THE DISEASES associated with hemoglobin S present a clinical spectrum. Most severe is sickle cell anemia (SS). Less severe, in general, in decreasing order, are hemoglobin S-D disease, S-thalassemia, S-C disease, S-F syndrome, and sickle cell trait (AS). Precise correlations cannot be made between the amount of hemoglobin S present, the number of sickle cells in the blood, and the clinical severity of the disease. It has been suggested that the greater the per cent of hemoglobin S, the greater the number of sickle cells and the more severe the disease. Exceptions to this have been described. The sickle phenomenon in a cell containing hemoglobin S has been postulated to be dependent on a variety of intra- and extracorpuscular factors including the type and amount of non-S hemoglobin present, the level of intracellular enzymes, blood oxygen tension, pH, osmolarity, temperature, and trauma.¹⁻⁸ This study was designed to investigate the sickle phenomenon and the oxygen dissociation curves in eight patients with hemoglobin S in association with other hemoglobin abnormalities in an attempt to provide at least a partial answer to the postulated correlations noted above.

METHODS

Hematologic Determinations

Routine hematology was done by standard methods. Glucose-6 PO₄-dehydrogenase (G-6-PD) was quantitated by the method of Marks¹⁰ and expressed as ΔO.D./min./mg. of hemoglobin (normal in our laboratory = 9–10u). Electrophoretic separation of hemoglobins was done on cellulose acetate in a discontinuous buffer as described by Petrakis.¹¹ Agar gel electrophoresis was carried out on glass slides at pH 6.2 and the hemoglobins quantitated as previously described.¹² Fetal hemoglobin was determined by the alkali denaturation technic of Singer and Chernoff.¹³ Intracellular fetal hemoglobin was examined by the technique of Kleihauer, Braun and Betke.¹⁴

Arterial and venous specimens were drawn simultaneously by two people into heparinized
oil sealed syringes. The blood was diluted and fixed in a 3 per cent formalin 0.9 per cent NaCl aqueous solution. The per cent of sickled cells in a sample was determined by counting the number of sickle cells per 1000 red cells in a hemocytometer. Cells were considered to be sickled if 2 or more distinct points were present on the cell.

In Vivo Studies
Subjects were placed at bed rest. Catheters were inserted in the right brachial artery and left antecubital vein. Samples for resting base line values were obtained. The subject was then given 100 per cent O2 by mask for 10 minutes. Blood samples were obtained and the subject allowed to return to baseline values as determined by measuring pO2, pCO2, and pH before and after each procedure.

The subject was then given 33.7 meq. of NaHCO3 intravenously and, after a five minute interval for equilibration, additional blood samples were obtained. One gram of MgSO4 was then given intravenously, and blood samples were again obtained after a five minute waiting period. The subject was then given 6 per cent CO2 in air by mask for ten minutes before the final blood specimens were obtained.

In Vitro Studies
The in vitro determinations of sickle cell formation were performed using fresh venous blood anticoagulated with sodium ethylenediaminetetraacetate. The blood was equilibrated with a continuous flow of moist warmed 95 per cent O2 and 5 per cent CO2 at 37 C. Equilibration was carried out in capped vials in a shaking water bath. The gas was introduced into the vial through a needle inserted through the cap and positioned so that the gas flow was directly above the surface of the blood. An outflow needle was inserted in the top of the vial. After exposure to this gas mixture for 10 minutes, the blood was equilibrated with a new mixture consisting of 5 per cent CO2, 1 per cent O2 and 94 per cent N2. Samples were withdrawn by syringe at 10 minute intervals, and the per cent of sickle cells determined. No decrease in the erythrocyte count of the samples was observed.

Magnesium sulfate (.005 and 1.0 mg. per cent) was added to blood specimens containing approximately 50 per cent sickle cells. The blood was kept at 20 C. for 1 hour and the per cent of sickle cells then determined.

Determination of Oxyhemoglobin Dissociation Curves
Fresh heparinized whole blood was used throughout this portion of the study. The blood was equilibrated in a tonometer at 37.5 C. with gas mixtures of 6 per cent CO2 and different concentrations of O2 and N2 using a continuous flow method.

Blood O2 and CO2 tensions were measured using appropriate electrodes (Instrumentation Laboratory Inc. Model 113). A model pH-27 Radiometer pH meter with a microelectrode was used for blood pH measurements. All measurements were made at 37.5 C.

Hemoglobin saturations were determined spectrophotometrically using a Beckman model DB spectrophotometer. The sample cuvette and the general procedure used have been described elsewhere.15

Saturated blood samples were prepared either by tonometry with a mixture of 95 per cent O2 and 5 per cent CO2 or by simply rotating the sample in room air. Both methods gave equivalent results. Deoxygenated, or reduced, samples were prepared by tonometry with a mixture of 95 per cent N2 and 5 per cent CO2.

The values of T50 (P50O2) at pH 7.40 were obtained by extrapolation of the experimental data assuming a normal Bohr effect. A few determinations were made at CO2 tensions of 20 and 60 mm. of Hg to determine the Bohr effect. One curve was determined on blood obtained after intravenous MgSO4 administration.

The data obtained are estimated to be accurate to ± 2 mm. oxygen tension and ± 1 to 1.5 per cent saturation. The mean normal T50 in our laboratory is 26.5 ± 2 (2 S.D.).

The Hill equation was solved for n (log \( \frac{y}{(1-y)} \) = n log pO2 at T50 y = 0.50). The normal range in our laboratory for n is 3.7 ± 0.4 (2 S. D.). Graphic solutions of the Hill equation and solutions using observed y and pO2 values most closely approximating y = 0.50 gave slightly lower but comparable values.
Table 1.—Analysis of Hemoglobins, Reticulocytes and G-6-PD
(Hemoglobin is expressed as per cent of total hemoglobin present)

<table>
<thead>
<tr>
<th>Patients No.</th>
<th>Type of Hgb.</th>
<th>S</th>
<th>D</th>
<th>C</th>
<th>F</th>
<th>A</th>
<th>A2</th>
<th>Retics %</th>
<th>G-6-PD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>SS†</td>
<td>87</td>
<td>—</td>
<td>—</td>
<td>8</td>
<td>—</td>
<td>5</td>
<td>13.3</td>
<td>26.6</td>
</tr>
<tr>
<td>2.</td>
<td>SD</td>
<td>76</td>
<td>22</td>
<td>—</td>
<td>—</td>
<td>Trace</td>
<td>2</td>
<td>9.7</td>
<td>29.8</td>
</tr>
<tr>
<td>3.</td>
<td>SD</td>
<td>44</td>
<td>54</td>
<td>—</td>
<td>—</td>
<td>Trace</td>
<td>2</td>
<td>14.0</td>
<td>30.1</td>
</tr>
<tr>
<td>4.</td>
<td>SD</td>
<td>25</td>
<td>73</td>
<td>—</td>
<td>—</td>
<td>Trace</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5.</td>
<td>S Thal</td>
<td>63</td>
<td>—</td>
<td>—</td>
<td>30</td>
<td>—</td>
<td>5</td>
<td>7.3</td>
<td>6.3</td>
</tr>
<tr>
<td>6.</td>
<td>S Thal</td>
<td>79</td>
<td>—</td>
<td>—</td>
<td>18</td>
<td>—</td>
<td>3</td>
<td>23.5</td>
<td>43</td>
</tr>
<tr>
<td>7.</td>
<td>SC</td>
<td>43</td>
<td>—</td>
<td>—</td>
<td>52</td>
<td>3</td>
<td>—</td>
<td>2.2</td>
<td>0</td>
</tr>
<tr>
<td>8.</td>
<td>SC</td>
<td>39</td>
<td>—</td>
<td>—</td>
<td>56</td>
<td>3</td>
<td>—</td>
<td>10.2</td>
<td>21.8</td>
</tr>
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</table>

RESULTS

Hematologic Studies and Analyses of Hemoglobins

The quantitative analyses of hemoglobins, reticulocyte counts and G-6-PD determination for the 8 patients included in this study are given in Table 1. G-6-PD deficiency in patient 4 was masked initially by a reticulocytosis of 40 per cent. At this time the G-6-PD was 40 u. Subsequent study of this patient during an aplastic crisis showed almost no detectable G-6-PD activity. Studies of the available family members showed G-6-PD deficiency in the mother and a half-brother. Patients 5 and 6 were classified as cases of sickle cell thalassemia rather than S-high fetal hemoglobin on the basis of the heterogeneous intracellular distribution of fetal hemoglobin, as well as on the basis of genetic studies.

In Vivo Studies

Relationship of hemoglobin S present, arterial pO2, T50, and per cent of sickled cells. The per cent of hemoglobin S, pAO2, T50, n values, and per cent of sickled cells in arterial blood is shown in Table 2. These data suggest that the per cent of sickled cells can be related to the amount of hemoglobin S present, and also may be influenced by the pO2, and the other type of hemoglobin present in the cell.

Table 2.—Relationship of % Hemoglobin S Present
Arterial pO2, T50, n value and % of Sickled Cells

<table>
<thead>
<tr>
<th>Patients No.</th>
<th>Type of Hgb.</th>
<th>% Hgb. S</th>
<th>pAO2</th>
<th>T50</th>
<th>n</th>
<th>% Sickled Cells Artery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>SS†</td>
<td>87</td>
<td>72</td>
<td>34</td>
<td>2.94</td>
<td>52</td>
</tr>
<tr>
<td>2.</td>
<td>SD</td>
<td>76</td>
<td>71</td>
<td>32</td>
<td>3.12</td>
<td>26</td>
</tr>
<tr>
<td>3.</td>
<td>SD</td>
<td>44</td>
<td>79</td>
<td>36.5</td>
<td>2.74</td>
<td>16.4</td>
</tr>
<tr>
<td>4.</td>
<td>SD †</td>
<td>25</td>
<td>75</td>
<td>34</td>
<td>2.94</td>
<td>39</td>
</tr>
<tr>
<td>5.</td>
<td>S Thal</td>
<td>65</td>
<td>72</td>
<td>30.5</td>
<td>3.28</td>
<td>5.3</td>
</tr>
<tr>
<td>6.</td>
<td>S Thal</td>
<td>79</td>
<td>72</td>
<td>34</td>
<td>2.94</td>
<td>0</td>
</tr>
<tr>
<td>7.</td>
<td>SC †</td>
<td>43</td>
<td>86</td>
<td>30</td>
<td>3.33</td>
<td>0</td>
</tr>
<tr>
<td>8.</td>
<td>SC</td>
<td>39</td>
<td>84</td>
<td>29</td>
<td>3.45</td>
<td>0</td>
</tr>
</tbody>
</table>

Normal ± 2 S.D. 26.5 ± 2 3.7 ± 0.4

* G-6-PD Deficiency.
† Patient in crisis at time of observation.
Effect of breathing 100 per cent \( O_2 \) on sickling. Seven of the eight subjects were tested for the effect of 100 per cent \( O_2 \) inhalation on arterial and venous \( pO_2 \) and the per cent of sickle cells. These data are summarized in Figures 1 and 2.

Fig. 1.—Per cent of sickle cells in arterial blood after the inhalation of 100 per cent of \( O_2 \).

Fig. 2.—Per cent of sickle cells in venous blood after the inhalation of 100 per cent \( O_2 \).
Fig. 3.—Rate of sickling on exposure of blood to 5 per cent CO₂, 1 per cent O₂, and 94 per cent N₂ at 24 C.

**Effect of NaHCO₃, MgSO₄, and CO₂ on the per cent of sickle cells.** Intravenous NaHCO₃ (33.7 meq.) increased mean arterial pH 0.05 and venous pH 0.048 units. Inhalation of 6 per cent CO₂ decreased mean arterial pH 0.14 and venous pH 0.09 units. No change in the per cent of sickle cells was noted with these changes in pH.

Serum magnesium values were slightly low in these patients with a mean of 1.41 mg. per cent (normal in this laboratory is 1.8 to 3 mg. per cent). The

Fig. 4.—Rate of sickling on exposure of blood to 5 per cent CO₂, 1 per cent O₂, 94 per cent N₂ at 24 C. and its reversal on exposure to room air.
injection of 1 Gm. of MgSO₄ intravenously increased the serum magnesium to approximately double the initial value without changing the number of sickle cells present.

**In Vitro Studies**

The rate of sickling in the blood of some of these subjects on exposure to a 5 per cent CO₂, 94 per cent N₂, and 1 per cent O₂ gas mixture at 24 C. is shown in Figure 3. As can be noted the most rapid rate of sickling was observed in the patient with SS hemoglobin. At this time the patient had 20 per cent AA cells from a transfusion, as evidenced by a failure to sickle of 20 per cent of these cells after 48 hours in 2 per cent sodium metabisulfite. The SD cells sickled more readily than the SF cells. Under these conditions AS and SC cells did not sickle.

Figure 4 illustrates the development of sickling in SF and SD cells. With deoxygenation, the SF cells sickled more slowly than the SD cells, and the sickling was more rapidly reversible by oxygenation.

At the concentrations used no effect of MgSO₄ on the per cent of sickle cells was observed in vitro.

*Relationship of type and amount of abnormal hemoglobin to shift in the oxygen dissociation curve.* The oxygen dissociation curves of the blood of these patients are shifted to the right of the normal curve. In two instances, the Bohr effect was determined and was found to be comparable to that present in normal hemoglobin. No change in the oxygen dissociation curve was observed in patient 4 after intravenous MgSO₄.

The oxygen tension at which the hemoglobin is fifty per cent saturated with oxygen (T₅₀) was determined in each case. The relationship between the per cent of hemoglobin S and T₅₀ is plotted in Figure 5. With the exception of two patients with SD hemoglobin there seems to be a straight line relationship between the T₅₀ and the per cent of hemoglobin S.

**DISCUSSION**

The per cent of sickle cells as measured in arterial and venous blood was roughly related both to the per cent of hemoglobin S present, the other type of
hemoglobin present, and to the pO₂. The exception to this was patient 4 who had a higher per cent of sickle cells than would have been predicted on the basis of only 25 per cent hemoglobin S and a pO₂ of 75 mm Hg. The blood pH was normal. Methemoglobinemia was not present. The patient was febrile, dehydrated, and had pneumococcal pneumonia. The patient was in aplastic crisis at this time and had no reticulocytes and no detectable G-6-PD. It is probable that all of these factors contributed to the high per cent of sickled cells. Restudy of the patient at a later date showed the same relative per cents of hemoglobins and a marked decline in the percent of sickle cells.

The in vitro rate of sickling on deoxygenation was greatest in SS cells, intermediate in SD cells, and low in S-thalassemia cells. Sickling did not occur under the conditions of this experiment in AS and SC cells. The rate at which sickling could be reversed by exposure to oxygen was compared in a patient with SD and S-thalassemia, both having approximately the same amount of hemoglobin S. Sickling was more rapidly reversed in the S-thalassemia cells with 30 per cent hemoglobin F. These observations are in agreement with the in vivo observations and clinical observations.16

The in vitro oxygen dissociation curves of these patients were all shifted to the right indicating a lower oxygen affinity. The values for T₅₀, ranged from 36.5 to 29 mm Hg. and were greater than the normal mean +2 S.D. These observations are consistent with those of other workers on blood from patients with hemoglobin SS and SC.17-20 The n values for patients with hemoglobin S, SD and S-Thal were below the normal in minus 2 S.D. which may indicate a decrease in heme-heme interaction although caution should be taken in attributing undue physiologic significance to Hill's equation.

The oxygen affinity for hemoglobin solutions of S hemoglobin has been reported by several workers.21-25 With the exception of the studies of Riggs and Wells the oxygen affinity of solutions of hemoglobin S has not been found to differ from that of hemoglobin A. It should be noted that the concentrations of hemoglobin S in these studies were less than 20 Gm. per cent, considerably below the 30 Gm. per cent solution found in the erythrocyte. The high protein concentration in the erythrocyte provides a unique environment for the hemoglobin molecule and should tend to maximize protein-protein interaction. Under such conditions subtle alterations in the tertiary structure of the molecule may lead to profound functional effects.

It has been observed that young cell populations have a lower oxygen affinity than do old cell populations.26 Benesch, Benesch and Yu have shown that the decrease in oxygen affinity of hemoglobin is related to the cell concentration of organic phosphates.27 Young cell populations have increased amounts of 2, 3 diphosphoglycerate and adenosine triphosphate, and the decrease in oxygen affinity observed in these patients may be due to the relative youth of the cell population rather than due to the abnormal hemoglobin.

SUMMARY

1. Data on pO₂, T₅₀, n values and per cent of sickle cells were obtained in patients with hemoglobins SS, SD, SF (S-thalassemia) and SC. In general the per cent of sickle cells was proportional to the per cent of hemoglobin S. The
number of sickle cells was, however, influenced by the other type of hemoglobin present and by the pO₂. Values for T₅₀ were greater than normal, and values for n were less than normal.

2. The effect of therapy with O₂, CO₂, NaHCO₃, and MgSO₄ on the per cent of sickle cells in arterial and venous blood was determined. Only inhalation of O₂ reduced the per cent of sickle cells.

3. The rate of sickle cell formation and its reversal were determined under conditions of deoxygenation and oxygenation. These were found to be a function of the type of hemoglobin combinations present.

4. The oxygen dissociation curves were found to be shifted to the right of the normal curve indicating a decreased affinity for oxygen. In two patients the Bohr effect was determined and found to be normal.

SUMMARIO IN INTERLINGUA

1. Datos relative al valores pO₂, T₅₀, e n e relative al procentage de cellulas falciforme eseva obtenite in patientes con le hemoglobinas SS, SD, SF (S-thalassemia), e SC. A generalmente parlar, le procentage de cellulas falciforme eseva proportional al procentage de hemoglobina S. Tamen, le numero del cellulas falciforme eseva influentiate per le altere typo de hemoglobina presente e per le valor de pO₂. Le valores de T₅₀ eseva plus grande que normal, e le valores pro n eseva minus grande que normal.

2. Le efecto de un therapia con O₂, CO₂, NaHCO₃, e MgSO₄ super le procentage de cellulas falciforme in sanguine arterial e venose eseva determinate. Solo le inhalation de O₂ reduceva le procentage del cellulas falciforme.

3. Le intensitate del formation de cellulas falciforme e su reversion eseva determinate sub conditiones de deoxygenation e de oxygenation. Esseva trovate que istos eseva un function del typo de combinationes de hemoglobina presente.

4. Le curvas de dissociation de oxygeno monstrava un displaciamento dextrorse relative al curva normal, indicante un reduceite affinitate pro oxygeno. In duo patientes, le efecto de Bohr eseva determinate, con le constatation que illo eseva normal.

REFERENCES


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A Study of the Sickling Phenomenon and Oxygen Dissociation Curve in Patients with Hemoglobins SS, SD, SF and SC

M. J. CAWEIN, R. P. O'NEILL, L. A. DANZER, E. J. LAPPAT and THOMAS ROACH