

Effect of 2,3-Diphosphoglycerate on Oxygen Affinity of Blood in Sickle Cell Anemia

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ABSTRACT Blood of patients with sickle cell anemia (SS) exhibits decreased affinity for oxygen, although the oxygen affinity of hemoglobin S is the same as that of hemoglobin A. SS red cells contain more 2,3-diphosphoglycerate (DPG) than normal erythrocytes. The oxygen affinity of hemolyzed red cells is decreased by added DPG, and hemolysates prepared from SS red cells do not differ from normal hemolysates in this regard. Reduction of oxygen affinity to the levels found in intact SS red cells required DPG concentrations in excess of those found in most SS patients. The same was true of oxygen affinity of patients with pyruvate kinase deficiency. Other organic phosphates, as well as inorganic ions, are known to alter the oxygen affinity of dilute solutions of hemoglobin. These substances, the state of aggregation of hemoglobin molecules, and cyto-architectural factors probably play roles in determining oxygen affinity of both normal and SS red cells.

INTRODUCTION

In 1938, Sidwell, Munch, Barron, and Hogness found that addition of phosphate ion to hemoglobin solutions caused a decrease in their oxygen affinity, and that the effect was more marked with phosphate than with citrate, sulfate, or chloride (1). In 1967, Benesch and Benesch (2) and Chanutin and Curnish (3) reported independently that organic phosphates alter the oxygen affinity of dilute solutions of human hemoglobin. The

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effects of 2,3-diphosphoglycerate (DPG) (and ATP, to a lesser extent) were exerted at concentrations comparable to those within human red cells. Greater affinity of DPG for deoxyhemoglobin than for the oxy-form (4, 5) was thought to account for the change in oxygen affinity produced by addition of DPG (6, 7).

Subsequently, Engel and Duc (8) reported that red cells which had been depleted of DPG by incubation with iodoacetate developed increased affinity for oxygen. Åkerblom, de Verdier, Garby, and Högman (9) and Bunn, May, Kochalaty, and Shields (10) found that the high oxygen affinity of stored blood could be related to its low content of DPG and ATP, and that restoration of DPG and ATP levels after incubation of stored red cells with inosine was associated with a return of oxygen affinity to normal levels. Decreased oxygen affinity of the blood of persons residing at high altitude was related to an increased concentration of DPG within their red cells (11, 12). Association between a decrease in oxygen affinity and elevated levels of DPG has also been documented in patients with pyruvate kinase (PK) deficiency (13, 14) and in patients with chronic hypoxemia (15).

The oxygen affinity of blood from patients with sickle cell anemia is known to be low (16, 17), although the oxygen affinity of hemoglobin S is the same as that of hemoglobin A (18). The present study attempts to answer two questions: (a) Are elevated levels of DPG present in red cells of patients with sickle cell anemia (SS), and (b) when DPG is added to concentrated solutions of hemoglobin in vitro, do the concentrations found in SS erythrocytes cause a decrease in oxygen affinity comparable to that found in intact SS red cells?

METHODS

Patients were selected from the inpatient and outpatient services of The Johns Hopkins Hospital. Hematologic studies were performed by standard techniques, and oxygen dissociation curves were constructed by the method of Hellegers, Meschia, Prystowsky, Wolkoff, and Barron (19).

DPG concentrations were measured on whole blood samples which had been frozen at -70°C within 30 min of collection. Microhematocrit values were measured before freezing, and DPG concentrations were expressed as micromoles per milliliter of packed red cells, since the DPG concentration of plasma is negligible. Pilot studies showed no difference between DPG concentrations measured by this technique and those measured in red cells which had been washed three times in 0.15 M NaCl solution. Samples from very anemic patients were centrifuged, and enough plasma was removed to raise the packed cell volume to approximately 40% before microhematocrit values were determined. Samples were shipped from Baltimore to Kansas City packed in dry ice, and were kept frozen at -20°C until the time of assay, which was within a few days. They were measured by an automated enzyme assay (20) in which the activity of phosphoglyceromutase is governed by the amount of DPG in samples added to an incubation mixture. It should be noted that both "free" and "hemoglobin-bound" DPG are measured by the assay system. Blood samples showed no change in DPG content after storage at -20°C for 1 wk, and recovery of DPG was complete when solutions prepared in Baltimore were assayed in Kansas City. Multiple analyses of coded samples with differing hematocrit values yielded values for a given sample which agreed within 0.6 $\mu\text{mole/ml}$; SS blood did not differ from that of normals in this regard.

The relationship between DPG concentration and oxygen affinity was studied by the addition of DPG to lysed red cells. Whole blood, collected in $\frac{1}{10}$ volume of a mixture of 2% potassium oxalate and 0.6% NH_4F , was frozen in a dry ice-acetone bath, and then thawed three times. From 20 to 2000 mg (24–240 μmoles) of the pentacyclohexylammonium salt of 2,3-diphosphoglyceric acid¹ was added to a 6.0 ml portion of the hemolyzed blood. In one experiment, free 2,3-diphosphoglyceric acid was prepared by passing 500 mg of the salt in approximately 2 ml of distilled water through a 4×7 cm column of Dowex 50.² The eluate was lyophilized, taken up in 4 ml of distilled water, and added to hemolysates after adjusting the pH to 7.4 with 2 N NaOH. Results were not different when free 2,3-DPG was used, rather than the salt, and the latter was used in all other experiments.

5-ml portions of these final mixtures were added to tonometers, and the remainder of the hemolysate-DPG mixture was frozen at -70°C for subsequent DPG assay. The tonometers were equilibrated with a gas mixture containing approximately 3.5% O_2 , 3.2% CO_2 , and 93.3% N_2 , at 38°C for 15 min. The pressure in the tonometers was then returned to atmospheric pressure, and a further 15-min period of equilibration followed. DPG concentrations did not change appreciably during the procedure. Oxygen contents were measured by the method of Van Slyke and Neill (21), oxygen tensions of the equilibrating gas were measured by the method of Scholander (22), and pH's on a Radiometer model 4 pH meter. After equilibration, hemo-

globin concentration of the hemolysates was 12–15 g/100 ml, and pH was 7.34–7.46. In other experiments, plasma was removed before the cells were frozen; the hemoglobin concentrations of these lysates were 30–32 g/100 ml.

Oxygen tensions are reported as the difference in mm Hg (ΔA) between the Po_2 which was found in these experiments and that which would have been obtained if samples of adult whole blood from pregnant women had been studied at the same pH and percentage oxygen saturation (23). The latter oxygen dissociation curve ($\log \text{Po}_2 = 4.734 - 0.45 \text{ pH} + 0.351 \log \text{S}/100 - \text{S}$) was used as a convenient standard for comparison; it is, in fact, quite similar to curves constructed in other laboratories using blood from nonpregnant adults.

The oxygen affinity of hemoglobin within red cells depends upon the intraerythrocytic pH, which is about 0.2 pH units below that of plasma, and comparisons between hemolysates and samples of whole blood must take into account that hydrogen ion gradient which exists across the intact red cell membrane. The magnitude of this gradient has been estimated by Battaglia, Behrman, Hellegers, and Battaglia (24). "Plasma" pH for a given point on the oxygen dissociation curve of the hemolysates here reported was calculated from their data. Oxygen affinity of the hemolysate was compared with that expected for whole blood at the calculated "plasma pH." This appears to be a reasonable comparison, for differences in oxygen affinity between normal whole blood and lysed packed red cells can be explained in this manner (25).³

The effect of DPG concentration on oxygen affinity of hemolysates of normal blood was compared with the effect on hemolysates from patients with sickle cell anemia (SS), using a similar technique. Approximately half the plasma was removed from SS blood before freezing and thawing in order to increase the hemoglobin concentration to about 10 g/100 ml. DPG (300 μmoles) was added to 30 ml of hemolysate, and oxygen affinity was compared with that of the same hemolysate to which no DPG had been added. Three or four points were determined for each of the two aliquots, the oxygen pressure required for half saturation was determined graphically, and these P_{50} 's were plotted against the DPG concentration of the aliquots.

RESULTS

The mean DPG concentration in blood from 34 healthy laboratory personnel was 4.47 $\mu\text{moles/ml}$ packed red cells (SD 0.53 $\mu\text{mole/ml}$) (Fig. 1). The mean concentration in blood from 32 patients with sickle cell anemia was 6.25 $\mu\text{moles/ml}$ (SD 0.91 $\mu\text{mole/ml}$); the difference is statistically significant ($P < 0.001$). DPG concentrations were not correlated with either hematocrit value ($r = 0.06$) or reticulocyte count ($r = 0.14$) (Fig. 2).

DPG was added to hemolyzed blood from four normal subjects, and oxygen affinity was measured (Fig. 3). There was considerable scatter between DPG concentrations of 2 and 8 $\mu\text{moles/ml}$. Allowing for such

³ For example, one sample of hemolysate was 42% saturated at a Po_2 of 25 mm Hg and a pH of 7.44. Normal red cells have an internal pH of 7.44 when the plasma pH is 7.66. At a plasma pH of 7.66, normal red cells are 42% saturated at a Po_2 of 18 mm Hg. ΔA for this sample, therefore, was reported as +7 mm Hg.

¹ Boehringer Mannheim Corp., New York.

² Bio-Rad AG 50W-X8, Bio-Rad Laboratories, Richmond, Calif.

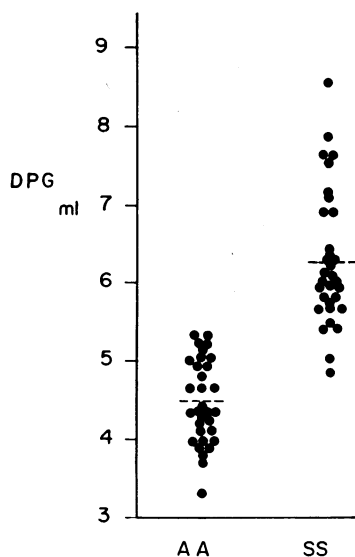


FIGURE 1 Concentration of 2,3-diphosphoglycerate in red cells of normal persons, and in red cells of patients with sickle cell anemia. Broken line (---) denotes mean for each group.

apparently random variation, oxygen affinity was related to DPG concentration in a curvilinear fashion, maximum decreases in affinity (ΔA , 8–10 mm Hg) being observed at concentrations of about 10 μ moles DPG/ml hemolysate. No further decrease in oxygen affinity was found at higher DPG concentrations. The oxygen affinity of hemolyzed packed red cells (hemoglobin concentration 30–32 g/100 ml) did not differ from that of hemolyzed blood (hemoglobin concentration 12–15 g/100 ml). Effects produced by the sodium salt of DPG were entirely equivalent to those produced by equimolar quantities of the commercially prepared pentacyclohexylammonium salt.

A hemolysate oxygen affinity similar to that of normal whole blood ($\Delta A = 0$) was found at a DPG level of about 5 μ moles/ml RBC, approximately the concentration which is found in normal red cells. A hemolysate affinity similar to that of SS blood (ΔA , 4–6) was observed at a DPG concentration of about 10 μ moles/ml RBC.

Oxygen affinity of lysed normal and SS blood was measured over a pH range from 6.99 to 7.59, with and without addition of a fixed amount of DPG (Fig. 4). Each entry in the figure represents the P_{50} derived from a 3 or 4 point oxygen dissociation curve. Since plasma contains a negligible amount of DPG, concentrations in hemolyzed blood before addition of that substance were somewhat less than half the normal concentration in red cells. There was considerable scatter when p_{50} was plotted against DPG concentration, and there was no convincing difference in oxygen affinity of samples with DPG concentrations from 2 to 8.5 μ moles/ml. SS blood did not differ from normal blood in this regard.

The hematocrit value of a patient with erythrocyte PK deficiency was 24.5%, the reticulocyte count was 59%, and the DPG concentration was 14.9 μ moles/ml RBC. The oxygen dissociation curve of her blood was approximately 12 mm to the right of the normal curve (Fig. 5). Oxygen affinity that low was not observed when DPG was added to hemolyzed blood, regardless of the amount added (Fig. 3).

DISCUSSION

The oxygen affinity of blood is decreased in persons residing at high altitudes, and in patients with hypoxic and anemic hypoxia (11, 12, 15, 26). It has been suggested that this shift in the position of the oxygen dissociation curve helps to maintain an adequate supply

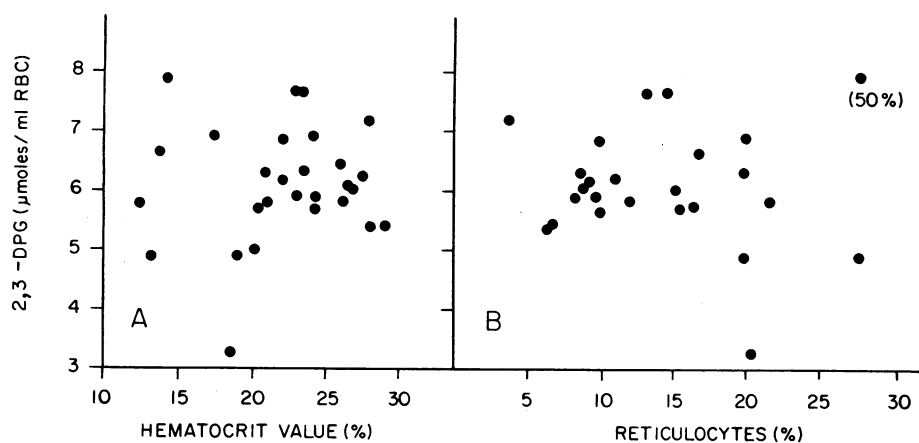


FIGURE 2 Relationship between concentration of 2,3-diphosphoglycerate in red cells and (A) hematocrit value, and (B) reticulocyte count of patients with sickle cell anemia.

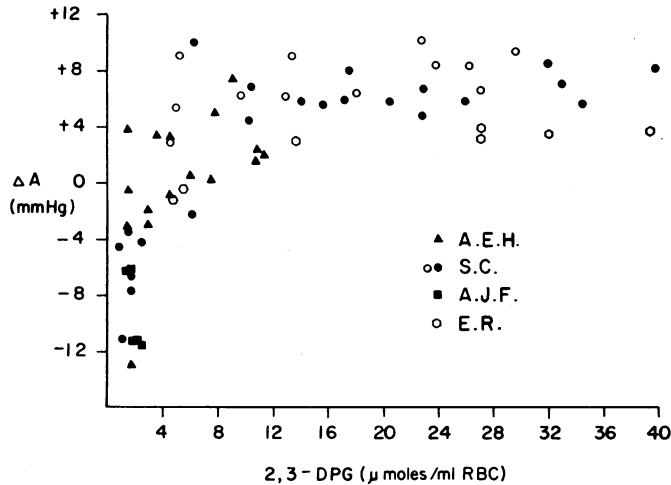


FIGURE 3 Relationship between concentration of 2,3-diphosphoglycerate and oxygen affinity of hemolyzed whole blood from four normal subjects. Oxygen affinity is expressed as the difference (ΔA) between the P_{O_2} of the sample studied and that of normal blood at the "same" pH, and the same per cent saturation (see text). ● = Hemoglobin concentration 12–15 g/100 ml; ○ = hemoglobin concentration 30–32 g/100 ml.

of oxygen to the tissues (27). DPG concentrations appear to be increased in the red cells of many of those persons (11, 12, 15, 28). In view of the effect of DPG on oxygen affinity, it seems reasonable to suggest that the two findings are related.

Elevated DPG in the red cells of hypoxic individuals may be produced by at least two mechanisms. According to one hypothesis (4) the average concentration of deoxyhemoglobin in their red cells (averaged over both sides of the circulation) is increased. Since deoxyhemoglobin binds more DPG than oxyhemoglobin (5, 29), the average concentration of unbound DPG is decreased. A decreased concentration of "free" DPG could relieve end product inhibition of the major enzyme of DPG synthesis, diphosphoglyceromutase (30–32), causing replenishment of the free pool and an increase in total DPG.

According to the second hypothesis (33), hypoxic individuals hyperventilate and tend to develop respiratory alkalosis. Increased pH within their red cells, produced by an elevated plasma pH and by an increased concentration of deoxyhemoglobin, is associated with an increased DPG concentration. The mechanism is not clear; it may involve stimulation of diphosphoglyceromutase (34) or decreased DPG catabolism (35), but does not appear to be a result of inhibition of diphosphoglycerate phosphatase⁴ (36). Therefore, hypoxemic, but *acidotic*, persons might not have increased amounts of DPG in their red cells.

⁴Rose, Z. Personal communication.

Studies in animals, and in some patients with anemia, have shown a correlation between severity of anemia and increase in DPG concentration (28, 37–39). Bromberg and Jensen related oxygen affinity to hemoglobin concentration in sickle cell anemia (17), and we ex-

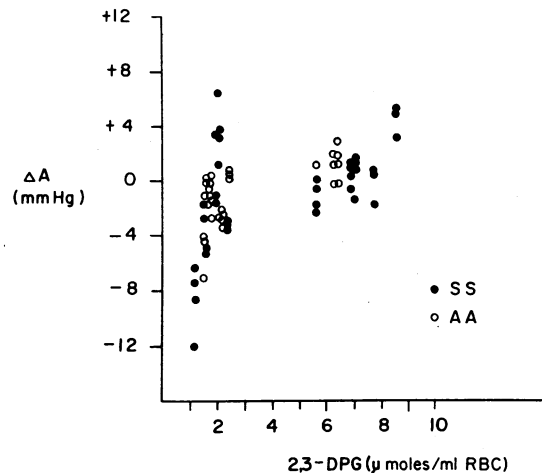


FIGURE 4 Comparison between concentration of 2,3-diphosphoglycerate and oxygen affinity of hemolyzed whole blood from normal subjects (○), and from patients with sickle cell anemia (●). DPG (300 μ moles/30 ml hemolyzate) was added to each of the samples on the right. Oxygen affinity is expressed as the difference (ΔA) between the P_{O_2} required for half saturation of the sample studied and that required for half saturation of normal blood at the "same" pH (see text).

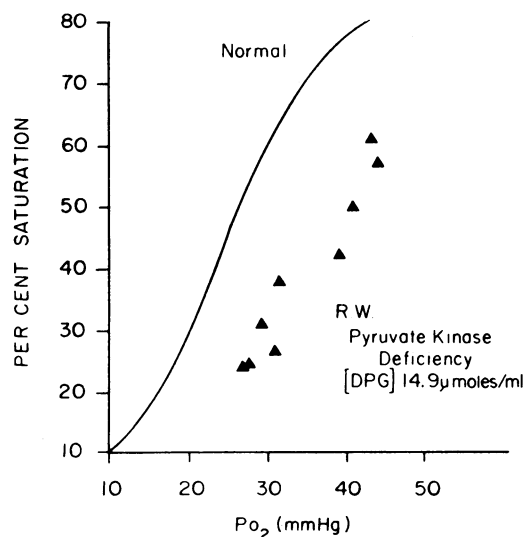


FIGURE 5 Oxygen affinity of the blood of a patient with erythrocyte pyruvate kinase deficiency (▲), compared with the normal oxygen dissociation curve (—).

pected to find the highest DPG levels in our most anemic patients. We did not find such a relationship (Fig. 2 A). The binding of DPG to hemoglobin and the activities of the enzymes of DPG metabolism are affected by DPG concentration, pH, and particularly by ionic strength of the system (29, 31, 34). Our patients with sickle cell anemia, although ambulatory, were not in good health, and it seems likely that unmeasured biochemical abnormalities of many types were the cause of the apparently random scatter of their DPG concentrations.

That view is supported by studies on two uremic patients without sickle cell anemia (Table I), who had anemia of similar magnitude, but widely differing DPG concentrations in their red cells. The plasma CO_2 combining power of the patient with the high DPG level, who was discharged on the day after blood was obtained, was almost twice that of the patient with the low DPG concentration. The difference in DPG concentrations probably was a result of differences in intracellular pH and ionic strength, and perhaps also of differences in activity of the acyl phosphatase of red cells, which regulates the metabolism of the precursor of DPG. A phosphatase with similar properties regulates metabolism of carbamyl phosphate (40, 41), the immediate precursor of urea, and it is conceivable that activity of the red cell phosphatase is affected by urea or related metabolites of the urea cycle which diffuse into erythrocytes from plasma.

Similar explanations may underlie our inability to correlate DPG levels with reticulocyte counts (Fig. 2 B). DPG concentrations are said to be increased in

reticulocyte-rich blood (10, 42), but that is not true of the reticulocytes of patients with hereditary spherocytosis (43). In that case, increased splitting of DPG was observed, and related to decreased pH within red cells.

Studies in which DPG was added to lysed normal red cells, and our comparisons of lysed normal and SS red cells, suggest that levels of DPG found in SS cells may be one cause of decreased oxygen affinity in sickle cell anemia (Figs. 3 and 4). Although the oxygen affinity of hemolysates was equivalent to that of normal blood at DPG concentrations found in normal red cells, an oxygen affinity as low as that found in SS patients ($\Delta A = 4-6$) was found only at DPG concentrations in excess of $8 \mu\text{mole/ml}$. Only one SS patient had a DPG level that high. Oxygen affinity of blood from our patient with pyruvate kinase deficiency, and that of other patients with the same disease (13, 14), is also difficult to explain on DPG concentration alone, for oxygen affinities as low as those found in these patients (ΔA , 12-14) were not found at the DPG levels found in their red cells ($6.6-14.9 \mu\text{mole/ml}$).

It may be argued that hemolysates are poor models of intact red cells. They *are*, for an essential feature of red cell cytoarchitecture, the cell membrane, has been disrupted. In addition, we have measured only DPG, and none of the other organic and inorganic ions which are known to alter oxygen affinity. Unequal concentrations of such unmeasured ions in the red cells of individual donors are probably the cause of the wide scatter of points in the "physiologic range" of DPG concentrations (Figs. 3 and 4). Unusual concentrations of such compounds in reticulocytes may also account for our inability to produce an oxygen affinity as low as that of patients with pyruvate kinase deficiency.

Abnormal aggregation of molecules of hemoglobin S, independent of DPG concentration, has been suggested as a cause of abnormal oxygen affinity of SS blood (44) and cytoarchitectural factors have been invoked as a cause of abnormal affinity of fetal red cells (45). It seems quite likely that a multiplicity of factors, in addition to the intracellular concentration of DPG, act

TABLE I
DPG Concentration in Two Patients with Uremia

Patient	Hemato- crit	Reticulo- cytes	Urea N	CO_2 comb- ining power	DPG
	%	%	mg/100 ml	mEq/liter	$\mu\text{moles}/$ ml RBC
E. T. 30-54-69	20	3.6	115	15.3	4.5
J. P. 127-97-68	17	4.0	150	28.5	12.3

in concert to govern oxygen affinity, not only in sickle cell anemia, but also in other pathophysiological states.

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