Antisickling Effects of 2,3-Diphosphoglycerate Depletion

By W.N. Poillon, 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 of deoxyhemoglobin S. The antisickling effect of 2,3-DPG depletion was evaluated after activation of the 2,3-DPG phosphatase activity of bisphosphoglycerate mutase by glycylate-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 (Δcum = 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 glycylate-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 vasocclusive severity of sickle cell disease. © 1995 by The American Society of Hematology.

A CONSTITUENT of the sickle erythrocyte (SS RBC), 2,3-bisphosphoglycerate (2,3-DPG) is not entirely benign. An earlier report1 showed that 2,3-DPG and intracellular pH (pH) are separate but interdependent determinants of the intrinsic solubility of deoxyhemoglobin 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 (cum) of fully deoxygenated, 2,3-DPG-saturated Hb S (Hb-2,3-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 cum. 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 normal1,3,4 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,5 that is erythroid cell-specific.6,5 The two enzymatic activities that govern 2,3-DPG metabolism reside in a single protein, bisphosphoglycerate 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.5 Because the unstimulated phosphatase activity of BPGM is low (approximately 1,000-fold lower than its other activities), the steady-state concentration of 2,3-DPG is very high in normal human erythrocytes (4 to 6 μmol/mL RBC).

An earlier study10 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.

Glycylate-2-phosphate (2-PG) was shown by Rose11 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,12 is an extremely potent stimulator of the 2,3-DPG phosphatase activity of BPGM at supranormal concentrations.13-16 As a nondiffusible polyanion, it must be generated inside the red cell by the action of pyruvate kinase on glycylate.16 We sought to establish, therefore, whether exposure of SS RBCs to glycylate 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 acid 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.16,16b,16c 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
affinities of control and treated cells were measured in a Hemoxy
Analyzer (TCS Medical Products Co., Huntingdon Valley, PA), which was also used to evaluate sickling as a function of pO2.

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 metabolic 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 suspended [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, 5 mmol/L NaCl, 2 mmol/L CaCl2, 1 mmol/L
MgCl2, 2 mmol/L Na2HPO4, 1 mmol/L inosine, 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
pCO2 of 40 mm Hg. Medium for treated cells contained 30 mmol/
L glycolic acid 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)
determination was done using the spin Hct, and the hemoglobin
concentration was measured with Drabkin's reagent.17 Hemoglobin F
was measured by alkaline denaturation.18 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 Hemoxy analyzer in an N-tris(hydroxy-
methyl)methyl]-2-aminooxetanesulfonic acid (TES)-based isosmotic
buffer, pH 7.4 at 37°C.39 Red blood cell density profiles were mea-
sured by phthalate ester flotation.20,21 Two parameters were extracted from each density profile: (1) D50, 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.7,8,14

Two observers, blinded to the identity of glutaraldehyde-fixed sam-
pies, 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.23 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
nominal O2 tension of the microcirculation,24 and (2) %RSC at half-
saturation with O2 (P50).

Depletion of 2,3-DPG by treatment with glycolate. Washed
erythrocytes were suspended to an Hct of 10% 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%), control and treated cells were washed
with control medium (pH 7.4), packed, and resuspended to the
original Hct. Washed RBCs were stored overnight at 4°C before equilibra-
tion at 37°C in the sample chamber of a TCS Hemoxy Analyzer.
Simultaneously with the generation of ODCs for control or treated
cells, samples at O2 tensions in the range of pathophysiologic interest
(5 to 60 mm Hg) were fixed in 2% glutaraldehyde in phosphate-
buffered saline, pH 7.4, for evaluation of morphologic sickling, as
described above.

Intracellular pH. In an experiment in HEPES-based medium,
31P-nuclear magnetic resonance (NMR) spectroscopy was used to monitor intracellular pH and 2,3-DPG levels during incubations at
37°C for 5 hours. Washed erythrocytes (normal or sickle) were sus-
pended in control buffer (isosmotic 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 Labotka and Kleps.27 The 3-PO2 signal was
used to monitor 2,3-DPG.28 Control and glycolate-treated cell sus-
pensions contained methylphosphonate (15 mmol/L) as an intracellu-
lar 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 (pHr), (3) hemoglobin composition, (4) O2 satu-
ration, and (5) total hemoglobin concentration (cT). Each of these
variables was measured directly for control and glycolate-treated SS
RBCs, except pHr, which was determined indirectly by use of appro-
imate 2,3-DPG concentrations and the relationship between pHr and
2,3-DPG deduced by Duhm29 for AA RBCs with supranormal levels
of 2,3-DPG (ΔpHr/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 (Δc3, = -1.6 g/dL; eq 1a) than
that of stripped deoxy-Hb S at pH 7.2.1 Using the slope of the c3, versus pH profile for deoxy-Hb S in isosmotic Bistris buffer over
the pH range of 6.8 to 7.6 (10.8 g/dL per pH unit), 1b, a value of
18.0 g/dL is obtained for c3, the intrinsic solubility of Hb-DPG
at pH 7.41, the predicted pH of fully deoxygenated SS RBCs.

This c3, value has been corrected for the solubilizing effect of
the buffering species (0.05 mol/L Bistris), which amounts to a decrea-
se of 2.2 g/dL; the value given by Poillon and Kim1 is higher by this
amount.) The sparing effect of Hb F and Hb A2 may be evaluated
from the dependence of c3, on %Hb (F + A2) (0.334 g/dL per 1%
non-S Hb; eq 2). The variation of c3, with O2 saturation was
estimated from an empirical relation (eq 3) derived by others.31 The
effect of each of these four variables on c3, can be summarized as:

\[
2,3-DPG/pHr = \Delta c_{3,}/\Delta pO2 = -1.6 \text{ g/dL (Hb-DPG - Hb)} \quad (1a)
\]

and \( \Delta c_{3,}/\Delta pH = 10.8 \text{ g/dL/pH unit} \) \quad (1b)

Non-S hemoglobins: \( \Delta c_{3,}/\Delta pH = 0.334 \text{ g/dL per 1% non-S Hb} \) (2)

Ligand saturation: \( c_{3,} = c_{3,}^{0} + 9.24y_{0} + 9.80y_{3} + 23.5y_{5} \) \quad (3)

where \( c_{3,}^{0} \) 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 cT, total hemoglobin concentration), influences polymerization by its nonideal
behavior at high Hb S concentration.

Knowledge of these five variables permits calculation of the mean
polymer fraction (fp) for individual cell samples without direct mea-
surement of c3, the equilibrium solubility of intraerythrocytic HbS.
This is achieved by substituting the appropriate values of c3, cT, and \( c_{3,}^{0} \) (polymer concentration)32 into the conservation of mass equa-
tion (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 P02 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 P02 = 20 mm Hg, the nominal O2 tension of the microcirculation25) 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 P02) was 11% lower for treated cells, indicative of inhibition of intracellular gelation independent of changes in O2 affinity.34 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 1. Effect of Loss of 2,3-DPG on pH, of Human Red Cells as Measured by 31P-NMR Spectroscopy

<table>
<thead>
<tr>
<th>Type of Cells and System</th>
<th>pH</th>
<th>% Loss of 2,3-DPG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (pH 7.4)</td>
<td>7.24</td>
<td>22</td>
</tr>
<tr>
<td>Glycolate (pH 7.0)</td>
<td>7.33</td>
<td>74</td>
</tr>
<tr>
<td>Sickle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (pH 7.4)</td>
<td>7.19</td>
<td>9</td>
</tr>
<tr>
<td>Glycolate (pH 7.0)</td>
<td>7.27</td>
<td>51</td>
</tr>
</tbody>
</table>

Table 2. Effect of 2,3-DPG Depletion by Incubation With Glycolate at Acid pH on O2 Affinity and Sickling Tendency of SS RBCs

<table>
<thead>
<tr>
<th>System</th>
<th>Incubation Time (h)</th>
<th>MCHC (g/dL)</th>
<th>ATP (µmol/mL RBC)</th>
<th>2,3-DPG (µmol/mL RBC)</th>
<th>P02 (mm Hg)*</th>
<th>Sickling Parameters†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>36.4</td>
<td>1.10</td>
<td>7.45</td>
<td>27.5</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>34.3</td>
<td>1.16</td>
<td>7.92</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Treated</td>
<td>0</td>
<td>35.0</td>
<td>1.15</td>
<td>6.57</td>
<td>19.5</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>30.7</td>
<td>1.23</td>
<td>1.34†</td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>

* 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 P02 = 20 mm Hg, the nominal O2 tension of the microvasculature,25 and (parameter 2) %RSC for O2 tension at half-saturation (P02).
‡ 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.
reflecting the sparing effect on polymerization of the ele-

R.

vated Hb F level for patient

E.

We evaluated in detail the osmotic and antisickling effects of

A.

Table

E.

show the following: (1) a reduction in 2,3-DPG concentra-

D.

The effect of oxygenation on solubility (2.4 g/dL) and pH produced by 2,3-DPG depletion would increase c_{in} for unliganded Hb S from 18.0 to 20.4 g/dL and pH from 7.41 to 7.48. The corresponding decrease in f_{p} is from 0.60 to 0.52. Cell swelling that accompanies adjustment of the Donnan equilibrium causes a decrease in MCHC from 32.3 to 30.0 g/dL and f_{p} from 0.52 to 0.45, a reduction of 25% in polymer content overall relative to the 2,3-DPG-replete species. Sunshine et al^{11} have shown that the O_{2} affinity of polymer is threefold lower than that of monomer and provided an empirical equation by which to

Direct and indirect effects of 2,3-DPG depletion on intra-
cellular polymerization. Table 4 quantitates all intra-
erythrocytic variables (2,3-DPG, pH_{i}, MCHC, and O_{2} satura-
tion) that interact to affect the polymerization of partially liganded Hb S in a 2,3-DPG–dependent fashion. Non-S hemoglo-

bions (Hbs F and A_{s}) also inhibit polymerization, as shown in our recent report,^{30} but this sparing effect is inde-
pendent 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 con-
centration is so low (3.0%) that its solubilizing effect (<1.0 g/dL^{30}) can be ignored.] Overall inhibition of polymer-
ization has been dissected into the component contributions
made by each of the four variables. Those due to DPG/pH_{i}
and MCHC (direct effects) have been calculated on the basis
of solubilizing or osmotic effects with Hb S in the unliganded
state (pO_{2} = 0). The effect of oxygenation on solubility
(an indirect effect) was then calculated for the measured O_{2}
saturations of control and glycolate-treated cells. A reason-
able estimate for the values of pH_{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^{30,31} would lower pH_{i} by 0.05 unit, while the compensa-
tory osmoregulatory chloride/proton shift necessary to main-
tain electroneutrality would raise pH_{i} by 0.12 unit; the net
change in pH_{i} would lower pH_{i} by 0.07 unit corresponds to a Δc_{in} of 0.8 g/dL, and the loss of 2,3-DPG per se produces a Δc_{in} of 1.6 g/dL (see Materials and Methods). Thus, the overall increment in solubility (2.4 g/dL) and pH_{i} produced by 2,3-DPG depletion would increase c_{in} for unliganded Hb S from 18.0 to 20.4 g/dL and pH_{i} from 7.41 to 7.48. The corresponding decrease in f_{p} is from 0.60 to 0.52. Cell swelling that accompanies adjustment of the Donnan equilibrium causes a decrease in MCHC from 32.3 to 30.0 g/dL and f_{p} from 0.52 to 0.45, a reduction of 25% in polymer content overall relative to the 2,3-DPG-replete species. Sunshine et al^{11} have shown that the O_{2} affinity of polymer is threefold lower than that of monomer and provided an empirical equation by which to
estimate cSat for partially oxygenated Hb S. Using this relationship (see Materials and Methods), we were able to calculate values of cSat 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 cSat so obtained were 20.5 and 26.5 g/dL. (Incorporated into these numbers are decrements in cSat for Hb and Hb-DPG due to the loss of Bohr protons arising from partial saturation and depolymerization.) The corresponding values of pHi, c., and cSat presented in Table 1, it was found that for both AA and SS RBCs, partial depletion

### 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

<table>
<thead>
<tr>
<th>Patient</th>
<th>Dense Cells (%)</th>
<th>% Hb F*</th>
<th>Δ2,3-DPG (mmol/L)</th>
<th>ΔpH (mm Hg)</th>
<th>ΔMCHC (g/dL)</th>
<th>ΔΔDp</th>
<th>Sickling Parameters†</th>
<th>Calculated Parameters</th>
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</thead>
<tbody>
<tr>
<td>A</td>
<td>3.2</td>
<td>17.2</td>
<td>-6.6</td>
<td>-8.0</td>
<td>-3.8</td>
<td>0.0123</td>
<td>-29 (71)</td>
<td>-11 (48)</td>
</tr>
<tr>
<td>B</td>
<td>12.1</td>
<td>3.0</td>
<td>-4.4</td>
<td>-10.5</td>
<td>-2.3</td>
<td>0.0095</td>
<td>-42 (95)</td>
<td>-13 (68)</td>
</tr>
<tr>
<td>C</td>
<td>8.7</td>
<td>0.8</td>
<td>-4.5</td>
<td>-8.0</td>
<td>-1.6</td>
<td>0.0073</td>
<td>-22 (48)</td>
<td>-6 (21)</td>
</tr>
<tr>
<td>D</td>
<td>21.8</td>
<td>1.9</td>
<td>-3.7</td>
<td>-10.0</td>
<td>-0.3</td>
<td>0.0057</td>
<td>-27 (46)</td>
<td>-4 (11)</td>
</tr>
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</table>

* Calculated from the conservation of mass relationship:

$$f_p = f_f - f_f(c_{sat} - c_{sat})$$

where $f_p$ is the polymer fraction and $c_{sat}$, $c_{sat}$ are the polymer, total hemoglobin, and saturation concentrations, respectively (see Table 3, footnote † for details).

† Release of Bohr protons on ligation caused by shift of allosteric equilibrium and depolymerization lowers pH by 0.10 and 0.13 units for control and treated cells, respectively.

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

### Table 4. Direct (cSat, $c_p$) 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>cSat (g/dL)</th>
<th>cSat* (g/dL)</th>
<th>$f_p$†</th>
<th>Intracellular Variable</th>
</tr>
</thead>
<tbody>
<tr>
<td>cSat</td>
<td>Control</td>
<td>Hb-DPG</td>
<td>7.41</td>
<td>6.16</td>
<td>32.3</td>
<td>18.0</td>
<td>0.60</td>
<td>DPG/pH</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>Hb</td>
<td>7.48</td>
<td>1.75</td>
<td>32.3</td>
<td>20.4</td>
<td>0.52</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Δ</td>
<td>0.07</td>
<td>-4.41</td>
<td>0</td>
<td>2.4</td>
<td>-0.08</td>
<td></td>
</tr>
<tr>
<td>cSat, $c_p$</td>
<td>Control</td>
<td>Hb-DPG</td>
<td>7.41</td>
<td>6.16</td>
<td>32.3</td>
<td>18.0</td>
<td>0.60</td>
<td>MCHC</td>
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<td>Treated</td>
<td>Hb</td>
<td>7.48</td>
<td>1.75</td>
<td>30.0</td>
<td>20.4</td>
<td>0.45</td>
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<td></td>
<td></td>
<td>Δ</td>
<td>0.07</td>
<td>-4.41</td>
<td>-2.3</td>
<td>2.4</td>
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<tr>
<td>cSat, $c_p$, pO2</td>
<td>Control (pO2 27.5 mm Hg)</td>
<td>Hb-DPG</td>
<td>7.31†</td>
<td>6.16</td>
<td>34.5</td>
<td>32.3</td>
<td>20.5†</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td>Treated (pO2 17.0 mm Hg)</td>
<td>Hb</td>
<td>7.35†</td>
<td>1.75</td>
<td>59.8</td>
<td>30.0</td>
<td>26.5†</td>
<td>0.19</td>
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<td>Δ</td>
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<td>-4.41</td>
<td>26</td>
<td>-2.3</td>
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<td>-0.33</td>
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</table>

These data pertain to patient B in Table 3. Values of 2,3-DPG concentration, %HbO2 (at pO2 = 20 mm Hg), and $c_p$ (ie, MCHC) were measured before and after incubation in isosmotic CO2/bicarbonate (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 pH, cSat, and $f_p$ 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 0.95 mol/L Bistris, 0.1 mol/L KCl, 0.02 mol/L NaCl, pH 7.2 (280 mosmol/kg H2O) at 37°C (see Materials and Methods for derivation of individual values) and have been corrected for the solubilizing effect of Bistris (48 g/dL per mol/L).

† Calculated from conservation of mass relationship:

$$f_p = c_{sat}(c_{sat} - c_{sat})$$

where $f_p$ is the polymer fraction and $c_{sat}$, $c_{sat}$ are the polymer, total hemoglobin, and saturation concentrations, respectively (see Table 3, footnote † for details).

‡ Release of Bohr protons on ligation caused by shift of allosteric equilibrium and depolymerization lowers pH by 0.10 and 0.13 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 Duham factor for AA RBCs enriched in 2,3-DPG (−0.023 pH unit/mmol/L 2,3-DPG). This finding allowed us to calculate pH, for our experiments, based on 2,3-DPG concentrations and the dependence of pH, 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 pH of 20 mm Hg play a role, because the reduction in sickling for patient A that depletion of 2,3-DPG to levels below 0.8 to 2.0 mmol/L 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, 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 pH of 20 mm Hg and at P50, and calculated ΔP50) 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 c50 are augmented by the compensatory osmotic swelling that lowers MCHC. Overall, then, four intraerythrocytic variables (2,3-DPG concentration, pH, 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 c50; (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, f50 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 hydroxypyruvate, whose phosphorylated form, 3-phosphohydroxypruvate (3-PHP), is 10 times more potent than 2-PG in activating BPGM, and the phosphate analogues of 2-PG and 3-PHP, which should be considerably more membrane-permeable than the parent compounds.

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 described 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. 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, 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. 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. 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. The degree to which the potential antisickling
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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Antisickling effects of 2,3-diphosphoglycerate depletion

WN Poillon, BC Kim, RJ Labotka, CU Hicks and JA Kark