THE EFFECT OF ALTERATION OF INTRACELLULAR 2,3-DPG CONCENTRATION UPON OXYGEN BINDING OF INTACT ERYTHROCYTES CONTAINING NORMAL AND MUTANT HEMOGLOBINS*

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Received July 27, 1971

SUMMARY

The oxygen dissociation and association characteristics of intact erythrocytes containing normal and mutant hemoglobins and variable concentrations of 2,3-DPG (0 to 25 mM) have been studied by a continuous-recording polarographic technique. The oxygen affinity of cells containing Hb AC and CC is normal, Hb SS is considerably lower while that of Hb AS and SC is intermediate. Cord blood and cells with Hb Köln have an increased affinity for oxygen. Plots of P_{50} against 2,3-DPG concentrations produce asymptotic curves in all cells studied. The effect of changes of 2,3-DPG upon oxygen binding in the types of cells studied expressed as $\Delta P_{50}/\Delta$ 2,3-DPG decreases in the following order: SS > AS and SC > AA, AC and CC > F and Köln. The molecular basis for the increased response to 2,3-DPG by cells containing Hb S as compared to the normal response of hemoglobin S in solution is briefly discussed.

INTRODUCTION

The oxygen dissociation curve of whole blood from patients with sickle cell anemia is markedly shifted to the right (1-7). On the other hand, the oxygen-binding affinity of dilute purified hemoglobin solutions or hemolysates of Hb S is generally accepted as being identical to that of Hb A and Hb C (3, 8-11), although there are conflicting reports (4, 12). It has been suggested that the decreased O_2-binding affinity in sickle cell anemia is due to an increased content of the dialyzable factor 2,3-DPG, which has been shown to decrease the O_2-binding affinity of solutions of both normal (13, 14) and S hemoglobins to the same degree (3, 11). However, the increase in cellular 2,3-DPG in the erythrocytes

* Supported by grants from the Veterans Administration, USPHS Grant # AM-09001-07 and Grant # 661 from the Council for Tobacco Research-USA.

in sickle cell disease is insufficient to account for the magnitude of the shift in P_{50} (3). Cameron (7) recently observed a biphasic O_2-dissociation curve in Hb SC fresh whole blood but it became monophasic in blood depleted of 2,3-DPG; he suggested that a differential binding of this cofactor to Hb S could account for such differences.

It is our purpose in this communication to report the differential effect of variable intracellular concentrations of 2,3-DPG upon the O_2-binding affinity of intact erythrocytes from patients homozygous and heterozygous for sickle cell hemoglobin, as compared to those containing Hb A, Hb CC, Hb AC, Hb Köln and cord blood. The molecular basis and physiological significance of the curves observed are briefly discussed.

METHODS

Venous blood was withdrawn from 25 normal persons, two with sickle cell anemia (Hb SS), three with Hb AS, four with Hb SC, one with Hb CC, one with Hb AC, one Hb Köln and two cord bloods, using heparin as anticoagulant. Hemoglobins were identified by electrophoresis on cellulose acetate (15) and hemoglobin F was quantitated by alkali denaturation (16).

Cells containing variable quantities of 2,3-DPG were prepared in the following manner: Plasma and buffy coat were removed by aspiration after centrifugation of freshly drawn heparinized blood. The erythrocytes were washed twice with Krebs-Ringer's buffer which had been adjusted to pH 7.6 and supplemented with 30 mM Pi and 10 mM glucose. The cells were suspended in the same buffer and a portion retained at 3°. A second portion of the cells was supplemented with 5 mM inosine and 5 mM pyruvate and incubated at 37°, pH 7.8, to allow accumulation of intracellular 2,3-DPG. Aliquots were removed and placed at 3° after ½, 1, 2, 4, and 8 hours. At 4 hours additional inosine and pyruvate were added and the pH readjusted to 7.8. Under these conditions 2,3-DPG levels rose in a nearly linear fashion to 20 to 25 mM after 8 hours. A third portion of the cells was depleted of 2,3-DPG by incubation at 37°, pH 7.2, in the presence of 20 mM sodium bisulfite. Samples were removed at ½, 1, 2, 4 and 8 hours and stored at 3°. In most

instances, these cells were completely devoid of 2,3-DPG after incubating for 4 hours, and invariably after 8 hours. There was no significant change in cellular ATP during these incubations.

The 2,3-DPG content of the cells stored at 3° remained nearly constant for as long as 12 hours. Just prior to measuring the oxygen dissociation curve, the cells were washed with 0.16 M sodium phosphate buffer, pH 7.40, and a portion was extracted with five volumes of 6% perchloric acid. The protein-free extracts were neutralized with saturated potassium carbonate and stored frozen until assayed for 2,3-DPG (17) and, in some cases, ATP (18). The results were expressed as μmoles/ml packed erythrocytes (designated mM without correction for cell water).

Continuous oxygen dissociation and association curves were measured at $_pCO_2$ 44, pH 7.40 and 37° on whole cell suspensions in 0.16 M sodium phosphate buffer in a 30 ml closed cell, according to the polarographic method of Longmuir (19). Buffers and blood samples were thoroughly equilibrated with gas mixtures prior to study. In order to ensure complete oxygenation of samples which contained Hb S, a gas mixture containing 29.6% O_2 instead of 19.6% was used. The dissociation curve was measured during the constant removal of oxygen by beef heart mitochondria. The association curves were performed by generating oxygen at a constant rate by electrolysis at 12 V and 3.5 mA D.C. after the complete inhibition of mitochondrial respiration by the addition of 0.6 ml 0.5 M oxalacetic acid. At the end of each experiment, samples were removed from the closed chamber for measurement of pH and $_pCO_2$, using a Corning pH and gas analyzer. The measurements were nearly unchanged from the starting conditions, i.e. pH 7.40 ± 0.015 and $_pCO_2$ 44 ± 1 mm Hg.

RESULTS

The P_{50} (the partial pressure of oxygen required to half saturate hemoglobin) of the blood of 25 normal persons was 26.9 ± S. D. 0.7 mm Hg when calculated from dissociation curves ($P_{50}\downarrow$) and 30 ± S. D. 1.3 mm Hg when calculated from association curves ($P_{50}\uparrow$). The 2,3-DPG levels in 21 normal samples of blood (males and females) was 5.01 ± S. D. 0.75 mM. The oxygen binding affinities of cells contain-

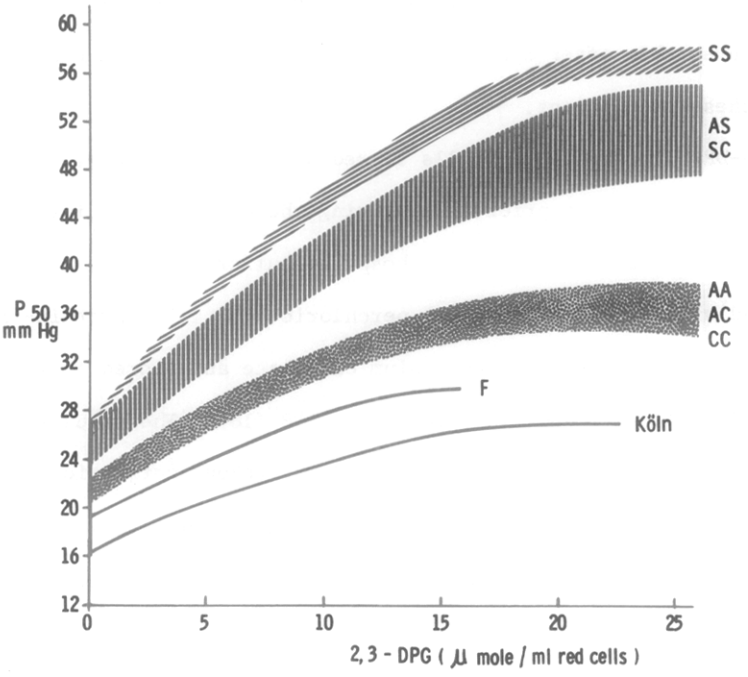

Fig. 1. The response of $P_{50}\downarrow$ to changes in 2,3-DPG concentration of cells containing normal and mutant hemoglobins.

The curves represent the ranges of values obtained on the following numbers of samples of blood: five normal, two SS, four SC, three AS, two F, and one each of AC, CC, and heterozygous Köln. All samples were studied at more than eight different levels of 2,3-DPG except the cord bloods which were each studied at four levels.

ing Hb AC and Hb CC were indistinguishable from that of normal cells. Blood possessing hemoglobin S, either Hb AS, Hb SC, or Hb SS, had a decreased affinity for oxygen ($P_{50}\downarrow$ = 33.3 ± 2, 38.6 ± 3.6 and 41.6 ± 2 mm Hg respectively).

When the $P_{50}\downarrow$ is plotted against the concentration of 2,3-DPG, an asymptotic curve results for each type of hemoglobin studied (Fig. 1.). At very low concentrations of 2,3-DPG (below 0.1 umole/ml red blood cells) all P_{50}'s dropped sharply to nearly the same value (18 to 20 mm Hg) with the exception of Hb Köln which was even lower. Because of the scale on the abscissa in Fig. 1 this portion of the curve cannot be shown in detail. The increment increase in P_{50} resulting from an increase in 2,3-DPG (expressed as $\Delta P_{50}/\Delta$ 2,3-DPG) decreased in the following order: Hb SS > Hb AS or SC > Hb AA, AC or CC > Hb F and Köln.

With the polarographic method used in these studies, for normal blood the P_{50} values measured by association ($P_{50}\uparrow$) are approximately 3 mm higher than those measured by dissociation ($P_{50}\downarrow$), and the shape of the curves is the same (Fig. 2.). In contrast the $P_{50}\uparrow$ of cells either heterozygous or homozygous

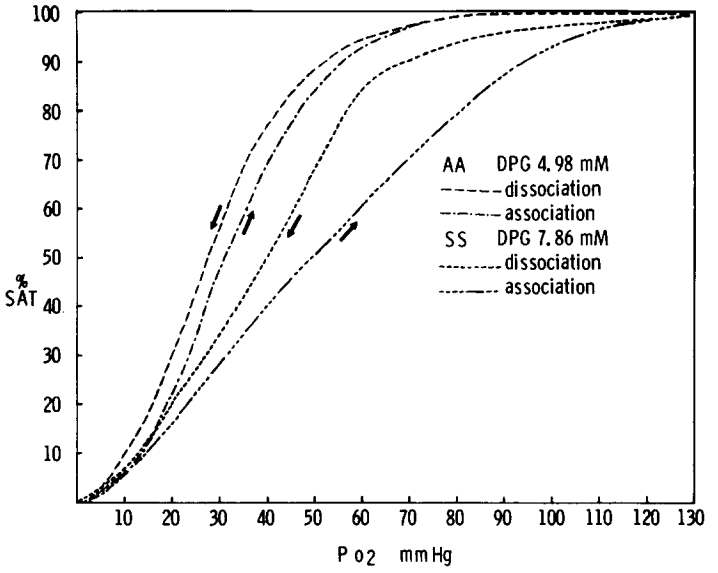

Fig. 2. Oxygen association and dissociation of normal and Hb SS blood.

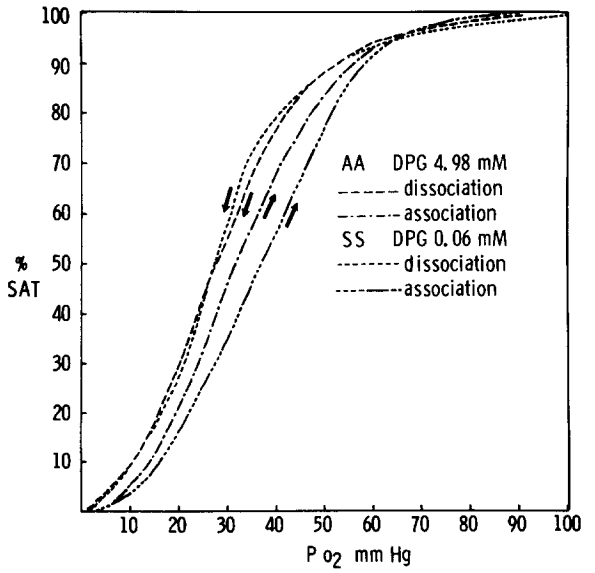

Fig. 3. Comparison of oxygen association and dissociation of Hb SS cells depleted of 2,3-DPG and normal cells.

for Hb S is much higher than $P_{50}\downarrow$, and the shapes of the two curves are significantly different (Fig. 2.). This difference in $P_{50}\uparrow$ and $P_{50}\downarrow$ is most apparent at higher levels of 2,3-DPG. As intracellular 2,3-DPG is diminished, both the shape and position of the association and dissociation curves become more nearly the same (Fig. 3.).

DISCUSSION

Our studies confirm previous reports that cells containing Hb CC and Hb AC have normal oxygen affinity and those with Hb F and Hb Köln have increased binding affinity (4, 5, 20). We further observed that the shift in position of the oxygen dissociation curve resulting from changes in 2,3-DPG is normal in cells containing Hb CC or Hb AC, while it is decreased in cells from the newborn and those containing Hb Köln. This decreased response of cord blood is almost the same as that observed in studies on solutions of hemoglobin F (11, 21).

The results presented here also agree with the observation that not only SS and SC, but also AS, cells have a lesser oxygen affinity than normal cells (1-7), even when measured at comparable intracellular 2,3-DPG levels. This deviation from normal becomes more marked as the intracellular 2,3-DPG increases, and nearly disappears when cells are completely depleted of 2,3-DPG (Fig. 1.). This is strikingly different from the behavior of these hemoglobins in dilute solution where the oxygen dissociation curves of hemoglobin A and S without 2,3-DPG are identical and are both shifted to the right to a similar degree upon addition of like quantities of 2,3-DPG (3, 11). Therefore, this enhanced response to 2,3-DPG in cells containing Hb S is most likely caused by the concentration-dependent intermolecular associations of the mutant hemoglobin. Since cells containing hemoglobin S which have been depleted of 2,3-DPG have an oxygen affinity nearly identical to that of normal cells (as observed by Bromberg and Andrade (22) and in these studies), polymerization of Hb S molecules within the cell cannot of itself explain this marked decrease in affinity for oxygen.

The discrepancy between the association and dissociation curves of normal blood is most likely due to our inability to completely paralyze mitochondrial

respiration and to non-uniform distribution of oxygen within the chamber. Since the oxygen is generated at a point source even rapid mixing does not completely abolish this problem. Both are errors in the ascending curve and we feel that the descending curves represent equilibrium conditions. At present we are unable to explain the greater difference between the two curves with cells containing S hemoglobin. However, we believe that this is related to preferential unsickling of many of the cells prior to combining with oxygen. We are reporting these data since they demonstrate a difference between cells which are able to sickle and those which cannot. Similar results could be obtained if some of the cells became irreversibly sickled; Gibbs, et al (23) have shown that such cells have a much lower oxygen affinity than reversibly sickled cells.

It has generally been accepted that the sickling phenomenon results from hydrophobic interactions between the region of the β chain altered by the mutation (β_6 glutamate → valine) and complementary sites on neighboring molecules (24, 25). Recently Bookchin has shown that electrostatic bonds may also play an important role in this aggregation of hemoglobin S molecules (26). Although the details underlying sickling are not fully understood, we wish to suggest several possible mechanisms which would explain the differences observed in oxygen affinity between intracellular and extracellular S hemoglobin: 1. The polymerization of Hb S molecules may facilitate the binding of 2,3-DPG; 2. The constraint upon one hemoglobin molecule induced by binding 2,3-DPG (27) may influence conformational changes in neighboring associated molecules; 3. The binding of 2,3-DPG to one deoxyhemoglobin S molecule may somehow make its complementary site more available to bind a second molecule of oxyhemoglobin or a hemoglobin without 2,3-DPG and increase the free energy of interaction with oxygen in the newly associated molecules. Each of these mechanisms would facilitate unloading of oxygen and thereby further subsequent intermolecular associations of deoxyhemoglobin.

Studies are in progress to test these proposals and to investigate the differences observed in the association and dissociation curves.

ACKNOWLEDGMENTS

We wish to thank Dr. Bruce Cameron for his helpful discussions and for the use of his laboratory where a portion of these studies were conducted and Miss L. Glazer for performing the hemoglobin electrophoreses.

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