Effects of Cyanate and 2,3-Diphosphoglycerate on Sickling

RELATIONSHIP TO OXYGENATION

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ABSTRACT Cyanate and 2,3-diphosphoglycerate (2,3-DPG) both influence the oxygen affinity of hemoglobin. The studies presented here concern the effects of these compounds on the sickling phenomenon. The inhibitory effect of cyanate on sickling is largely due to the fact that it increases the percentage of oxyhemoglobin S at a given oxygen tension. In addition, cyanate inhibits sickling by a mechanism that is independent of oxygenation. In this paper, we have demonstrated that the viscosity of carbamylated sickle blood was lower than that of non-carbamylated controls at the same oxygen saturation. Furthermore, carbamylation resulted in an increase in the minimum concentration of deoxy-sickle hemoglobin required for gelation.

Like cyanate, 2,3-DPG affected sickling of intact erythrocytes by two mechanisms. Since 2,3-DPG decreases the percentage of oxyhemoglobin S at a given oxygen tension, sickling is enhanced. In addition, 2,3-DPG had a direct effect. When the intracellular 2,3-DPG concentration was increased in vitro, a greater percentage of cells were sickled at a given oxygen saturation. Conversely, sickling was inhibited in cells in which 2,3-DPG was artificially lowered. These data indicate that the enhancement of sickling by 2,3-DPG is in part independent of its influence on oxygen affinity.

INTRODUCTION

Aggregation of hemoglobin S molecules, the basis of the sickling phenomenon, is favored when hemoglobin is deoxygenated. Compounds that alter the oxygen affinity of hemoglobin therefore affect the degree of sickling at a given partial pressure of oxygen. This report deals with the effects on sickling of two compounds, cyanate and 2,3-diphosphoglycerate (2,3-DPG), which, by different mechanisms, shift the oxygen dissociation curve and also influence sickling directly.

Cyanate irreversibly carbamylates the N-terminal amino groups of hemoglobin chains. Carbamylated hemoglobin has increased oxygen affinity (1). Therefore, the inhibition of sickling by cyanate (2) appears to be mostly due to the presence of more oxygenated hemoglobin at a given P_{O_2} (3–5). However, if carbamylation and thus the overall inhibition of sickling is extensive, it can be demonstrated that a portion of the effect of carbamylation on sickling is independent of the increase in oxygen affinity (3, 6). The present studies confirm and extend this observation.

In contrast, 2,3-DPG is known to decrease the oxygen affinity of hemoglobin by a specific electrostatic interaction that stabilizes the deoxy conformation. 2,3-DPG can therefore be expected to favor sickling at a given P_{O_2}. Recent experiments suggest, in fact, that 2,3-DPG enhances the viscosity of hemoglobin S in solution (6, 7). The experiments presented here were designed to ascertain if, like cyanate, 2,3-DPG can influence sickling by a mechanism independent of its effect on oxygen affinity.

METHODS

Heparinized blood specimens were obtained from patients who were homozygous for hemoglobin S. Reagent grades of sodium cyanate (Pfaltz & Bauer, Inc., Flushing, N. Y.), inosine (Sigma Chemical Co., St. Louis, Mo.) and pyruvate were used without further purification.

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The red cells were carbamylated by incubation at a hematocrit of 25% in a Krebs-Henseleit buffer containing 1% albumin, 10 mM glucose, and 50 mM cyanate (final concentrations). Controls were incubated with equimolar concentrations of sodium chloride, substituted for sodium cyanate. The incubations were performed at 37°C for 1 h under a stream of 5% CO_2 in air (300 ml/min). To determine the degree of carbamylation, the erythrocytes were incubated in the presence of $^{14}CNO^2$ (final specific activity: 29.5 μCi/mmol). The cells were then subjected to hypotonic lysis, and the lysate was passed over a Sephadex[1] G-25 column, which separated the hemoglobin from unbound CNO. 0.4 ml of the effluent hemoglobin was added to 1.5 ml of a 1:1 mixture of isopropanol: protosol,[2] followed by 1 ml of 30% H_2O_2 to bleach the hemoglobin. 30 min later, each sample was mixed with 10 ml of Aquasol.[2] The vials were placed in a liquid scintillation counter[3] and precooled for several hours before counting. Hemoglobin concentration was determined from the absorbance at 540 nm of the cyanmethemoglobin derivative ($\epsilon = 1.1 \times 10^4$ [heme]).

In order to raise the intracellular 2,3-DPG concentration, cells were incubated at a hematocrit of 25% for 30–45 min at 37°C in Krebs-Henseleit buffer, to which phosphate buffer pH 7.4, inosine, and pyruvate (IPP) were added to achieve final concentrations of 20, 6.25, and 6.25 mM, respectively (8). The 2,3-DPG content of red cells was lowered by incubation for 4 h in Krebs-Henseleit buffer containing 10 mM sodium metabisulfite (hematocrit 25, 37°C, pH 7.4) (9). Variations in pH in these incubations did not exceed 0.1 pH units. Red cell 2,3-DPG content was measured by the method of Rose and Liebowitz (10).

After the incubation with sodium cyanate, IPP, or sodium metabisulfite, cells were washed three times in Krebs-Henseleit buffer and resuspended in autologous serum.

Viscosity of carbamylated and control sickle blood was measured at selected oxygen tensions with the use of a Wells-Brookfield cone plate viscometer.[4] The hematocrit was adjusted to 43±0.3%. A sheer rate of 46 s^{-1} was employed. The instrument was operated in an air-tight plastic bag previously equilibrated with the same gas mixture and through which a stream of hydrated gas was maintained.

Minimum gelling concentrations were determined as described by Singer and Singer (11). In order to determine the time required for complete deoxygenation, some specimens were placed in tonometers to which cuvettes with a 2 mm light path were attached, and the change in spectra of the concentrated hemoglobin solutions was followed with a Perkin-Elmer 350 recording spectrophotometer.[5] Deoxygenation was complete when no further change of the spectrum (700–900 nm) could be induced by continuing exposure to N_2. The temperature was kept at 20°C by placing the tonometers into a constant temperature water bath throughout the experiment.

To compare the percentage of cells in the sickled form at various oxygen tensions, 10 ml of 25% cell suspensions containing different intracellular concentrations of 2,3-DPG were placed in 50-cm^3 Erlenmeyer flasks. The oxygen tension was then progressively reduced by incubation at 37°C under a stream of hydrated nitrogen plus 5% CO_2 delivered at the rate of 100 ml/min, while the flasks were shaken at the rate of about 80 oscillations/min. During the deoxygenation period of about 2 h, samples were removed at intervals for the immediate determination of P_{O_2}, oxygen saturation, and the percentage of sickled forms. For the latter, a small amount of red cell suspension was anaerobically transferred into 10% formaldehyde in saline for fixation. 400 cells were then counted using previously described criteria (12). The reproducibility of this method in our hands is approximately 5%. Oxygen saturations of hemoglobin were measured spectrophotometrically in a CO-oximeter, Model IL 180.[6]

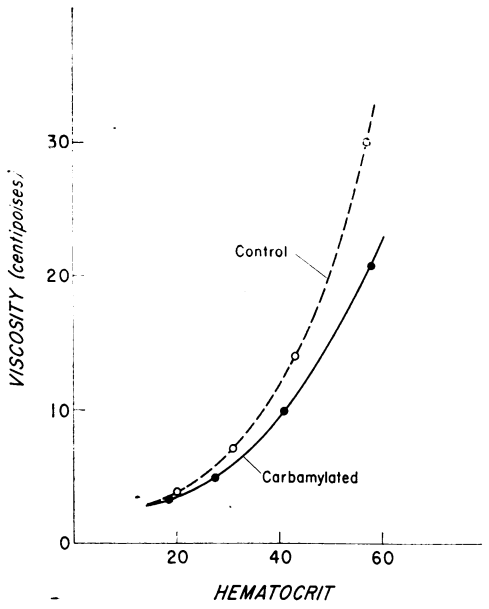

FIGURE 1 Viscosity of carbamylated (●) and untreated (○) sickle blood at different hematocrits. All samples were equilibrated with 1.95% O_2, 5% CO_2, balance N_2. Shear rate: 46 s^{-1}.

RESULTS

After a 1 h incubation with 50 mM cyanate, 3–4 mol of cyanate was bound to 1 mol of hemoglobin tetramer. This result is in agreement with data obtained by others (2). At a given hematocrit the viscosity of sickle blood is a function of the percentage of sickled forms present (13). Fig. 1 shows the relationship between hematocrit and viscosity of partially deoxygenated sickled blood with and without previous carbamylation. The blood was completely equilibrated with the same gas mixture, producing an equal P_{O_2} in all samples. Viscosity was decreased at all hematocrits by carbamylation. To demonstrate the oxygen affinity independent aspect of the action of 50 mM cyanate, samples of hemoglobin S blood were equilibrated with gas mixtures containing between 0.5 and 3.5% oxygen. The relationship between viscosity and oxygen saturation of car-

[1] Pharmacia Fine Chemicals, Inc., Piscataway, N. J.
[2] New England Nuclear Corp., Boston, Mass.
[3] Packard Tri-Carb, Model 3375; Packard Instrument Co., Inc., Downers Grove, Ill.
[4] Brookfield Engineering Laboratories, Inc., Stoughton, Mass.
[5] Perkin-Elmer Corp., Norwalk, Conn.
[6] Instrumentation Laboratory, Inc., Lexington, Mass.

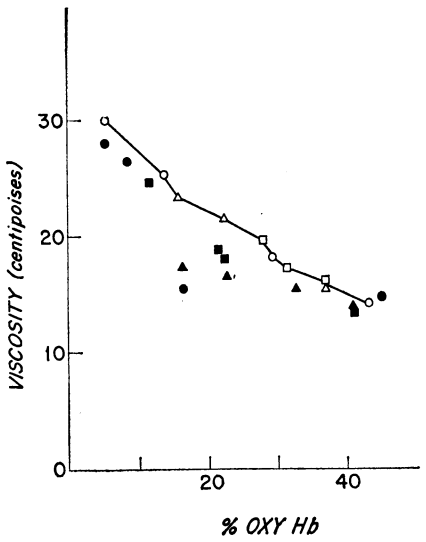

FIGURE 2 Viscosity of carbamylated and non-carbamylated sickle blood at different oxygen contents of hemoglobin. Data obtained from the blood of three patients are combined. Each patient is represented by a differnt symbol (circles, squares, or triangles). Samples carbamylated with 50 mM CNO (closed symbols) and non-carbamylated samples (open symbols) were equilibrated with gas mixtures containing different amounts of oxygen. Hematocrit of all samples: 43±0.3. Shear rate: 46 s^{-1}.

bamylated and control blood is shown in Fig. 2. The viscosity of carbamylated samples was lower at the same oxygen saturation. This indicated a small, direct effect of carbamylation on sickling.

The sickling properties of membrane-free hemoglobin S solutions were similarly affected by cyanate. To demonstrate the influence of carbamylation on hemoglobin itself, gelling concentrations of hemoglobin solutions with increasing amounts of bound carbamyl groups were determined. The concentration of hemoglobin S at the gelling point has been shown to be a sensitive indicator of the sickling propensity of various hemoglobin mixtures (11, 14). To obtain different degrees of carbamylation, advantage was taken of the fact that carbamylation is a time-dependent reaction. Hemoglobin S red cells were incubated with 50 mM ^{14}CNO as described under Methods. Samples were removed after 20, 50, 65, 90, and 120 min, and revealed the amounts of bound carbamyl groups shown in Fig. 3. Care was taken to insure that all these hemoglobin solutions were completely deoxygenated. The minimum gelling concentration increased with the degree of carbamylation. In the experiment shown here, the minimum gelling concentration of non-carbamylated hemoglobin was higher (28 g/100 ml), then the 24 g/100 ml usually found with hemoglobin S (14). This patient's hemoglobin contained 7.2% hemoglobin F at the time of the experiment as determined by alkaline denaturation, which may account for an increase in minimum gelling concentration. In addition, this method shows considerable variation from one laboratory to another, and even higher gelling points for sickle hemoglobin have been reported (15). The samples were examined for their methemoglobin content directly following the gelling experiments, and in no case was methemoglobin found to exceed 2%.

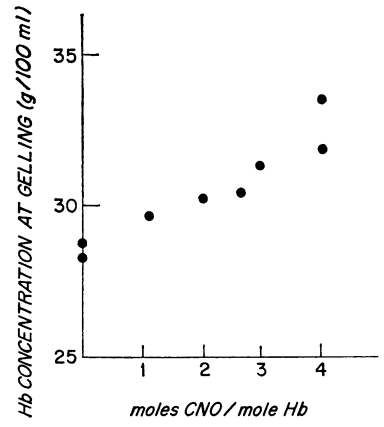

FIGURE 3 Minimum gelling concentrations of Hb S with increasing carbamylation. Cells were exposed to 50 mM [^{14}C]cyanate, and samples were removed after 20, 50, 65, 90, and 120 min. After hypotonic lysis of the cells, the hemoglobin specimens were dialysed against 0.15 M phosphate buffer, pH 7.35. Then the gelling concentrations and the CNO/Hb ratios were determined.

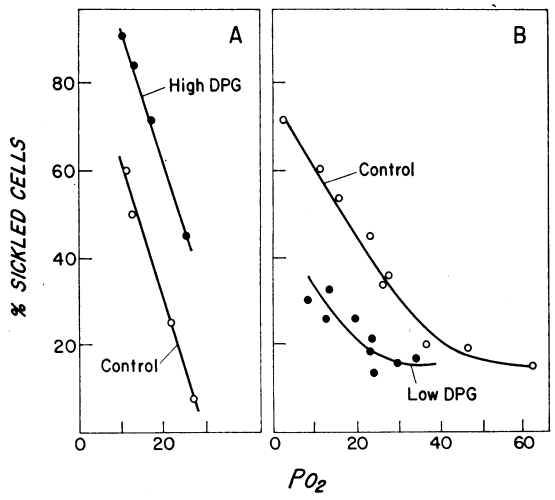

FIGURE 4 The effect of increased (*A*) and decreased (*B*) intracellular 2,3-DPG concentration on sickling. Sickling is related to the oxygen tension. Experiments *A* and *B* were performed on blood of different patients, having 7% and 20% irreversibly sickled cells, respectively. 2,3-DPG concentrations (mmol/liter cells): (*A*) high DPG 11.8, control 6.1; (*B*) low DPG 0.71, control 7.05. Po_2 is in millimeters of mercury.

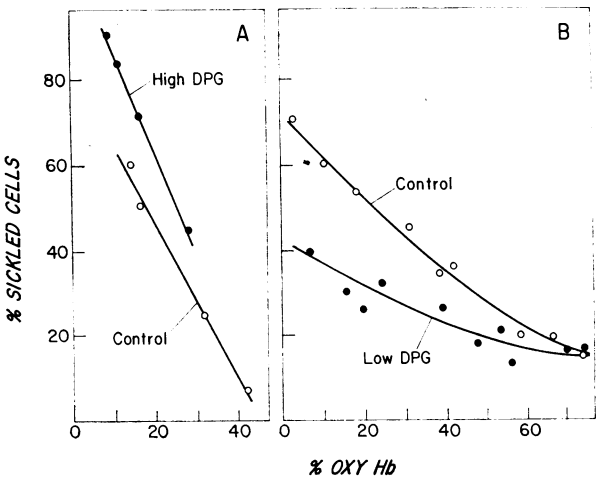

FIGURE 5 The oxygen affinity independent effect of 2,3-DPG on sickling. Sickling is related to the measured hemoglobin oxygen saturation. (Same data as shown in Fig. 4.)

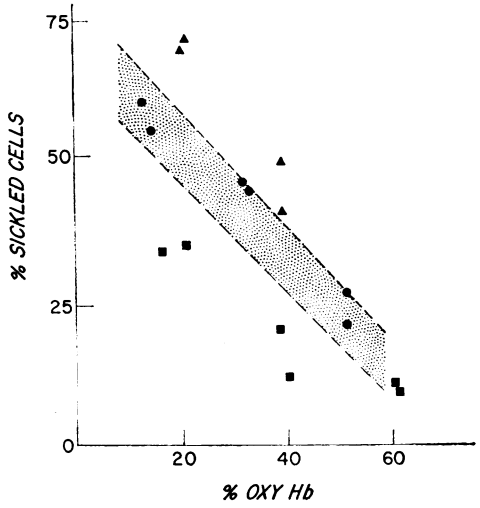

FIGURE 6 The combined effects of cyanate and 2,3-DPG on sickling. ▲ = non-carbamylated blood, 2,3-DPG = 8.1 mmol/liter cells; ■ = carbamylated blood, 2,3-DPG = 5.0 mmol/liter cells; ● = carbamylated blood, 2,3-DPG = 8.5 mmol/liter cells. The shaded area represents the normal range for this patient. All of the percentages of sickled cells obtained on three different occasions on untreated cells of this patient fell within this area. Representative 2,3-DPG concentration after 1 h incubation in buffer alone: 5.3 mmol/liter cells.

Experimentally induced alterations of the 2,3-DPG levels were used in another approach to alter hemoglobin-oxygen affinity and thereby to influence the sickling properties of intact erythrocytes. Incubations with IPP produced an approximately two-fold increase in 2,3-DPG. Incubation with sodium metabisulfite caused a decrease in 2,3-DPG to 0.1–1.0 mmol/liter cells. 2,3-DPG concentrations prior to deoxygenation for individual experiments are given in the legends to the figures. These normal, high, and low DPG cells were then deoxygenated as described. Fig. 4 shows that sickling is increased in high DPG cells and markedly reduced in low DPG cells, as compared with the controls. This was expected from the known effect of the compound on hemoglobin-oxygen affinity. Fig. 5 demonstrates that the effect of 2,3-DPG is partly independent of its influence on oxygen affinity; the same sickling data are shown, but the partial pressure of oxygen on the abscissa is replaced by the measured oxygen saturation. There remained a greater number of sickled forms at a given oxygen saturation in the high DPG sample. Conversely, there was still clear inhibition of sickling in the low DPG cells. It was thus confirmed that 2,3-DPG favors sickling by at least two mechanisms, only one of which is related to the effect of the compound on oxygen affinity of hemoglobin.

Fig. 6 shows the result when sickle cells were altered by a combination of carbamylation and an artificial increment in intracellular 2,3-DPG concentration. After a 1 h incubation with 50 mM cyanate, the cells were washed and resuspended in Krebs-Henseleit buffer containing IPP. Sickling of such cells was compared with those that had been exposed to only one of the incubation systems. Whereas carbamylation decreases sickling and high 2,3-DPG increases sickling at any given oxygen saturation, sickling of the carbamylated high 2,3-DPG cells was similar to that of completely untreated cells of the same patient. Thus, these two oxygen affinity independent effects on sickling counterbalanced one another.

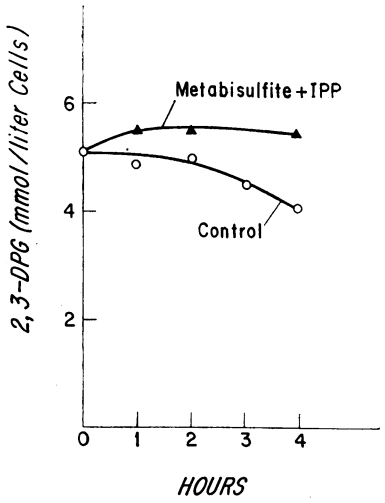

FIGURE 7 The effect on the red cell 2,3-DPG concentration of sodium metabisulfite (10 mM) together with inosine (6.25 mM), pyruvate (6.25 mM), and phosphate (20 mM) (IPP).

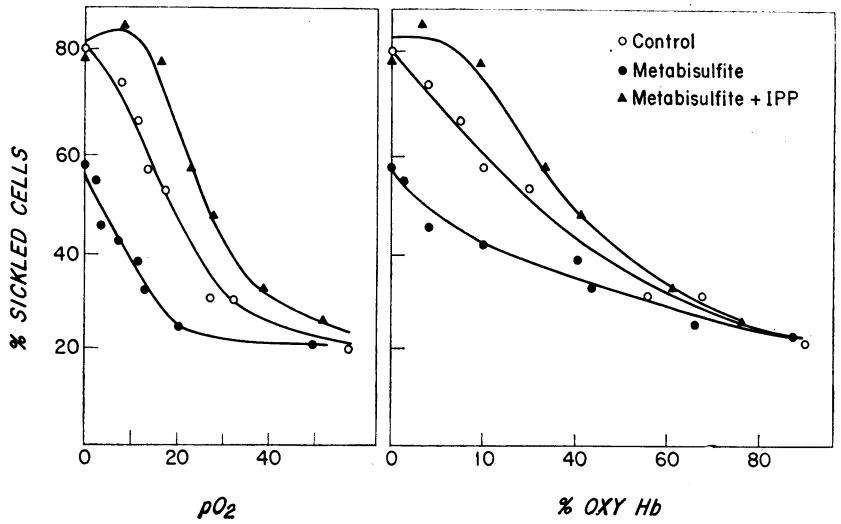

FIGURE 8 Sickling of erythrocytes preincubated for 4 h with 10 mM sodium metabisulfite (●, 2,3-DPG = 0.4 mmol/liter cells), 10 mM sodium metabisulfite together with IPP (▲, 2,3-DPG = 7.4 mmol/liter cells), and controls (○, 2,3-DPG = 4.1 mmol/liter cells). P_{O_2} is in millimeters of mercury.

The rapid decrease of the intracellular 2,3-DPG concentration upon exposure to metabisulfite is due to a stimulation of the 2,3-DPG phosphatase (9, 16). Therefore, if enough substrates are added to the incubation to insure resynthesis of 2,3-DPG, no depletion occurs. Fig. 7 shows 2,3-DPG concentrations in an incubation in which 10 mM metabisulfite and IPP were added together. It is demonstrated that under these conditions the DPG concentration after 4 h is as high as initially, whereas cells incubated without any additive have a 20–25% lower concentration. Hemoglobin S red cells were treated in such a way, and their sickling properties were tested. The results are shown in Fig. 8; they demonstrate that cells, in which the high DPG concentration is maintained despite the presence of 10 mM metabisulfite during the 4 h preincubation, sickled to a slightly greater extent than the controls, in which a moderate fall of DPG occurred during the preincubation without additive.

DISCUSSION

Cyanate is presently being administered to sickle cell anemia patients in limited clinical trials (17). Therefore, it is important to understand its mechanism of action. We have confirmed previous observations (3) that indicate that the inhibition of sickling by cyanate is, in part, independent of the increase in oxygen affinity. This additional effect is small. The doses of cyanate that are required to detect this oxygen-independent effect in terms of a decrease in sickled forms or a decrease in viscosity exceed by far those that are necessary to influence sickling at a physiologic P_{O_2} or that have been obtained in vivo. It seems unlikely that this direct cyanate effect is due to carbamylation of other cell components, such as membrane proteins, because gelling experiments performed on membrane-free lysates demonstrated that the effect of carbamylation is on hemoglobin itself. The method used for quantitation of bound cyanate does not distinguish between carbamylation of N-terminal groups and of other residues. It is possible that the direct effect of cyanate may not be related specifically to carbamylation of N-terminals.

2,3-DPG is the most important intracellular factor in the control of oxygen affinity of hemoglobin. The sickling of hemoglobin S red cells containing different amounts of 2,3-DPG was compared because sickling is dependent upon the oxygen saturation of the hemoglobin. Inhibition of sickling at a given P_{O_2} in cells with decreased 2,3-DPG content could readily be demonstrated. Conversely, cells with increased 2,3-DPG sickled to a greater extent at the same P_{O_2}. In order to evaluate whether the effect of 2,3-DPG is entirely due to the shift in the oxygen dissociation curve or whether 2,3-DPG as well as cyanate plays an independent role in sickling, sickling was also related to the hemoglobin oxygen saturation. It was found that sickling is increased in cells with high 2,3-DPG content and decreased in cells with low 2,3-DPG at equal oxygen saturations.

At the extracellular pH of 7.4, which was well controlled in our incubation system, the intracellular pH in the high 2,3-DPG cells can be expected to be of the order of 7.0–7.1 (8), and that of the low 2,3-DPG cells 7.3–7.35 as compared with 7.2 in normal 2,3-DPG cells.

The mechanism by which increasing hydrogen ion concentration favors sickling is probably based to a great extent upon the decrease in oxygen affinity of hemoglobin induced by acidification (Bohr effect). This part of the pH-influence does not affect the results, if sickling is compared at the same oxygen content of hemoglobin. It is unknown whether small alterations in the intracellular hydrogen ion concentration would influence sickling per se. The available experimental data are contradictory (18–20).

In order to rule out the possibility that the 4 h preincubation with metabisulfite causes a membrane alteration that would make it impossible for the cell to sickle when deoxygenated, we have produced cells in which high 2,3-DPG levels were maintained despite the exposure to metabisulfite. These cells sickled extensively, consistent with their high 2,3-DPG content. Sickling in this system is therefore dependent on intracellular 2,3-DPG and not upon the effects of metabisulfite on the cell membrane.

The mode of action of 2,3-DPG on sickling is not established. It has been suggested that since 2,3-DPG stabilizes the deoxy conformation, it may favor the aggregation of hemoglobin S molecules at a given oxygen saturation (21). This interpretation is supported by experiments of Panicker, Ben-Bassat, and Beutler (6) who found increasing viscosity of partially deoxygenated hemoglobin S solutions when the 2,3-DPG concentration was raised.

Although the influence of 2,3-DPG as well as cyanate on oxygen affinity appears to be very important for the effects of these compounds on sickling, it is clear that they both also influence sickling directly. Because of the dual effect of 2,3-DPG, a decrease of its concentration results in a strong inhibition of sickling in vitro. Since this inhibition was demonstrated in intact erythrocytes that were incubated under physiological conditions, it can be expected that a therapy that lowers red cell 2,3-DPG in patients would be beneficial.

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