ignored.] Overall inhibition of polymerization has been dissected into the component contributions made by each of the four variables. Those due to DPG/pH\textsubscript{i} and MCHC (direct effects) have been calculated on the basis of solubilizing or osmotic effects with Hb S in the unliganded state (pO\textsubscript{2} = 0). The effect of oxygenation on solubility (an indirect effect) was then calculated for the measured O\textsubscript{2} saturations of control and glycolate-treated cells. A reasonable estimate for the values of pH\textsubscript{i} corresponding to 2,3-DPG–replete and 2,3-DPG–depleted sickle erythrocytes at each stage can be made as follows: for unliganded Hb S, loss of that portion of the Bohr effect due to 2,3-DPG binding\textsuperscript{30,35} would lower pH\textsubscript{i} by 0.05 unit, while the compensatory osmoregulatory chloride/proton shift necessary to maintain electroneutrality would raise pH\textsubscript{i} by 0.12 unit; the net loss of that portion of the Bohr effect due to 2,3-DPG binding\textsuperscript{30,35} would lower pH\textsubscript{i} by 0.05 unit, while the compensatory osmoregulatory chloride/proton shift necessary to maintain electroneutrality would raise pH\textsubscript{i} by 0.12 unit; the net loss of that portion of the Bohr effect due to 2,3-DPG binding\textsuperscript{30,35} would lower pH\textsubscript{i} by 0.05 unit, while the compensatory osmoregulatory chloride/proton shift necessary to maintain electroneutrality would raise pH\textsubscript{i} by 0.12 unit; the net loss of that portion of the Bohr effect due to 2,3-DPG binding\textsuperscript{30,35} would lower pH\textsubscript{i} by 0.05 unit, while the compensatory osmoregulatory chloride/proton shift necessary to maintain electroneutrality would raise pH\textsubscript{i} by 0.12 unit; the net
Treatments in columns 4 through 9 refer to the measured difference between glycolate-treated and control cells after incubation in isosmotic CO2bicarbonate (high-K) medium for 6 hours at 37°C; those in columns 10 and 11 have been calculated by the treatment given in Materials and Methods by which the four variables that affect polymerization in a 2,3-DPG–dependent manner are quantitated.

* Values are for whole blood, before treatment.

† See notes to Table 2 for definition of sickling parameters 1 and 2; numbers in parentheses refer to percentage reduction in each parameter relative to controls.

‡ Calculated from the conservation of mass equation as the difference in f, for treated v control cells with the measured values of c, (MCHC) and estimated values of c sat corresponding to the partial saturation at PO2 = 20 mm Hg; the value of 69.3 g/dL was used for c, the polymer concentration.

estimate c sat for partially oxygenated Hb S. Using this relationship (see Materials and Methods), we were able to calculate values of c sat for stripped Hb and 2,3-DPG–saturated Hb S (Hb-DPG) under conditions of O2 saturation that correspond to a PO2 of 20 mm Hg (34% and 59%, respectively). Values of c sat so obtained were 20.5 and 26.5 g/dL. (Incorporated into these numbers are decrements in c sat for Hb and Hb-DPG due to the loss of Bohr protons arising from partial saturation and depolymerization.) The corresponding values of f, were 0.52 and 0.19, an overall reduction in polymer content of 63% for 2,3-DPG–depleted cells.

### DISCUSSION

**Interplay of cellular variables that inhibit polymerization and retard sickling of 2,3-DPG–depleted SS RBCs.** Because incubation of treated cells at pH 7.4 led to incomplete depletion of their 2,3-DPG, it was necessary to reduce the pH to 7.0 and change the extracellular cation from Na+ to K+ to prevent potassium efflux. Under these conditions, 2,3-DPG was maximally depleted with minimal cell shrinkage.

In a preliminary experiment using the noninvasive technique of 31P-NMR spectroscopy to monitor pH (Table 1), it was found that for both AA and SS RBCs, partial depletion

| Table 3. Osmotic and Antisickling Effects of 2,3-DPG Depletion on Erythrocytes From Four SCA Patients With Varying Levels of Hb F |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Patient        | Dense Cells (%) | % Hb F (mmol/L)| Δ2,3-DPG (mg/dL)| ΔMCHC (g/dL) |
| A              | 3.2             | 17.2            | -6.6            | -3.6           |
| B              | 12.1            | 3.0             | -4.4            | -2.3           |
| C              | 8.7             | 0.8             | -4.5            | -1.6           |
| D              | 21.8            | 1.9             | -3.7            | -0.3           |

### Table 4. Direct (c sat, c) Versus Indirect (oxygenation-dependent) Effects of 2,3-DPG Depletion on Intracellular Polymerization

<table>
<thead>
<tr>
<th>Parameter Affected</th>
<th>Species</th>
<th>Estimated pH,</th>
<th>2,3-DPG (mmol/L)</th>
<th>% HbO2</th>
<th>c (g/dL)</th>
<th>c sat (g/dL)</th>
<th>f, (mmol/L)</th>
<th>Intracellular Variable</th>
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<tr>
<td>c sat</td>
<td>Hb-DPG</td>
<td>7.41</td>
<td>6.16</td>
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<td>1.75</td>
<td>595</td>
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</tbody>
</table>

**These data pertain to patient B in Table 3. Values of 2,3-DPG concentration (%HbO2 and pO2 = 20 mm Hg), and c (ie, MCHC) were measured before and after incubation in isosmotic CO2bicarbonate (high-K) medium for 6 hours at 37°C. Because the 2,3-DPG assay tends to overestimate low concentrations, depletion was assumed to be complete for the glycolate-treated species (despite its measured value of 1.75 mmol/L). Values of pH1, c sat, and f were calculated according to the treatment outlined in Materials and Methods.**

**Based on the physiologic solubility of deoxy-Hb S presented in an earlier report.** Measurements were made for stripped Hb and Hb-DPG in 9.05 mol/L BisTris, 0.1 mol/L KCl, 0.02 mol/L NaCl, pH 7.2 (290 mOsm/kg H2O) at 37°C (see Materials and Methods for derivation of individual values and have been corrected for the solubilizing effect of BisTris (46 g/dL per mol/L). Calculated from conservation of mass relationship: f, = c sat/ c - c sat/ cMCHC - c sat) where f, is the polymer fraction and c sat, c, and c sat are the polymer, total hemoglobin, and saturation concentrations, respectively. (See Table 3, footnote f for details).**

**Release of Bohr protons on titration caused by shift of allosteric equilibrium and depolymerization lowers pH by 0.10 and 0.15 units for control and treated cells, respectively.**

**Oxygen saturation at the nominal pO2 of the tissue capillary bed (20 mm Hg).**

**Estimated from the empirical relationship between c sat and fractional O2 saturation presented in Materials and Methods (based on Fig 4 in Sunshine et al).**
of 2,3-DPG resulted in alkalinization of the cell, the magnitude of which agreed well with that estimated using the Duhm factor for AA RBCs enriched in 2,3-DPG (−0.023 pH unit/mmol/L, 2,3-DPG).28 This finding allowed us to calculate pH i for our experiments, based on 2,3-DPG concentrations and the dependence of pH i on 2,3-DPG concentration.

The principal observation of this study was the finding that depletion of 2,3-DPG to levels below 0.8 to 2.0 mmol/L (probably overestimates; see notes to Table 2) resulted in a considerable reduction in the sickling tendency of erythrocytes from four different patients. Results indicate that the antisickling effect at the pO2 of the microcirculation (20 mm Hg) is considerably greater for patients A and B (71% and 95%, respectively) than for patients C and D (46% and 48%). Values for the antisickling effect at half-saturation (column 9 in Table 3) are similarly affected. Although there is no obvious explanation for this variation in effects on reversible sickling for the four patients, Hb F levels do not appear to play a role, because the reduction in sickling for patient A is less than for patient B (71% v 95%, respectively). This disparity somehow involves departure from an assumption necessary to this analysis—that the four variables affecting polymerization in a 2,3-DPG–dependent manner (2,3-DPG/ pH i, O2 saturation, and MCHC) are distributed homogeneously throughout the red blood cell population. The actual heterogeneous distribution of these variables may account for the individual variations in antisickling observed. Nevertheless, the order of the three measures of antisickling (%RSC at pO2 of 20 mm Hg and at pO2, and calculated Δf s) are identical for the four patients, showing that both measured and calculated indices of sickling and polymer content are internally consistent.

The data in Table 3 show that the depletion of 2,3-DPG from SS RBCs by manipulation of glycolysis significantly retards their propensity to sickle after deoxygenation. Furthermore, the data in Table 4 show that the relatively modest solubilizing effect of 2,3-DPG depletion per se is amplified into a much larger one by the concomitant alkalinization and increase in O2 affinity of the cell. These effects on c sat are augmented by the compensatory osmotic swelling that lowers MCHC. Overall, then, four intraerythrocytic variables (2,3-DPG concentration, pH i, O2 saturation, and MCHC) interact to inhibit polymerization and substantially reduce sickling in SS RBCs devoid of 2,3-DPG.

Therapeutic potential of metabolic intervention in sickle erythrocytes. Three strategies would produce a therapeutic inhibition of the intracellular polymerization that underlies sickling: (1) direct inhibition by elevation of c sat; (2) indirect inhibition by increasing O2 affinity, thereby stabilizing the more soluble oxy conformer; and (3) decrease of intracellular hemoglobin concentration (MCHC). It is evident from Table 3 that 2,3-DPG depletion evokes changes in all three of these variables, the sum of which leads to the overall inhibition of polymerization and reduction in sickling observed. For the most favorable patient (patient B) in Table 3, f s is reduced by 63% to a value (0.19) that approaches that of sickle trait erythrocytes at 25% O2 saturation (0.16).

What are the prospects for achieving such a profound antisickling effect in SCA patients? The major obstacles to inducing the removal of 2,3-DPG from SS RBCs in vivo are (1) the nonpenetrating nature of 2-PG, the activator of the 2,3-DPG phosphatase activity of BPGM, and (2) the concentration of glycolate (30 mmol/L) in our experiments, which is too high for safe human use. Analogues of 2-PG could be examined to find a nontoxic compound that similarly stimulates the phosphatase activity of BPGM at micromolar concentrations. Among the candidates would be hydroxypropyruvate, whose phosphorylated form, 3-phosphohydroxypropyruvate (3-PHP), is 10 times more potent than 2-PG in activating BPGM,41 and the phosphonate analogues of 2-PG and 3-PHP, which should be considerably more membrane-permeable than the parent compounds.42

An alternative approach would be genetic therapy to reduce concentrations of 2,3-DPG in vivo to negligible levels. A mutant BPGM expressed in Escherichia coli has recently been described43 whose phosphatase activity was greatly enhanced and synthase activity significantly decreased. Thus, loss of 2,3-DPG from the sickle erythrocyte can be effected by molecular genetic or metabolic alterations of the enzyme that controls its intracellular concentration. Either approach is inherently simpler than those based on manipulation of the β-globin–like gene complex. Our metabolic intervention strategy is especially attractive because it involves no recombinant DNA technology to achieve amelioration of sickling.

Loss of 2,3-DPG from SS RBCs produced a variable decrease in MCHC (Table 3, column 6). Because incubations were short (6 hours), it is unknown whether such cell volume changes would persist during the life span of the erythrocyte in vivo. A single case has been reported in which neither 2,3-DPG nor the mutase/phosphatase activities of BPGM were detectable in human erythrocytes.44 The RBCs of the propositus had an increased O2 affinity (ΔP50 = −9 mm Hg) and a moderate secondary polycythemia (Hct 54%). The individual had no physical disability and showed no signs of hypoxemia. Although it was not emphasized in the original report,44 based on the directly measured whole blood Hb and spun Hct values given therein, a small but significant degree of osmotic swelling was evident in this individual’s red cells compared with those of his two children, who were heterozygotes (ΔMCHC = −1.2 g/dL). This decrease of MCHC is in the range of the four patients in Table 3 and suggests that increased erythrocyte hydration was permanent in the natural form of congenital absence of red blood cell 2,3-DPG.

The increased O2 affinity produced by 2,3-DPG depletion [O2 saturations ranging from 45% (patient C) to 59% (patient B)] accounts for the largest component of the reduction in polymer content (38% and 55%, respectively, for these patients). Thus, circulation of 2,3-DPG–depleted SS RBCs with a left-shifted O2 equilibrium curve could reduce O2 delivery per unit volume of blood flow through the microcirculation.42 However, the improvement in blood rheology when polymer content is reduced in sickle erythrocytes might be enough to facilitate O2 delivery at lower cardiac output despite this potential disadvantage.45 Thus, cyanate, which had antisickling effects accompanied by increased O2 affinity, was not rejected for clinical trials based on this fact or the minor increase in red blood cell mass it consistently produced.46 The degree to which the potential antisickling
The effect of 2,3-DPG depletion would offset the negative features of increased O₂ affinity will have to be tested empirically by an in vivo study.

The findings of this in vitro study provide compelling evidence that 2,3-DPG is not a benign component of the sickle erythrocyte, but rather plays an essential role in the pathogenesis of polymer formation. Sustained reduction of this glycolytic intermediate could, therefore, substantially decrease Hb S polymerization in vivo, providing a therapeutically useful modality for ameliorating the vasoocclusive complications of sickle cell disease.

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