Studies of the Interaction of 2,3-Diphosphoglycerate and Carbon Dioxide with Hemoglobins from Mouse, Man, and Elephant*

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SUMMARY

The oxygen affinity of mouse hemoglobin is greatly lowered by 2,3-diphosphoglycerate (2,3-DPG). The higher the pH, the smaller the size of the effect. The pH dependence of this shift in log \( p_{\text{O}_2} \) resembles a titration curve with an apparent \( pK \) of 7.76. This value is close to that reported for the \( \alpha\text{-NH}_2 \) groups of deoxyhemoglobin.

The addition of 1 to 2 moles of 2,3-DPG per mole of hemoglobin decreases the value of \( n \) in Hill's equation. Further addition causes \( n \) to return to normal values. However, estimation of the overall free energy of interaction shows that 2,3-DPG does not affect interaction. The changes in \( n \) are therefore believed either to reflect the presence of mixtures of hemoglobins combined to varying extents with 2,3-DPG or the fact that the free 2,3-DPG concentration is continuously increasing during oxygenation.

The Bohr effects at 20°, between pH 7.0 and 7.5, of hemoglobins from mouse, man, and elephant are approximately -0.52, -0.48, and -0.30, respectively; these values become -0.9, -0.7, and -0.44, respectively, in the presence of 1 mole of 2,3-DPG per mole of hemoglobin.

Carbon dioxide abolishes the effect of 2,3-DPG on the oxygen equilibrium of mouse hemoglobin at \( p\text{CO}_2 = 60 \text{ mm Hg} \), pH 7.3, and 20°. The higher the \( p\text{CO}_2 \) the lower is the pH at which CO₂ abolishes the 2,3-DPG effect.

Mouse hemoglobin digested with carboxypeptidase A is devoid of a 2,3-DPG effect. In contrast, the oxygen equilibrium of hemoglobin digested with carboxypeptidase B shows a 2,3-DPG effect.

We conclude that 2,3-DPG binds at the same sites as does CO₂, namely at the NH₂-termini of the \( \alpha \) and \( \beta \) chains, but that interaction of 2,3-DPG with the \( \beta \) chain is functionally the most important.

Mechanisms are proposed to explain both the effects of CO₂ and of 2,3-DPG. The CO₂, bound as carbamate (-NHCOO⁻), and the 2,3-DPG anion are both believed to reduce the oxygen affinity because the introduction of the negative charge interferes with the conformation change associated with oxygenation.

We conclude that hemoglobins from different mammals are not physiologically equivalent and do not by themselves provide support for the theory of selectively neutral mutations. Instead, the properties of the oxygen equilibria of mammalian hemoglobins appear to reflect an adaptation of the metabolic needs of the animal.

The substantial decrease of the oxygen affinity of human hemoglobin in the presence of organic phosphates, such as 2,3-diphosphoglyceric acid and adenosine triphosphate has been demonstrated by many investigators (1-11). Such an effect must result from the binding of the phosphate to the hemoglobin. However, the number and nature of the binding sites are not yet clear.

In an earlier paper (12) the Bohr effect of hemoglobins from different mammals was reported to be related to the body weight or metabolic rate of the animal, the smaller the mammal and the larger the metabolic rate, the larger the Bohr effect. Thus, mouse hemoglobin was reported to have a Bohr effect almost twice that of human hemoglobin. Later work by Smith et al. (13) failed to show any significant difference between the Bohr effects of mouse and human hemoglobins. These results have been interpreted to mean that mammalian hemoglobins were functionally all alike (14) and their supposed identical functional properties were even taken as evidence for selectively neutral mutations (15). The earlier experiments on the Bohr effect (12) of various hemoglobins were done with hemolysates dialyzed only against distilled water which does not remove 2,3-diphosphoglycerate (10, 11). The earlier results (12) might therefore have been due to the presence of different quantities of diphosphoglycerate or to differences in the sensitivity of the hemoglobins to this substance. The purpose of this paper is to determine the nature of the effects of 2,3-diphosphoglycerate on the oxygen equilibrium of mouse hemoglobin, the amino acid sequence of which is largely known (16) and to resolve the question of the existence of functional differences among mammalian hemoglobins.
MATERIALS AND METHODS

Animals—Mice of strain C57BL/6J were purchased from the Jackson Laboratory, Bar Harbor, Maine. The sample of Indian elephant blood was obtained through the San Antonio Zoological Gardens, San Antonio, Texas.

Hemoglobin Preparation—Fresh citrated blood from decapitation of five mice was washed 3 times with 0.9% NaCl (w/v). The washed, packed red blood cells were lysed with an equal volume of deionized water, to which 2 volumes of toluene were added, and then shaken vigorously. The stroma was removed after 1 hour at 40°C by centrifugation at 3000 rpm for 25 min.

The clear hemoglobin solution was applied to a Sephadex G-25 column (1.5 x 45 cm) equilibrated with 0.1 M NaCl. Elution was carried out with 0.1 M NaCl at a flow rate of 25 ml per hour. The stripped hemoglobin so prepared was freed of NaCl by dialysis against deionized water overnight. All steps were carried out at 4°C. Elephant blood was obtained by syringing from an ear vein. Mouse, elephant, and human bloods were processed identically.

Determination of 2,3-Diphosphoglycerate— Determination of 2,3-DPG1 was carried out colorimetrically (17) and by column chromatography (18) without significant changes. The total phosphorus was determined as described by Bartlett (18). The 1,2,4-aminonaphtholsulfonic acid used in this procedure was purified by crystallization as described by Fiske and SubbaRow (19). The 2,3-DPG was prepared as described (20).

Oxygen Equilibria—Determinations were made spectrophotometrically at 20°C as previously described (21). The hemoglobin concentration was 0.2%, which was made by mixing the hemoglobin solution with an equal volume of Tris-HCl buffer (ionic strength, 0.025) or by adjusting the pH with dilute NaOH prior to the experiments. The hemoglobin solution (4.0 ml) was pipetted into a tonometer (volume, 270 to 300 ml; cell light path, 10 mm) and deoxygenated by evacuation and flushing with nitrogen which was purified by passage through chromous chloride (22). The absorbance of the hemoglobin was measured at 540, 550, and 580 nm with a Beckman model DU spectrophotometer. The value of \( n \) was taken as the slope of the line relating log \( y/1 - y \) to log \( p_{O_2} \) between \( y = 0.25 \) and 0.75, where \( y \) is the fractional degree of oxygenation and \( p_{O_2} \) is the oxygen pressure in mm Hg; the log \( p_{O_2} \) value is the log \( p_{O_2} \) corresponding to half-oxygenation.

Oxygen equilibria in the presence of \( CO_2 \) were also measured. Water-saturated \( CO_2 \) was injected into the tonometer after deoxygenation of the hemoglobin to give the desired \( p_{O_2} \). The \( p_{CO_2} \) was not corrected for dissolved and bound \( CO_2 \). After rotating the tonometer for 30 min in a water bath, a solution of 2,3-DPG was injected when appropriate.

The absorbance at 540, 550, or 580 nm was unchanged by the injection of either \( CO_2 \) or 2,3-DPG when correction was made for the dilution by the 2,3-DPG solution. The Bohr effect was measured from pH 6.0 to pH 8.0. The pH after each experiment was measured with a Beckman research pH meter, model 1010, without exposure to atmospheric \( CO_2 \). Methemoglobin was determined before and after each experiment by the cyanmethemoglobin method (23). The amount was always under 5%.

Digestion of Oxyhemoglobin and Deoxyhemoglobin with Carboxypeptidase A and B solutions were injected by a microsyringe into the tonometer after making the gas pressure ~1 atmosphere with oxygen-free nitrogen for deoxyhemoglobin experiments and with air for oxyhemoglobin experiments. Digestions were carried out at pH 8.0 in 0.1 M Tris-HCl buffer at 25°C essentially as described (24-26). Residual carboxypeptidase A activity in the carboxypeptidase B preparations was inhibited by making the enzyme solution 1 x 10^-9 m in hydroxynamic acid (25). Similarly, traces of carboxypeptidase B in the A preparations were inhibited with 1.5 x 10^-4 m arginine acid (25). Aliquots (0.5 ml) were withdrawn from the tonometer at intervals and squirted into a cold mixture (2.0 ml) of 5% trichloracetic acid (1.6 parts) and 0.2 M sodium citrate (1.0 part), pH 2.0. After removing precipitated protein by centrifugation at 4°C, the released amino acids were analyzed with a Beckman amino acid analyzer.

Digestion with carboxypeptidase A was done with an enzyme concentration of 0.9 x 10^-4 M; the hemoglobin concentration was 2.8 x 10^-4 M. The digestion with carboxypeptidase B was done with a concentration of enzyme, 1.11 x 10^-4 M, and of hemoglobin, 2.94 x 10^-4 M. The enzyme concentrations were estimated by using published extinction coefficients (27).

Carboxypeptidase digestion of those DPG-free hemoglobin samples used for the oxygen equilibria was judged essentially complete by the determination of the liberated amino acids, histidine or arginine. The digested hemoglobin was dialyzed against deionized water overnight. After centrifugation, the hemoglobin was applied to a Sephadex G-25 column (1.5 x 45 cm) and eluted with 0.1 M NaCl.

RESULTS

Red Cell Phosphates

Analysis of phosphate compounds isolated chromatographically from the fresh mouse hemolysate (stroma free, undialyzed) showed that 2,3-DPG contained 44.6% of the total phosphorus in the red blood cells; this corresponded to 1.71 moles of 2,3-DPG per mole of hemoglobin. Adenosine triphosphate was only 0.11 mole per mole of hemoglobin. The other organic phosphate compounds together (adenosine diphosphate, monophosphoglycerate, fructose diphosphoglycerate, and glucose diphosphate) totaled about 0.5 mole per mole of hemoglobin. Extensive dialysis of the hemoglobin solution against water (2 liters changed 3 times over 24 hours) removed over 80% of the inorganic phosphate and the hexose monophosphates but only 4.7% of the 2,3-DPG and virtually none of the ATP. The 2,3-DPG to Hb ratios in mouse hemolysates prepared in different ways are given in Table I together with data obtained from human and elephant hemolysates. The results show that the amount of 2,3-DPG in the mouse hemolysate is about twice as great as that in human and elephant hemolysates. Not all of the 2,3-DPG is removed by dialysis against 0.1 M NaCl. Even though the hemoglobin was passed through a Sephadex G-25 column equilibrated with 0.1 M NaCl, an extremely small amount of 2,3-DPG (0.088 mole per mole of Hb) was retained in the hemolysate. Similar residual amounts have been obtained by others with human hemoglobin (20, 28).

peptidase in Presence of 2,3-Diphosphoglyceric Acid—Stripped hemoglobin was mixed with a solution of 2,3-DPG to give the appropriate 2,3-DPG to Hb ratio. The hemoglobin was deoxy- genated as described above. Deoxygenated carboxypeptidase A and B solutions were injected by a microsyringe into the tonometer after making the gas pressure ~1 atmosphere with oxygen-free nitrogen for deoxyhemoglobin experiments and with air for oxyhemoglobin experiments. Digestions were carried out at pH 8.0 in 0.1 M Tris-HCl buffer at 25°C essentially as described (24-26). Residual carboxypeptidase A activity in the carboxypeptidase B preparations was inhibited by making the enzyme solution 1 x 10^-9 m in hydroxynamic acid (25). Similarly, traces of carboxypeptidase B in the A preparations were inhibited with 1.5 x 10^-4 m arginine acid (25). Aliquots (0.5 ml) were withdrawn from the tonometer at intervals and squirted into a cold mixture (2.0 ml) of 5% trichloracetic acid (1.6 parts) and 0.2 M sodium citrate (1.0 part), pH 2.0. After removing precipitated protein by centrifugation at 4°C, the released amino acids were analyzed with a Beckman amino acid analyzer.

1 The abbreviation used is: 2,3-DPG, 2,3-diphosphoglyceric acid.
Comparison of 2,3-diphosphoglycerate content of hemolysates from mouse, man, and elephant

<table>
<thead>
<tr>
<th>Hemolysate</th>
<th>Molar ratio (2,3-DPG:Hb)</th>
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<tbody>
<tr>
<td>Mouse</td>
<td></td>
</tr>
<tr>
<td>Undialyzed b.</td>
<td>1.69 ± 0.023</td>
</tr>
<tr>
<td>Dialyzed versus water b.</td>
<td>1.56 ± 0.044</td>
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<tr>
<td>Dialyzed versus 0.1 M NaCl b.</td>
<td>0.183 ± 0.006</td>
</tr>
<tr>
<td>Stripped b.</td>
<td>0.038 ± 0.020</td>
</tr>
<tr>
<td>Undialyzed b.</td>
<td>1.71</td>
</tr>
<tr>
<td>Dialyzed versus water b.</td>
<td>1.63</td>
</tr>
<tr>
<td>Man</td>
<td></td>
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<tr>
<td>Undialyzed b.</td>
<td>0.879 ± 0.081</td>
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<tr>
<td>Dialyzed versus water b.</td>
<td>0.865 ± 0.034</td>
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<tr>
<td>Elephant</td>
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<td>Undialyzed b.</td>
<td>0.833 ± 0.008</td>
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<tr>
<td>Dialyzed versus water b.</td>
<td>0.044 ± 0.003</td>
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a Molar ratio with the standard deviation resulting from six determinations. 
b 2,3-DPG was determined calorimetrically. Hemoglobin concentration was 0.270 to 0.414 mg in 1.0 ml.
c 2,3-DPG was calculated from total phosphorus of Fraction V separated by column chromatography.

dependence of n on 2,3-DPG concentration at 20°. Mouse hemoglobin concentration, 0.2%. See text for discussion.

Effect of 2,3-Diphosphoglyceric Acid on Oxygen Equilibrium

Apparent Effect on Cooperativity—The data in Fig. 1 show that the observed value of n depends on the 2,3-DPG to Hb ratio. The value of n decreases to a minimum when the 2,3-DPG ratio is 1.0. Further increase in the 2,3-DPG results in a gradual return to normal values so that n = 2.6 to 2.8 when the 2,3-DPG to Hb ratio is greater than 20. Such an effect of 2,3-DPG on n is most pronounced at very low salt concentrations; n = 1.4 to 1.5 when the hemoglobin is in 4 × 10⁻³ M NaCl. This effect does not occur in the presence of inorganic phosphate (>5 × 10⁻³ M); n = 2.7 to 2.8 for all values of the 2,3-DPG to Hb ratio. Fig. 2 shows an effect of pH on n in the presence of 2,3-DPG. The data show that n is normal at values greater than 7.5 but that n decreases to a minimum value at pH 5.3 to 6.2. At these low pH values the oxygen equilibrium curve is smooth, sigmoid, and is not biphasic. An estimate of the overall free energy of interaction by Wyman's graphical procedure (29) is shown in Fig. 3. In the presence of 1 mole of 2,3-DPG per mole of hemoglobin, the slope of the curve, log y/(1 - y) versus log pO₂, is much lower than that found in the absence of 2,3-DPG. However, the overall free energy of interaction is almost the same in both curves (about 2500 cal per mole of Hb). Evidently the decrease in n does not result from a lowered subunit interaction. The amount of methemoglobin (<5%) is too low...
Diphosphoglycerate, Carbon Dioxide, and Hemoglobin

PH 15

6.5

i

o

o

o

DPG/Hb

FIG. 4. Dependence of the log $p_{50}$ value of mouse hemoglobin on the 2,3-DPG concentration in Tris-HCl buffer (ionic strength, 0.025) at 20°C. O, hemolysate, dialyzed versus water, which was found to contain 1.6 moles of 2,3-DPG per mole of hemoglobin. Hemoglobin concentration, 0.2%.

100 -

pH 8.90 pH 6.35

FIG. 5. Oxygen equilibria of mouse hemoglobin (0.297%) at two pH values. The different filled symbols for the pH 8.90 data are for the same DPG to Hb ratio as given for the corresponding open symbols for data at pH 6.95. Buffer, Tris-HCl (ionic strength, 0.025) at pH 6.95 and 0.05 M Tris-HCl at pH 8.9.

to have a significant effect on these results. We have also measured the sedimentation velocity of hemoglobin at a concentration of 0.2%. The $p_{50}$ values do not change significantly when the 2,3-DPG to Hb ratio is increased between 0 to 10 at pH 6 or 8 in 0.01 M Tris-maleate buffer. The apparent decrease in $n$ could arise from the presence of hemoglobins with different oxygen affinities. The lowered values of $n$ must result in part from the fact that a constant low free 2,3-DPG concentration cannot be maintained throughout the oxygenation procedure. Thus each successive point in an oxygen equilibrium is, in fact, determined at a slightly greater 2,3-DPG concentration. Unlike the normal measurement of the Bohr effect where the pH is kept essentially constant with a buffer, measurements with small amounts of 2,3-DPG lack any DPG-buffering ability.

Oxygen Affinity—Fig. 4 shows the effect of 2,3-DPG on the oxygen affinity of stripped mouse hemoglobin at various pH values. Fig. 4 also shows data on the oxygen affinity of non-stripped mouse hemoglobin which had been dialyzed against water and was found to contain 1.6 moles of 2,3-DPG per mole of hemoglobin. The oxygen affinity (log $p_{50}$) of this water-dialyzed preparation fits the experimental curve of log $p_{50}$ versus 2,3-DPG per mole of hemoglobin for the stripped hemoglobin, even though some other organic phosphate compounds are present in the solution. This means that 2,3-DPG in the red blood cells plays the major role in causing the decrease in the oxygen affinity. At a given pH, the oxygen affinity decreases with an increase of 2,3-DPG concentration, in agreement with reports on the behavior of other hemoglobins (1-9). However, the magnitude of the decrease varies with pH as was found for human adult and fetal hemoglobin (7). The higher the pH the smaller is the effect of 2,3-DPG; a decrease of about 0.5 in log $p_{50}$ requires approximately 1, 3, 10, and 100 moles of 2,3-DPG per mole of Hb at pH 6.5, 7.0, 7.5, and 8.0, respectively. If the decrease in the oxygen affinity results from the electrostatic bonding of 2,3-DPG with positively charged groups of hemoglobin (10, 11), the 2,3-DPG effect should disappear at a sufficiently high pH. The data obtained at pH 8.9 (Fig. 5) show that the oxygen equilibrium is unaffected by 0, 1, and 10 moles of 2,3-DPG per mole of hemoglobin. Fig. 6 shows the pH dependence of the shift in log $p_{50}$ resulting from the addition of 10 moles of 2,3-DPG per mole of hemoglobin. This curve resembles a titration curve with an apparent pK of approximately 7.76.

Bohr Effect—Fig. 7 shows a comparison of the Bohr effects for the oxygen equilibria of hemoglobins from mouse, man, and elephant at various 2,3-DPG concentrations. In the absence of 2,3-DPG, the Bohr effect of mouse hemoglobin is almost the same as that of human hemoglobin, about -0.5, but the Bohr effect of elephant hemoglobin is much lower (-0.3). The similarity of the Bohr effect in mouse and human hemoglobins is in agreement with the results obtained by Smith et al. (13). However, in the presence of low concentrations of 2,3-DPG the Bohr effect of mouse hemoglobin differs greatly from that of human and elephant hemoglobins. This is completely consistent with
the earlier findings (12). The magnitude of the Bohr effect of mouse hemoglobin increases very steeply to a maximum at 1 to 2 moles of 2,3-DPG per mole of hemoglobin whereas the Bohr effect of human hemoglobin reaches its maximum at 3 to 4 moles of 2,3-DPG per mole of hemoglobin. The Bohr effect of mouse hemoglobin decreases at high 2,3-DPG to Hb ratios but never to the value obtained with the stripped hemoglobin, in contrast to the results with human hemoglobin (10, 30). The pH dependence of n does not greatly influence the pattern of the Bohr effect of mouse hemoglobin in the pH range, 7.0 to 7.6, although the Bohr effect at 20 and 80% oxygenation becomes slightly lower and higher, respectively, than at 50% oxygenation.

Effect of 2,3-Diphosphoglyceric Acid in Presence of CO₂

A carbamino compound is formed by reaction of CO₂ with uncharged α- and ε-amino groups of hemoglobin (31-33). Fig. 8 shows the oxygen equilibria of mouse hemoglobin in the presence and absence of CO₂ and of 2,3-DPG. The deoxygenated 2,3-DPG was injected after rotating the tonometer containing deoxyhemoglobin under pCO₂ g 60 mm Hg for 30 min. At this pCO₂ the 2,3-DPG effect is completely abolished; 2,3-DPG has no effect whatever on the oxygen equilibrium. Such a CO₂ effect is found at a pH greater than 7.3. However, at a pH less than 7.3, the 2,3-DPG effect is present but much smaller than in the absence of CO₂. The results are similar at pCO₂ = 40 mm Hg except that CO₂ abolishes the 2,3-DPG effect only above pH 7.5. The absence of the 2,3-DPG effect on human hemoglobin at a pH greater than 7.5 has been found by Bauer (34). The magnitude of the Bohr effect of stripped mouse hemoglobin, measured between pH 7.3 and 7.8 at pCO₂ = 60 mm Hg is only about -0.10 compared with the value of -0.52 obtained in the absence of both CO₂ and 2,3-DPG. A similar result was found by Rossi-Bernardi and Roughton in studies of human hemoglobin (32). They showed that at pCO₂ = 50 mm Hg oxygenation caused no shift in pH at pH values greater than 7.4. In the presence of CO₂, the Bohr effect of mouse hemoglobin with 2,3-DPG is exactly the same as that with no 2,3-DPG at pH values greater than 7.4. The value of n is also unaffected by CO₂ in this pH range (n = 2.8 to 2.9). Oxygen equilibria were also carried out after injecting CO₂ (pCO₂ = 60 mm Hg) after binding 2,3-DPG to deoxyhemoglobin. The results are identical with those obtained above. These results suggest that the binding sites of CO₂ and 2,3-DPG are identical and that binding of CO₂ is stronger than that of 2,3-DPG. Only the α-NH₂ groups are likely to be involved because the pK (10.2 to 10.5) of an ε-NH₂ group is too high (35).

Effect of 2,3-Diphosphoglyceric Acid on Carboxypeptidase A- and B-digested Hemoglobins

Carboxypeptidase A removes the last two amino acids, tyrosine and histidine, of the β chain and carboxypeptidase B removes the last residue, arginine, of the α chain (25). Figs. 9 and 10 show the oxygen equilibria of mouse hemoglobin digested by carboxypeptidase A and B in the presence and absence of 2,3-DPG. The oxygen affinity of both carboxypeptidase A Hb and carboxypeptidase B Hb in the absence of 2,3-DPG is drastically increased. This confirms the results reported previously (24). The log p50 value for carboxypeptidase A Hb is lowered by 1.1 at pH 7.5, n decreases to 1.0, and the Bohr effect (−0.34) is slightly higher than that of carboxypeptidase B Hb. Low concentrations of 2,3-DPG greatly lower the oxygen affinity of carboxypeptidase B Hb; the effect is very similar to that obtained with the unmodi-
Fig. 10. The Bohr effect of mouse hemoglobin after digestion either with carboxypeptidase A (CPA-Hb) for 270 min at 30° (hemoglobin to carboxypeptidase A ratio, 25) or with carboxypeptidase B (CPB-Hb) (hemoglobin to carboxypeptidase B ratio, 270) for 90 min at 30°. Hemoglobin concentration, 0.2%. Buffer, Tris-HCl, ionic strength, 0.025.

Fig. 11. Digestion of oxyhemoglobin and deoxyhemoglobin by carboxypeptidase A in the absence and presence of 2,3-DPG; measurement of the release of histidine. The digestion was carried out at 25° in 0.1 M Tris-HCl, pH 8.0. The concentrations of hemoglobin and carboxypeptidase A were 2.75 PM and 8.8 PM, respectively.

If 2,3-DPG is bound to some positively charged amino acid residues near the central cavity (10, 37, 38) we might expect the action of carboxypeptidase to be inhibited by 2,3-DPG. The lower rate of digestion by carboxypeptidase A and B of human deoxyhemoglobin compared with oxyhemoglobin (25) might be explained by hydrogen bond formation between the COOH-terminal arginines and the β-NH₃⁺ groups of the α chain and between the COOH-terminal histidine and the β94 aspartyl residue of the β chain in the deoxy form (39). Furthermore, the binding of 2,3-DPG to α-NH₃⁺ groups might interfere with this reaction. We have measured the rate of digestion of the α and β chains in mouse hemoglobin by carboxypeptidase A and B. As can be seen in Fig. 11, oxyhemoglobin is digested much faster than deoxyhemoglobin by carboxypeptidase A. The presence of 2,3-DPG does not change the rate of digestion with carboxypeptidase A of either oxyhemoglobin or deoxyhemoglobin at all. However, this experiment was done at pH 3.0 where the 2,3-DPG effect is low. A difference might exist at a lower pH or at higher DPG concentrations. However, Fig. 12 shows that the rate of digestion of deoxyhemoglobin by carboxypeptidase B is significantly reduced by this 2,3-DPG concentration; the digestion of oxyhemoglobin is unaffected by 2,3-DPG.

2,3-DPG but that carboxypeptidase B Hb is not. However, this observation does not appear to be consistent with their report that hemoglobin heated with both enzymes is still sensitive to 2,3-DPG.

**Rate of Digestion of Mouse Hemoglobin by Carboxypeptidases in Presence of 2,3-Diphosphoglyceric Acid**

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**DISCUSSION**

Our measurements of the behavior of mouse, elephant, and human hemoglobins show that mouse hemoglobin has the largest Bohr effect and that of the elephant hemoglobin the smallest (Fig. 6) in the presence of physiological concentrations of 2,3-DPG, while that of human hemoglobin occupies an intermediate position. Thus, the early results (12) which showed differences in the Bohr effects can be explained. It now appears that mammalian hemoglobins differ greatly in the sensitivity of their oxygen equilibria to 2,3-DPG. These differences must reflect variations in the binding constants for 2,3-DPG. Such varia-
tions presumably result from small changes in the environment and the nature of the binding groups or both. The results do not support the conclusion of Antonini (14) that mammalian hemoglobins are all functionally equivalent and do not provide support for the contention of King and Jukes (15) that mammalian hemoglobins are selectively neutral.

The dependence of binding of organic phosphates on pH suggests that positively charged groups on the hemoglobin are involved in the binding of negatively charged organic phosphates. The 2,3-DPG molecule has five ionizable groups, three with a pK of 2.8 and two groups with a pK of 7.1 (10). Therefore, 2,3-DPG has at least three negatively charged groups above pH 7.1, one carboxyl and two phosphate groups. It appears probable that the pK values for 2,3-DPG itself change as a result of binding of the anion to hemoglobin. If the pK of 7.1 were raised by binding to the hemoglobin, release of 2,3-DPG would be associated with an additional contribution to the measured Bohr effect. This may explain the increase in Bohr effect actually observed in the presence of low concentrations of 2,3-DPG. The decrease in the Bohr effect upon the further addition of 2,3-DPG is presumably associated with binding at a different site.

Benesch et al. (10, 11, 20, 37) have provided evidence that the stoichiometry of 2,3-DPG binding to tetrameric deoxygenated hemoglobin is 1:1. This suggests that its binding site is on the diad axis in the central cavity (34, 38). Garby, Gerber, and de Verdiér (40) have suggested that β 143 histidine might be a major binding site on the basis of the pH dependence of 2,3-DPG binding. They found evidence for at least two binding sites in agreement with the results obtained by Chanutin and Hermann (41) but in contrast to the results of Benesch, Benesch, and Yu (20). The lower oxygen affinity of human fetal hemoglobin compared with adult hemoglobin A may result from the replacement of β 143 histidine by serine (40, 42). This substitution does not abolish the effect of organic phosphates but their effect is different for the two hemoglobins (7). Furthermore, if ATP combines with hemoglobin at the same site as does 2,3-DPG (43), the much larger size of ATP suggests that it would not be accommodated as well within the central cavity. Part of the molecule might be external.

We suggest that the primary sites of 2,3-DPG binding are the α-NH₂ groups of the α and β chains for the following reasons. (a) CO₂ completely inhibits the 2,3-DPG effect. The higher the pCO₂ the lower the pH at which CO₂ inhibits. This result can be explained by assuming that CO₂ and 2,3-DPG bind at the same site. (b) CO₂ is known to react with the uncharged α-NH₂ groups of human hemoglobin to form carbamates, R-NH-CO₂⁻ (33). (c) The pH dependence of the shift in log pO₂ by 2,3-DPG resembles a titration curve with an apparent pK of approximately 7.76; this is close to the pK values reported for the relevant α-NH₂ groups of deoxyhemoglobins (35, 44, 45). This correlation implies that 2,3-DPG exerts its effect by binding only with the charged α-NH⁺ groups. Since CO₂ combines only with the uncharged α-NH₂ groups, the higher the pCO₂ at constant pH, the more will the equilibrium, RNNH₂⁺ ⇌ RNNH₂ + H⁺, be shifted to the right, so that less RNH⁺ will be available for reaction with 2,3-DPG. This scheme explains why CO₂ can abolish the 2,3-DPG effect at high but not at low pH and also explains why the 2,3-DPG effect decreases with increasing pH.

The complete absence of an effect of 2,3-DPG on hemoglobin digested with carboxypeptidase A which removes the COOH-terminal residues of the β chain suggests that 2,3-DPG binds to the β chain. The COOH terminus of each β chain is about 12 A from the NH₂ terminus of the neighboring β chain in deoxyhemoglobin; oxygenation is accompanied by a shortening of this distance to about 6 A (46). This fact indicates a possible mechanism of action of both CO₂ and 2,3-DPG. We suggest that the reaction of the β chain α-NH₂ group with CO₂ might affect oxygenation by the introduction of the negative charge in the resulting carbamate, R-NHCO₂⁻. Electrostatic repulsion between this group and the —COO⁻ of the other β chain might retard normal movement of the —NII⁺ of β toward the —COO⁻ of β. Such a retardation would be expected to lower the oxygen affinity because the deoxy conformation would be favored.

The primary effect of 2,3-DPG can be explained on a similar basis. We suppose that 2,3-DPG is bound primarily (although not entirely) by forming a bridge between the α-NH⁺ groups of each β chain. This would explain the very approximate 1:1 stoichiometry which has been reported (1, 20). The presence of the large negative DPG anion would prevent any salt linkage between —NH⁺ (α) and —COO⁻ (β) and would tend to favor the deoxy state. Such a mechanism would explain our results with carboxypeptidase A which removes the —COOH-terminal segment, —Tyr-His. Not only does this enzyme remove the residue with which the β-NH₂⁺ would form a salt bridge, but the loss of tyrosine results in a “dislocation extending through most of the β-subunits” (39). Cooperativity of oxygen binding is completely lost (24). It is not surprising, therefore, for carboxypeptidase A Hb to be devoid of any 2,3-DPG effect in spite of the fact that the α-NH₂ groups of the α chain are still intact. Since we suppose that the largest effect of 2,3-DPG is normally with the β chains, it is to be expected that an effect of 2,3-DPG would still exist in carboxypeptidase B Hb where the COOH-terminal arginines have been removed from the α chains. The α-NH₂ groups of the α chains are not far from the guanidine groups of the COOH-terminal arginines. Thus, binding of 2,3-DPG at or between the two α-NH₂⁺ groups of the α chain or with the positively charged guanidine groups would explain the inhibition of carboxypeptidase B action in the presence of 2,3-DPG.

Our conclusion that the α-NH₂ groups of the β chains are primarily involved in 2,3-DPG binding in mouse hemoglobin is supported by "the 2 of Figure 13. The difference in log pO₂ values for mouse hemoglobin with and without 2 moles of 2,3-DPG per mole of hemoglobin at various concentrations of inorganic phosphate buffer, pH 6.9, at 20°C."
consistent with the recent report (47) that pyridoxal phosphate has an effect similar to that of 2,3-DPG and can be covalently bound to the NH2-terminal valine of the human β chain. It is also most significant that human hemoglobins A2 and A, which have blocked —NH2 termini in their non-α chains have depressed binding of 2,3-DPG (48).

In conclusion, our observations emphasize the need to re-examine the comparative studies of the oxygen equilibria of hemoglobins from different animals carried out before the discovery of the 2,3-DPG effect (1, 2). Most such studies utilized inorganic phosphate as a buffer. We have measured the 2,3-DPG effect as a function of inorganic phosphate concentration (Fig. 13). The results show that the higher the inorganic phosphate concentration the smaller is the observed 2,3-DPG effect. Thus, a 2,3-DPG effect can easily be seen in 0.04 m phosphate but vanishes at phosphate concentrations at or above 0.1 m at 20°. The earlier experiments (12) were done in 0.1 m phosphate but at 35° rather than 20°. It is clear that measurements of the oxygen affinity and the Bohr effect in different hemoglobins need to be carried out both in the presence and absence of inorganic phosphate and of appropriate organic phosphates.

Note Added in Proof—After submission of this paper the work of J. E. Bailey, J. G. Beetlestone, and D. H. Irvine (49) came to our attention. They also conclude that the variability of the Bohr effect in hemolysates results in part from differences in the affinity with which 2,3-DPG is bound to the hemoglobins of different species. The recent paper by M. F. Perutz (50) which appeared after submission of our work provides strong support for the site of 2,3-DPG binding. However, he concludes that the variability of the 2,3-DPG effect as a function of inorganic phosphate concentration (Fig. 13) is consistent with the recent report (47) that pyridoxal phosphate has an effect similar to that of 2,3-DPG and can be covalently bound to the NH2-terminal valine of the human β chain. It is also most significant that human hemoglobins A2 and A, which have blocked —NH2 termini in their non-α chains have depressed binding of 2,3-DPG (48).

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Studies of the Interaction of 2,3-Diphosphoglycerate and Carbon Dioxide with Hemoglobins from Mouse, Man, and Elephant
Susumu Tomita and Austen Riggs


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