**Stabilizing Interactions in Hemoglobin**

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**SUMMARY**

A detailed re-examination of the relationship between the value of the Hill constant, \( n \), and the partial pressure necessary for oxygenation of half the hemoglobin, \( P_{50} \), indicates that there is a slight dependence of \( n \) on \( P_{50} \) for normal human hemoglobin. The value of \( n \) varies between 2.3 and 3.1 over a 50-fold variation in the value of \( P_{50} \) (from 0.5 to 25 mm Hg). Above values of 40 mm Hg for \( P_{50} \), the value of \( n \) decreases below 2. However, there is no obvious relationship between oxygen affinity and cooperativity. In fact, the Bohr effect and cooperativity are largely independent phenomena which means that the stabilizing interactions in hemoglobin are not related in any simple fashion to oxygen affinity.

Recent progress on the chemistry of hemoglobin (1-4) has renewed interest in the mechanism of ligand binding and the cooperative effects in this molecule. The x-ray studies have progressed to the point that Perutz (5) has proposed a detailed stereochemical mechanism for the heme-heme interactions, a mechanism which receives some support from the work of Ogawa and Shulman (6) and from the theoretical consideration of Edelstein (7). The main feature of this mechanism is the postulate that there are two conformational states for hemoglobin in equilibrium (the Monod-Wyman-Changeux model (8)). According to the simplest version of this model, which is not necessarily the one endorsed by Perutz, or by Ogawa and Shulman, the system is defined by the allosteric constant \( L \), which indicates the relative stability of the two conformations; by the value of \( \alpha_1 \), the ratio of the activity of ligand necessary to bring the system to 50% saturation as compared to that required to saturate 50% of the high affinity form; and by \( c \), the ratio of ligand affinities for the two states. All the properties of the system are envisaged as related to shifts in the equilibrium between the two states, that is, to changes in the values of \( L \) (which are proportional to the values of \( \alpha_1 \)) while the actual ligand affinities of the two conformations (\( K_R \) and \( K_F \)) are thought to be invariant. Thus, the effects of low pH (the Bohr effect) (9) and of organic phosphates (10) (primarily 2,3-diphosphoglycerate) on the oxygen affinity are simply due to stabilization of the low affinity conformation of hemoglobin. And the high ligand affinity of mutant hemoglobins (11) and of chemical derivatives (12) is caused by the increased stability of the high affinity form of these molecules. These two forms are presumably the ones elucidated by the x-ray studies (5).

One consequence of such a state of affairs (that is, of the simplest two-state model with \( L \) variable, and \( K_R \) and \( K_F \) invariant) is that the shape of the ligand-binding curve must vary with changes in pH and in the concentration of organic phosphates, to say, the stabilizing interaction energy between the heme groups will depend on the actual value of the ligand affinity. Such a situation has not been commonly found in experiments (13, 14). However, the article of Rubin and Changeux (9) and the more recent one by Edelstein (7) explain how the apparent invariance in shape of the ligand-binding curve is compatible with a two-state model. The explanation is that the observed variation in the ligand affinity of hemoglobin is too small (only one order of magnitude in a critical range of values (7, 15)) to result in an observable change in the value of \( n \), the interaction constant. This is a consequence of the "buffering" of cooperativity in the critical range of values for the affinity.

We wish to point out here that the simplest two-state model, with \( L \) variable and \( K_R \) and \( K_F \) invariant, can be considered to be an inadequate explanation of the behavior of hemoglobin because the stabilizing interactions between the ligand-binding sites persist to a considerable extent even when the ligand affinity of hemoglobin is very high and similar to that of the isolated chains, or very low. That is to say, cooperativity is not related in any simple way to ligand affinity, and the shape of the binding curves is indeed largely invariant with changes in solvent conditions. This would mean that the value of \( L \) is invariant, while \( K_R \) and \( K_F \) vary with solvent condition, which of course rules out the simplest two-state model.

A review of the literature concerning the relationship between oxygen affinity and cooperativity of normal hemoglobin is shown in Table I. The important parameter for the present purpose is the value of \( \Delta \log P_{50} \), the difference between the ligand affinity of the hemoglobin under consideration and that of the isolated chains (Table II). The smaller the value of \( \Delta \log P_{50} \), the closer the affinity of hemoglobin is to that of the
TABLE I

Relationship between oxygen affinity and cooperativity of normal hemoglobin

$\Delta \log p_{50}$ indicates the difference in affinity between the given hemoglobin and the isolated chains (see Table II). The affinity of the chains is taken as the average of the values for the $\alpha$ and $\beta$ chains. $p_{50}$ is the partial pressure of $O_2$ (in mm Hg) necessary to saturate 50% of the hemoglobin.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Solvent</th>
<th>pH</th>
<th>$\log p_{50}$</th>
<th>$\Delta \log p_{50}$</th>
<th>$n$</th>
<th>Reference</th>
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<tbody>
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<td>$10^\circ$</td>
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<td>6.7</td>
<td>0.60</td>
<td>1.60</td>
<td>2.8</td>
<td>11</td>
</tr>
<tr>
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<td>0.15</td>
<td>1.15</td>
<td>2.8</td>
<td>11</td>
</tr>
<tr>
<td></td>
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<td>2.7</td>
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</tr>
<tr>
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<td>-0.64</td>
<td>0.36</td>
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<td>17</td>
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<td>-0.01</td>
<td>0.37</td>
<td>2.56</td>
<td>17</td>
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<td>1.116</td>
<td>1.49</td>
<td>2.90</td>
<td>13</td>
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<td>0.4 M phosphate</td>
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<td>0.820</td>
<td>1.19</td>
<td>2.90</td>
<td>13</td>
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<td>0.69</td>
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<td>1.56</td>
<td>2.8</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>6.8</td>
<td>1.02</td>
<td>1.36</td>
<td>2.65</td>
<td>19</td>
<td></td>
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<tr>
<td></td>
<td>9.2</td>
<td>0.21</td>
<td>0.56</td>
<td>2.8</td>
<td>18</td>
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<td>1.0</td>
<td>1.34</td>
<td>2.8</td>
<td>20</td>
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<td>1.12</td>
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<td></td>
<td>7</td>
<td>0.94</td>
<td>1.31</td>
<td>2.1-2.4</td>
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<td></td>
<td>9.2</td>
<td>0.09</td>
<td>0.40</td>
<td>2.1-2.4</td>
<td>22</td>
<td></td>
</tr>
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<td>0.18</td>
<td>0.55</td>
<td>3.05</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>0.05 M bis-tris stripped, 0.01 M NaCl</td>
<td>7.3</td>
<td>0.11</td>
<td>0.46</td>
<td>3.05</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>0.05 M bis-tris stripped, 0.1 M NaCl</td>
<td>7.3</td>
<td>0.60</td>
<td>0.95</td>
<td>3.06</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>0.05 M bis-tris stripped, 0.01 M NaCl + $2.5 \times 10^{-4}$ M DPG</td>
<td>7.3</td>
<td>(-0.15)</td>
<td>(0.20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tris-HCl buffer $\Gamma/2 = 0.025$, stripped</td>
<td>6.95</td>
<td>0.55</td>
<td>2.8</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tris-HCl buffer $\Gamma/2 = 0.025$, stripped + $3 \times 10^{-4}$ M DPG</td>
<td>7.5</td>
<td>0.28</td>
<td>2.7</td>
<td>24</td>
<td></td>
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<td>$25^\circ$</td>
<td>0.01 M Tris-HCl stripped</td>
<td>7.4</td>
<td>0.30</td>
<td>0.70</td>
<td>2.52</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>0.01 M Tris-HCl stripped + $2 \times 10^{-4}$ M DPG</td>
<td>7.4</td>
<td>1.2</td>
<td>1.6</td>
<td>3.02</td>
<td>25</td>
</tr>
<tr>
<td>$30^\circ$</td>
<td>0.1 M phosphate</td>
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<td>1.22</td>
<td>1.37</td>
<td>2.7</td>
<td>26</td>
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<tr>
<td></td>
<td>7.53</td>
<td>0.88</td>
<td>1.03</td>
<td>2.7</td>
<td>14</td>
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</tr>
<tr>
<td></td>
<td>0.01 M NaCl stripped</td>
<td>7.0</td>
<td>0.43</td>
<td>0.57</td>
<td>2.4</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>0.01 M NaCl stripped + $10^{-4}$ M DPG</td>
<td>7.0</td>
<td>1.13</td>
<td>1.27</td>
<td>2.1</td>
<td>14</td>
</tr>
</tbody>
</table>

On this basis, we thought it might be of interest to investigate further the relationship between oxygen affinity and cooperativity in normal hemoglobin, in a range of oxygen affinity where $\Delta \log p_{50}$ approaches zero, and where it is close to 2. The concept of the experiments derives from the discovery that hemoglobin stripped of organic phosphates and at a low ionic strength has a high oxygen affinity and a Bohr effect, and from the observation that inositol hexaphosphate greatly decreases the oxygen affinity of hemoglobin. Thus high and low values of affinity can be reached.

METHODS AND MATERIALS

Specimens of normal human blood were collected in heparin. Hemoglobin was prepared as described by Drabkin (29). Organic phosphates were "stripped" from the hemolysate by dialysis in a stretched cellulose membrane as described by Benesch, Benesch, and Yu (10). Phosphate analyses of the "stripped"
hemoglobin, done as previously described (30), showed that at least 95% of the total phosphate had been removed. \( \alpha \) and \( \beta \) chains were prepared from human hemoglobin \( \text{A} \) by the method of Bucci and Fronticelli (31). Mercury was removed from globin and psO is the poZ at which hemoglobin is half-saturated. (Y and p
hemoglobin, done as previously described (30), showed that at

\[
\frac{Y}{1-Y} = \left( \frac{P_{02}}{P_{50}} \right)^n
\]

where \( Y \) is the fractional saturation of hemoglobin with oxygen and \( P_{50} \) is the \( P_{02} \) at which hemoglobin is half-saturated.

RESULTS AND DISCUSSION

The oxygenation studies done on stripped hemoglobin in the presence and absence of organic phosphates and as a function of pH and ionic strength are summarized in Tables IV and V. Some representative oxygen-binding curves are shown in Fig. 1.

The three curves for tetrameric hemoglobin on the left-hand side of this figure clearly indicate that the shifts in \( P_{50} \) with solvent conditions are fully reversible changes. In this experiment, after oxygen equilibration was measured on a solution of stripped hemoglobin in 0.01 m Cl\(^-\), pH 8.8, sufficient 3.0 m NaCl and 1.0 m bis-tris buffer (pH 7.0) were added to the solution to make it 0.1 m Cl\(^-\), pH 7.5. This solution had the same oxygen saturation curve as a fresh solution of identical composition (Fig. 1). This suggests that the very high oxygen affinity which is observed at high pH, low ionic strength is entirely reversible. The figure also shows that the value of \( P_{50} \) for normal hemoglobin can be made to vary by two orders of magnitude.

All the data are collected in Fig. 2, where they show the dependence of \( n \) on the affinity of hemoglobin for oxygen. The results indicate that the value of \( n \) varies from 2.4 to 3.1 over a variation in log \( P_{50} \) of 1.8. At very high values of \( P_{50} \) (in the presence of inositol hexaphosphate) log \( P_{50} \) increases above 1.6, the value of \( n \) decreases to a value of 1.5. This decrease is probably not associated with a decrease in the interaction energy between the heme groups, but it most likely indicates the introduction of intramolecular heterogeneity into the system (33).

In any event even when the affinity of hemoglobin is as high as that of the isolated \( \alpha \) chains (\( \Delta \log P_{50} = 0.07 \)) the value of \( n \) is large (\( n = 2.4 \)) and it changes only slightly as the value of \( P_{50} \) increases. One should also notice the slight but certain dependence of the value of \( n \) on the value of \( P_{50} \). This dependence would have escaped us if only the data from log \( P_{50} = 0 \) to log \( P_{50} = 1.2 \) were available. The additional data clearly indicate that there is slight dependence between \( n \) and the value of \( P_{50} \) for normal human hemoglobin.

An important feature of the data shown in Fig. 2 is that while the value of \( n \) is related to the value of \( P_{50} \), it seems to be independent of the solvent conditions used to obtain the value of \( P_{50} \). Thus low ionic strength and low pH produce the same results as high ionic strength and high pH (see the left-hand points). This
TABLE IV
Effect of solvent conditions on value of p50 and n obtained at 20° with 0.2 mM human hemoglobin

<table>
<thead>
<tr>
<th>Solvent</th>
<th>pH</th>
<th>p50</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.03 M bis-tris</td>
<td>6.8</td>
<td>2.16</td>
<td>2.99</td>
</tr>
<tr>
<td>0.01 M Cl^-</td>
<td>7.2</td>
<td>1.38</td>
<td>2.76</td>
</tr>
<tr>
<td>0.03 M Tris</td>
<td>7.6</td>
<td>0.94</td>
<td>2.57</td>
</tr>
<tr>
<td>0.01 M Cl^-</td>
<td>8.0</td>
<td>0.80</td>
<td>2.60</td>
</tr>
<tr>
<td>0.03 M Tris</td>
<td>8.0</td>
<td>0.69</td>
<td>2.74</td>
</tr>
<tr>
<td>0.01 M Cl^-</td>
<td>8.4</td>
<td>0.56</td>
<td>2.45</td>
</tr>
<tr>
<td>0.03 M Tris</td>
<td>8.8</td>
<td>0.52</td>
<td>2.55</td>
</tr>
<tr>
<td>0.01 M Cl^-</td>
<td>8.8</td>
<td>0.49</td>
<td>2.35</td>
</tr>
<tr>
<td>0.03 M bis-tris</td>
<td>5.5</td>
<td>0.44</td>
<td>2.32</td>
</tr>
<tr>
<td>0.1 M Cl^-</td>
<td>6.0</td>
<td>0.42</td>
<td>2.50</td>
</tr>
<tr>
<td>0.03 M Tris</td>
<td>6.4</td>
<td>0.26</td>
<td>2.50</td>
</tr>
<tr>
<td>0.1 M Cl^-</td>
<td>7.0</td>
<td>0.19</td>
<td>2.70</td>
</tr>
<tr>
<td>0.03 M Tris</td>
<td>7.0</td>
<td>0.14</td>
<td>2.50</td>
</tr>
<tr>
<td>0.1 M Cl^-</td>
<td>7.2</td>
<td>0.10</td>
<td>2.60</td>
</tr>
<tr>
<td>0.03 M Tris</td>
<td>8.0</td>
<td>0.07</td>
<td>2.50</td>
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<tr>
<td>0.1 M Cl^-</td>
<td>8.4</td>
<td>0.05</td>
<td>2.30</td>
</tr>
<tr>
<td>0.03 M Tris</td>
<td>8.8</td>
<td>0.04</td>
<td>2.20</td>
</tr>
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</table>

TABLE V
Effect of solvent conditions on value of p50 and n obtained at 20° with 0.2 mM α and β chains

<table>
<thead>
<tr>
<th>Hb</th>
<th>Solvent</th>
<th>pH</th>
<th>p50</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>α^SH</td>
<td>0.03 M bis-tris</td>
<td>6.8</td>
<td>0.45</td>
<td>1.0</td>
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<tr>
<td>α^SH</td>
<td>0.1 M Cl^-</td>
<td>8.4</td>
<td>0.37</td>
<td>1.0</td>
</tr>
<tr>
<td>α^SH</td>
<td>0.03 M Tris</td>
<td>8.4</td>
<td>0.37</td>
<td>1.0</td>
</tr>
<tr>
<td>α^SH</td>
<td>0.01 M Cl^-</td>
<td>6.8</td>
<td>0.24</td>
<td>1.0</td>
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<tr>
<td>β^SH</td>
<td>0.03 M bis-tris</td>
<td>8.4</td>
<td>0.19</td>
<td>1.0</td>
</tr>
<tr>
<td>β^SH</td>
<td>0.1 M Cl^-</td>
<td>8.8</td>
<td>0.19</td>
<td>1.0</td>
</tr>
<tr>
<td>β^MB α</td>
<td>0.03 M bis-tris</td>
<td>6.8</td>
<td>3.00</td>
<td>1.0</td>
</tr>
<tr>
<td>β^MB α</td>
<td>0.1 M Cl^-</td>
<td>6.8</td>
<td>3.00</td>
<td>1.0</td>
</tr>
</tbody>
</table>

* PMB, p-mercuribenzoate.

means that, for normal hemoglobin at a given temperature, the value of n can be predicted with the data shown in the figure from the value of p50. This also means that abnormal or chemically altered hemoglobins which have a relationship between n and p50 different from that shown here must have conformations which differ from that of normal hemoglobin.

For example, hemoglobins Kansas and Chesapeake which have a large Bohr effect but a low value of n (1.3) which is insensitive to the value of p50 (see Table III), cannot represent hemoglobins in which the normal oxygenated or the deoxygenated conformation, respectively, is destabilized with respect to that of normal hemoglobin. If they were examples of such shifts in the conformational equilibrium, the values of p50 at the observed p50 would fall on the line defined by the observed data points in Fig. 2. Therefore, one should use the information obtained from abnormal and chemically modified hemoglobins with great caution to support a proposed mechanism for hemoglobin unless the data are in agreement with the "standard" curve shown in Fig. 2.
The major conclusion deduced from this work is that these data are in obvious disagreement with the relationship between \( n \) and \( p_{50} \) (\( q \)) expected from a simple two-state model and depicted by the dotted line shown in Fig 2. This line shows the relationship between \( q \) (\( p_{so} \)) and \( n \) on the left-hand ordinate, and between \( cQ \) and the interaction energy, \( AG_{1,3} \) on the right-hand ordinate, and it illustrates the relationship between \( n \) and \( AG_{I} \) expected according to the model of Monod-Wyman-Changaux (8). Thus the observed dependence of \( n \) on \( p_{so} \) must be accounted for by some other mechanism. We cannot, at this time indicate what this mechanism might be because we do not have sufficient data to obtain the interaction energy between the heme groups for all cases (that is, the values of \( K_{4} \) and \( K_{1} \)). Therefore, we cannot judge to what extent the variation in the value of \( n \) is due to a change in the ratio of \( K_{4}:K_{1} \) as compared to a variation in the values of \( K_{2}:K_{1} \) and \( K_{3}:K_{1} \). It is instructive, however, to notice that the data of Roughton and Lyster (17) indicate that the difference in the value of \( n \) for human hemoglobin at \( pH 9.1 \) (\( n = 2.56 \)) as compared to \( pH 6.96 \) (\( n = 3.18 \)) is due not to a decrease of the ratio \( K_{4}:K_{1} \) at high \( pH \) but to a change in the values of \( K_{2}:K_{1} \) and \( K_{3}:K_{1} \). It is also pertinent that Tyuma, Imai, and Shimizu (34) have recently demonstrated that \( AG_{I} \) is not proportional to \( n_{max} \) for hemoglobin in the presence of organic phosphates. These observations are not compatible with the model of Monod Wyman Changeux (8).

In any event, our data indicate that there is no clear relationship between oxygen affinity and cooperativity, that the Bohr effect and cooperativity are largely independent phenomena, and that a simple two-state model cannot be correct for hemoglobin. This means that the linkage between oxygen affinity and subunit association is not simply related to the stabilizing interactions between the heme groups. That is to say, there is

\[
\Delta G_{I} = RT \ln \frac{K_{4}}{K_{1}}
\]

The Number 16 is the statistical factor which relates the observed to the intrinsic constants. In terms of the allosteric model \( \Delta G_{I} \) is given by

\[
\Delta G_{I} = RT \ln \frac{(1+Lc^{4})(1+L)}{(1+Lc^{3})(1+Lc)}
\]

Since \( L \) is related to \( a_{I} \) by

\[
L = \frac{(a_{I/2}^{2} - 1)(1+a_{I/2}^{2})}{(1-a_{I/2}c)(1+a_{I/2}c)}
\]

then at any given value of \( c \), \( \Delta G_{I} \) is directly related to the value of \( a_{I} \) and hence to the value of \( n \). In other words, in a simple two-state system the value of \( \Delta G_{I} \) can be calculated from the value of \( n \). This is not so for a system in which there are more than two states.

If \( K_{1}, K_{2}, K_{3}, \) and \( K_{4} \) are the measured binding constants for four identical sites in a molecule, the value of \( n \) is related to the constants by the following relationship.

\[
n = \frac{16 + 6K_{1}P_{50} - 2K_{1}K_{2}K_{3}P_{50}^{3}}{4 + 3K_{1}P_{50} + 2K_{1}K_{2}P_{50}^{2} + K_{1}K_{2}K_{3}P_{50}^{3}}
\]

where \( K_{1}K_{2}K_{3}P_{50}^{3} = \frac{2 + K_{1}P_{60}}{1 + 2K_{4}P_{50}} \)

or \( K_{1}K_{2}K_{3}K_{4}P_{50}^{4} = \frac{2 + K_{1}P_{60}}{2 + 1/K_{4}P_{50}} \)

This relationship shows that for any given ratio of the values of \( K_{4}:K_{1} \) the value of \( n \) will also depend on the values of \( K_{2} \) and \( K_{3} \). In a two-state system, the values of \( K_{2} \) and \( K_{3} \) are fixed in a definite proportion to those of \( K_{1} \) and \( K_{4} \), and therefore \( n \) depends only on the ratio \( K_{4}:K_{1} \). For a system with more than two states, \( K_{2} \) and \( K_{3} \) are not so related to \( K_{4} \) and \( K_{1} \), and the value of \( n \) cannot be used to give the value of the ratio \( K_{4}:K_{1} \) and thus the value of \( \Delta G_{I} \).
no simple relationship between the free energy change for the
tetramer-dimer dissociation reaction and cooperativity, since the
former is linked to oxygen affinity but the latter property is not.
Thus we must distinguish between passive linkage and active
linkage in the hemoglobin system.

Let us consider first the linkage between subunit-binding sites
and ligand-binding sites. From the data (see Tables IV and V)
for the binding of O₂ to hemoglobin at 20° and pH 7 we can write the
following cycle of reactions.

\[ Hb_4 \rightarrow L_4 \rightarrow a \rightarrow 4 Hb L \]

\[ -24 \]

\[ Hb_4 \rightarrow a + 8 \rightarrow 4 Hb \]

\[ -32 \]

The numbers indicate the values of the standard free energy
change for the reactions (ligand binding and dissociation reactions)
in kilocalories per mole. This reaction scheme points out the
well known facts that the affinity of the isolated chains for
O₂ is greater than that of the tetrameric molecule, and recipro-
cally that the bonds holding the deoxy chains together in a
tetramer are stronger than those involved with the oxygenated
chains by about 8 kcal per mole of tetramers. The question we
are concerned with is whether or not this free energy change (8
cal per mole of tetramers) is related to the interaction energy
between the ligand-binding sites which can be estimated by the
methods of Wyman (35) to be approximately 10 kcal per mole of
tetramer. The evidence presented in Table IV indicates clearly
that the answer is no. At high pH and at low ionic strength, the
behavior of stripped hemoglobin can be written as follows:

\[ Hb_4 \rightarrow \text{L}_4 \rightarrow a \rightarrow 4 Hb L \]

\[ -32 \]

\[ Hb_4 \rightarrow \text{a} + \text{8} \rightarrow 4 Hb \]

which indicates that there is no difference between the free energy
change of subunit association of oxyhemoglobin as compared to
that of deoxyhemoglobin. However, the value of the interaction
constant between the ligand-binding groups, nᵢ, is still 2.4 and
there is little change in the interaction energy. It seems clear
that cooperativity (interaction energy or active linkage) and the
difference in affinity for ligand between tetramer and
dimer dissociation reaction and cooperativity, since the
well known facts that the affinity of the isolated chains for
O₂ is greater than that of the tetrameric molecule, and recipro-
cally that the bonds holding the deoxy chains together in a
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