Studies on the Oxidation-Reduction Potentials of Heme Proteins

VI. HUMAN HEMOGLOBIN TREATED WITH VARIOUS SULPHHYDRYL REAGENTS*

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SUMMARY

The oxidation-reduction equilibrium of human hemoglobin treated with various sulphydryl reagents has been studied. The following modified hemoglobins have been characterized: hemoglobin treated with cystine, hemoglobin treated with iodoacetamide, and hemoglobin treated with 2 eq of p-mercuribenzoate.

In all cases the modified hemoglobins have an oxidation-reduction potential which is more negative than normal hemoglobin (by about 15 to 30 mv) at pH 6, but about the same as normal hemoglobin at pH 9.

The value of n in the oxidation-reduction equilibrium is much less sensitive to pH than in the case of normal hemoglobin, and is very near to 2 from pH 6 to 9. The titration potentiometric data have been substantiated by differential (acid-base) measurements. Moreover, some data on the kinetics of oxidation of the various modified hemoglobins by ferricyanide have been collected.

Previous papers of this series have reported studies of the oxidation-reduction properties of normal and modified human hemoglobin (1-2). These properties were determined by measurements of the oxidation-reduction potentials as a function of pH or of the proton binding of the systems as a function of oxidation. With both these methods it was shown that in human hemoglobin the heme-heme interaction in the oxidation-reduction equilibrium is pH-dependent, the value of the interaction coefficient a1 changing from about 1.2 at pH 6 to about 2.5 at pH 9. The kinetics of the oxidation of hemoglobin by ferricyanide (3)

was found to be in qualitative agreement with the equilibrium data; the time course of the reaction was complex, and the ratio of the velocity constant, measured at the initial and final stages, was found to be pH-dependent in accordance with what might be expected from the equilibrium behavior.

Studies on hemoglobin modified by digestion with carboxypeptidases and on hemoglobin in the presence of bromothymol blue (4, 5) revealed a striking similarity between the oxidation-reduction and the oxygen equilibria, in spite of the different nature of the two chemical processes.

As a sequence to these studies, we have now investigated the oxidation-reduction potentials of human hemoglobin modified in the —SH groups at position 93 of the B chains. These two sulphydryl groups, although adjacent in the 2 histidines at position 92, do not appear to play an essential role in ligand equilibria; but when they are blocked, the oxygen equilibrium is affected in a manner depending on the nature of the reagent (6-9). Hemoglobin in which the reactive —SH groups are covered, with iodoacetamide or with disulfides, such as cystine or cystamine, showed a diminished oxygen Bohr effect, whereas the heme-heme interaction appeared unchanged. In contrast, treatment with p-mercuribenzoate and some other —SH reagents significantly alters the shape of the O2 equilibrium curve, more so at some pH values than at others, and also affects the kinetic behavior in a manner suggesting a sort of heterogeneity.

In this paper we report the results on the oxidation-reduction potentials of hemoglobin treated with cystine, cystamine, or iodoacetamide, and also some observations on hemoglobin in the presence of p-mercuribenzoate. The data, which include potentiometric equilibrium measurements and differential pH measurements, emphasize once more the similarity between oxygen and oxidation equilibrium in hemoglobin, but reveal also more specific effects of the modification on the oxidation-reduction equilibrium.

EXPERIMENTAL PROCEDURE

Preparation of Hemoglobin—Human hemoglobin was prepared from freshly drawn red blood cells according to the standard procedure previously described (1). Hemoglobin concentrations were determined spectrophotometrically (10). The amount of ferrihemoglobin in the presence of ferrohemoglobin was measured by the cyanide method (11).
FIG. 1. Oxidation Bohr effect for cystine-Hb and cystamine-Hb. Experimental points obtained from potentiometric measurements by the dye titration method are indicated by: O, cystine-Hb; , cystamine-Hb. Experimental points obtained from potentiometric measurements of an equimolar mixture of ferro- and ferrihemoglobin are indicated by : , cystine-Hb in phosphate or borate buffers; , cystine-Hb in NaCl. The continuous line which connects these experimental points is a smooth curve traced through the potentiometric measurements (in phosphate or borate) on cystine Hb. The oxidation Bohr effect obtained from differential pH measurements on cystine-Hb is indicated by .---. The oxidation Bohr effect for normal hemoglobin under the same experimental conditions is reported for comparison, -----. Results at 30°, in solvents at ionic strength 0.10 to 0.30. Ordinates give the potential (in volts) referred to the standard hydrogen electrode.

FIG. 2. Oxidation Bohr effect for IAA-Hb and PMB-Hb. Experimental points obtained from potentiometric measurements by the dye titration method are indicated by: O, IAA-Hb; , PMB-Hb. The oxidation Bohr effect obtained from differential pH measurements (on IAA-Hb) is indicated by .---. The oxidation Bohr effect for normal Hb under the same experimental conditions is reported for comparison, ----- . Results at 30°, in solvents at ionic strength 0.10 to 0.30. Ordinates give the potential (in volts) referred to the standard hydrogen electrode.

With blocked -SH groups were prepared as previously described (6), with a 50-fold excess of disulfide and a 10-fold excess of iodoacetamide.

Determination of Oxidation-Reduction Potentials—Oxidation-reduction potentials were determined at 30° either by the dye titration method as previously described (1) or as follows. A mixture containing nearly equal amounts of ferri- and ferrohemoglobin was prepared by adding 0.5 eq of ferricyanide to a solution of oxyhemoglobin in water. The solution was then deoxygenated, and the exact proportions of ferri- and ferrohemoglobins were determined both spectrophotometrically and potentiometrically. Aliquots of the same mixture were then added anaerobically to deoxygenated buffer solutions of different pH, containing a suitable oxidation-reduction mediator, and the potential of the system was measured. By this simple and quick procedure several points on the E-pH curve could be obtained with the same stock solution of hemoglobin.

A similar procedure was used to study the effect of NaCl or of potassium phosphate on the potential of cystine-treated hemoglobin. These experiments were performed in a cell equipped with a platinum and a glass electrode, enabling measurements of both E and pH. The oxidation-reduction potential of a 1:1 mixture of ferri- and ferrohemoglobin in 0.2 M NaCl (at 30°) was measured; salt was then added and the potential of the system was followed as a function of the salt concentration (in these experiments, phosphate); the pH was kept constant by the addition of acid or alkali.

Differential Titrations—Differential titrations were performed at 30° following the procedure previously described (2). An unbuffered solution of ferrohemoglobin was titrated with ferricyanide and the pH was measured; at the end of the titration the pH was brought back to the initial value by the addition of measured amounts of acid or alkali.

Ionization in Ferrihemoglobins—The transition between "acid" and "alkali" ferrihemoglobins due to the ionization of the hemin-like water molecule was determined spectrophotometrically in phosphate and borate buffers, at ionic strength 0.1 and ~25°, following the method described by George and Hanania (13).

Kinetics of Oxidation by Ferricyanide—The kinetics of the oxidation of hemoglobin by ferricyanide was studied as described earlier (3) with a Gibson-Milnes stopped flow apparatus (12).

RESULTS

Equilibrium Measurements—Measurements of the oxidation-reduction equilibrium were performed on all the modified hemoglobins, namely, cystine-treated hemoglobin, cystamine-treated hemoglobin, iodoacetamide-treated hemoglobin, and p-mercuribenzoate-treated hemoglobin. The effect of pH on $E_0$ (oxidation Bohr effect) is shown in Figs. 1 and 2 in comparison with that for normal hemoglobin. The results were obtained either from the midpoint of full titration curves or by the procedure described under "Methods." When both methods were used (for normal hemoglobin and cystine-Hb), they gave the same results. Figs. 1 and 2 also show, in the cases of cystine-Hb and IAA-Hb, a comparison of the oxidation Bohr effect as measured directly or by the differential titration method. This comparison is discussed later.

The abbreviations used are: cystine-Hb, cystine-treated hemoglobin; IAA-Hb, iodoacetamide-treated hemoglobin; PMB-Hb, p-mercuribenzoate-treated hemoglobin; cystamine-Hb, cystamine-treated hemoglobin.
All the modified hemoglobins show a similar change in the value of $E_+$ as compared with normal hemoglobin; the potentials are very similar to that of untreated hemoglobin at pH 9 and more negative by about 15 to 30 mV at pH 6, as can be seen from Figs. 1 and 2.

Fig. 3 shows the oxidation-reduction titration curves for cystine Hb, IAA-Hb, and PMB-Hb at different pH values. The equilibrium curves for IAA-Hb correspond almost exactly to $n = 2$ (solid lines) at every pH from ~6 to ~9. In the case of cystine-Hb (and also cystamine-Hb) the value of $n$ is equal to 2 in the acid and neutral regions, but becomes significantly less than 2 (~1.6) at pH 9. In order to check the validity of these values of $n$ and $E_+$, an analysis of the results on cystine-Hb at pH 5.95 was carried out by the objective method of Reed and Berkson (14). The results are shown in Table I. It is evident that the titration is fitted almost exactly by a value of $n = 2$ for values of $P$ (fractional oxidation) from ~0.05 to ~0.95. Hemoglobin treated with p-mercuribenzoate shows changes in the shape of the curves with pH; the value of $n$ varies at the different pH values.

It should be noted that the equilibrium curves of these modified hemoglobins are in every case quite symmetrical, in contrast to what was found to be the case for the normal hemoglobin of either horse (15) or man (1), which gives asymmetrical titration curves by the dye titration method.

**Table I**

<table>
<thead>
<tr>
<th>$x$</th>
<th>$x - d$</th>
<th>Reduction</th>
<th>$E_+$</th>
<th>$E_-$</th>
<th>Deviation from $+0.1508$</th>
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<tr>
<td>0.10</td>
<td>0.02</td>
<td>2.09</td>
<td>0.2087</td>
<td>0.1586</td>
<td>+0.0078</td>
</tr>
<tr>
<td>0.15</td>
<td>0.12</td>
<td>12.50</td>
<td>0.1765</td>
<td>0.1012</td>
<td>+0.0004</td>
</tr>
<tr>
<td>0.20</td>
<td>0.22</td>
<td>23.00</td>
<td>0.1662</td>
<td>0.1305</td>
<td>-0.0003</td>
</tr>
<tr>
<td>0.40</td>
<td>0.32</td>
<td>33.45</td>
<td>0.1595</td>
<td>0.1505</td>
<td>-0.0003</td>
</tr>
<tr>
<td>0.50</td>
<td>0.42</td>
<td>43.90</td>
<td>0.1599</td>
<td>0.1507</td>
<td>-0.0001</td>
</tr>
<tr>
<td>0.60</td>
<td>0.52</td>
<td>54.30</td>
<td>0.1455</td>
<td>0.1507</td>
<td>-0.0001</td>
</tr>
<tr>
<td>0.70</td>
<td>0.62</td>
<td>64.75</td>
<td>0.1385</td>
<td>0.1517</td>
<td>+0.0009</td>
</tr>
<tr>
<td>0.80</td>
<td>0.72</td>
<td>75.20</td>
<td>0.1360</td>
<td>0.1505</td>
<td>-0.0003</td>
</tr>
<tr>
<td>0.90</td>
<td>0.82</td>
<td>85.60</td>
<td>0.1264</td>
<td>0.1496</td>
<td>-0.0012</td>
</tr>
<tr>
<td>0.95</td>
<td>0.87</td>
<td>95.90</td>
<td>0.1190</td>
<td>0.1490</td>
<td>-0.0018</td>
</tr>
<tr>
<td>1.00</td>
<td>0.92</td>
<td>96.10</td>
<td>0.1085</td>
<td>0.1502</td>
<td>-0.0006</td>
</tr>
</tbody>
</table>

**Fig. 4.** Difference in proton bound between hemoglobin and Hb+ as a function of pH, at 20°C and ionic strength 0.20. Results on cystine-Hb, C, and IAA-Hb, O. The difference in proton bound between the two forms in the case of normal Hb is also reported for comparison, -----. The contribution of the ionization of the heme-linked water molecule is also included, ---, with a $pK'$ of 8.05.
hemoglobin, the vertical position was fixed to give the best fit to all the measured values of $E_1$ plotted against pH. In the case of cystine-Hb, where a good fit by this procedure was impossible, the absolute vertical position of the differential curve was obtained by fitting the $E_1$ value of a mixture of ferrohemoglobin and ferrihemoglobin 50:50 measured in the same solvent as that used for the differential measurements (0.2 M NaCl); the values at pH 5.9 and 6.5, which are in close agreement with $E_1$ values in phosphate buffer, were weighted strongly.

The change in pH as a function of the fractional oxidation is reported in Figs. 5 and 6 for cystine-Hb and IAA-Hb, respectively. It should be recalled that in the case of normal hemoglobin the change of $E$ with pH is reflected in the nonlinear relation between $\Delta \mathrm{pH}$ and $P$ (2). In the case of hemoglobin treated with $\text{--SH}$ reagents at pH values higher than $\sim 6.5$, there is a linear relationship between $\Delta \mathrm{pH}$ and $P$ corresponding to the invariance of $n$ with pH. Such linearity seems to break down at lower pH values, although the nonlinearity is much less pronounced than in the case of normal Hb.

Evaluation of Ion Effects Associated with Solvents Used—It was mentioned above that the measurements of the half-potentials included in the same figure. The oxidation Doehl effect in terms of changes of $E$ with pH can be obtained by graphical integration of the differential curves. The results for cystine-Hb and IAA-Hb are reported in Figs. 1 and 2, where they are compared with the values directly obtained by measurements of the potential in buffers of different pH. In the case of IAA-Hb, as well as normal

<p>| TABLE II |
| PK' values for ionization of heme-linked water molecule in normal and modified ferrihemoglobins |
| Hemoglobin concentration $= 6 \times 10^{-4}$ M; $\gamma/2 = 0.2$; temperature $= 25^\circ$. |</p>
<table>
<thead>
<tr>
<th>Hemoglobin</th>
<th>PK'</th>
<th>n</th>
</tr>
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<tr>
<td>Hemoglobin</td>
<td>8.05</td>
<td>1.0</td>
</tr>
<tr>
<td>IAA-Hb</td>
<td>8.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Cystine-Hb</td>
<td>8.12</td>
<td>1.0</td>
</tr>
<tr>
<td>Cystamine-Hb</td>
<td>8.17</td>
<td>1.0</td>
</tr>
<tr>
<td>PMB-Hb</td>
<td>8.15</td>
<td>0.9</td>
</tr>
</tbody>
</table>

<p>| TABLE III |
| Velocity constants for reaction of normal and modified ferrohemoglobins with ferricyanide |
| Hemoglobin concentration $= 1 \times 10^{-4}$ M; ferricyanide $= 1 \times 10^{-4}$ M; $\lambda = 500 \text{ m\mu}$; temperature $= 20^\circ$. |</p>
<table>
<thead>
<tr>
<th>pH</th>
<th>Buffer</th>
<th>$\Delta E/\Delta t \times 10^3$</th>
<th>$\Delta E/\Delta t \times 10^3$</th>
<th>$\Delta E/\Delta t/\Delta E/\Delta n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.2</td>
<td>Phosphate, 0.1 M</td>
<td>Hemoglobin</td>
<td>4.85</td>
<td>3.27</td>
</tr>
<tr>
<td></td>
<td>IAA-Hb</td>
<td>3.80</td>
<td>5.06</td>
<td>1.32</td>
</tr>
<tr>
<td></td>
<td>Cystine-Hb</td>
<td>4.15</td>
<td>5.30</td>
<td>1.28</td>
</tr>
<tr>
<td></td>
<td>PMB-Hb</td>
<td>4.02</td>
<td>4.01</td>
<td>1.00</td>
</tr>
<tr>
<td>7.5</td>
<td>Phosphate, 0.1 M</td>
<td>Hemoglobin</td>
<td>1.90</td>
<td>1.44</td>
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<tr>
<td></td>
<td>IAA-Hb</td>
<td>2.01</td>
<td>2.50</td>
<td>1.25</td>
</tr>
<tr>
<td></td>
<td>Cystine-Hb</td>
<td>1.96</td>
<td>2.31</td>
<td>1.21</td>
</tr>
<tr>
<td></td>
<td>PMB-Hb</td>
<td>2.01</td>
<td>2.30</td>
<td>1.15</td>
</tr>
<tr>
<td>9.1</td>
<td>Borate, 0.05 M</td>
<td>Hemoglobin</td>
<td>0.81</td>
<td>1.20</td>
</tr>
<tr>
<td></td>
<td>IAA-Hb</td>
<td>1.03</td>
<td>1.44</td>
<td>1.40</td>
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<tr>
<td></td>
<td>Cystine-Hb</td>
<td>1.15</td>
<td>1.27</td>
<td>1.10</td>
</tr>
<tr>
<td></td>
<td>PMB-Hb</td>
<td>0.92</td>
<td>1.24</td>
<td>1.35</td>
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</tbody>
</table>
were made in buffer solutions; those of differential proton-binding
capacity, in 0.2 M NaCl. An exact comparison of the Bohr effect
by the two methods calls for evaluation of specific ion effects asso-
ciated with the different solvents used. A set of experiments
to investigate this point was therefore performed on cystine-Hb.
The potential of an equimolar mixture of ferrohemoglobin and
ferrihemoglobin in a solvent of a given salt composition was fol-
lowed as a function of the amount of a second salt present at
constant pH. These experiments were performed either by
adding NaCl to a solution of ferrohemoglobin-ferrihemoglobin in
phosphate at pH 6 or borate at pH 9, or vice versa. The data
obtained are also reported in Fig. 1 and are indicated by the
triangles. They indicate that the actual value of $E_1$ at pH 7.6
is relatively independent of the nature of the salt present, pro-
vided the ionic strength does not vary, since the same value is
obtained in phosphate or in NaCl. At more alkaline pH values
(8 to 9) the data show a definite dependence of the half-potential
on the type of salt, i.e. phosphate (or borate) and NaCl. More-
over, at alkaline pH values the values of potential obtained in
NaCl do not agree with the values expected from the differential
masurements in the same solvent. This is not surprising, how-
ever, because the curve obtained by differential titrations is sub-
ject to the basic assumption that the only ion differentially bound
between the two forms of hemoglobin is the proton, and this
might not be true in the particular case.

Ionization in Ferrihemoglobins—The apparent $pK$ values for
the ionization of the water molecule coordinated with the heme
iron determined under conditions similar to those used for the
oxidation-reduction equilibrium experiments are reported in
Table II.

No attempts to correct for the low pH ionizations reported by
George and Hanania (13) were made in the calculation of the $pK$
values.

Kinetics of Oxidation by Ferricyanide—The second order ve-
locity constants for the reaction of normal and modified hemo-
globins with ferricyanide ($O^+$) are reported in Table III. In
general the results do not correspond with a simple second order
process: this is reflected by the ratio of the final value of the
velocity constant ($O^+$) to the initial value ($O^+$), given in the
last column of the table. While normal hemoglobin at pH 6
shows a decrease in the apparent rate constant with the progress
of the reaction, both cystine-Hb and IAA-Hb show an increase. It
should be noted that the values of the constants obtained for
normal hemoglobin are remarkably consistent with those reported
before (3).

**DISCUSSION**

It was long ago pointed out by Wyman and Ingalls (16) that
the quantity $k_{rot}/k_{rot}$ in the oxidation process is equivalent to in
$p$ ($O_2$) in the oxygenation process, and that similarities or diffe-
rences in the two equilibria are reflected in the behavior of these
twquantities. The question now arises as to whether chemical
modifications of the hemoglobin molecule have the same or
different effects on the two equilibria. This question is particu-
larly pertinent in view of the fact that the same conformational
change appears to occur in both the oxygenation and oxidation
processes. The experimental results reported in Table IV for a
number of modifications of human hemoglobin reveal the extent
to which the two processes are equally affected. These results
are given in terms of differences in $E_1$ between normal hemoglobin and
any one of the modified hemoglobins. $AE$ is for the oxida-
tion-reduction equilibrium; $AE'$ for the oxygenation equilibrium.
It will be seen that, whatever the nature of the modification, it
produces very similar changes in the over-all free energy of both
processes over a wide pH range. This brings out clearly the
phenomenological similarity of the two equilibria in the case of
human hemoglobin.

A particularly striking instance of this similarity is the effect of
salt. In the case of both the oxidation and oxygenation equilibria a large salt effect is only found in the case of hemoglobin.

### Table IV

<table>
<thead>
<tr>
<th>Compound</th>
<th>pH 6.0</th>
<th></th>
<th></th>
<th>pH 9.0</th>
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<th></th>
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<tbody>
<tr>
<td></td>
<td>$E_1$</td>
<td>$AE$</td>
<td>$AE'$</td>
<td>$E_1$</td>
<td>$AE$</td>
<td>$AE'$</td>
</tr>
<tr>
<td>Normal hemoglobin</td>
<td>0.175</td>
<td>0</td>
<td>0</td>
<td>0.030</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Carboxypeptidase B-Hb</td>
<td>0.108</td>
<td>68</td>
<td>0</td>
<td>-0.009</td>
<td>39</td>
<td>36</td>
</tr>
<tr>
<td>Carboxypeptidase A-Hb</td>
<td>0.079</td>
<td>96</td>
<td>0</td>
<td>-0.027</td>
<td>57</td>
<td>62</td>
</tr>
<tr>
<td>Hb (carboxypeptidase A + B)</td>
<td>0.040</td>
<td>135</td>
<td>110</td>
<td>-0.045</td>
<td>75</td>
<td>56</td>
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<tr>
<td>Hemoglobin + bromthymol</td>
<td>0.170</td>
<td>-1</td>
<td>0</td>
<td>0.028</td>
<td>-29</td>
<td>-30</td>
</tr>
<tr>
<td>LAA-Hb</td>
<td>0.149</td>
<td>26</td>
<td>30</td>
<td>0.021</td>
<td>9</td>
<td>11</td>
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<td>Cystine-Hb</td>
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<td>0.052</td>
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<td>Cystamine-Hb</td>
<td>0.156</td>
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<td>6</td>
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<td>Hemoglobin + PMBc</td>
<td>0.163</td>
<td>12</td>
<td>17</td>
<td>0.037</td>
<td>-7</td>
<td>6</td>
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<tr>
<td>Mb (horse)</td>
<td>0.053</td>
<td>122</td>
<td>72</td>
<td>0.003</td>
<td>27</td>
<td>23</td>
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<tr>
<td>Mb (whale)</td>
<td>0.061</td>
<td>114</td>
<td>114</td>
<td>0.024</td>
<td>6</td>
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</table>

$a$ One equivalent of bromthymol blue per heme.
$b$ Differences were calculated from oxygen equilibrium experiments performed at 20°C.
$\text{Two equivalents of } p\text{-mercuribenzoate per tetramer.}$
$\text{E. Antonioini, M. Brunori, and J. Wyman, unpublished data.}$
treated with cystine (see Fig. 1). For this hemoglobin the approximation that the effect of proton binding largely dominates that of the binding of any other ion in solution, which is valid for normal or iodoacetamide-treated hemoglobins, is simply not valid.

When it comes to the heme-heme interactions the situation is more complex. It is well known that the value of $n$ for the oxygen equilibrium of normal human hemoglobin is essentially invariant (in dilute buffers) from pH 5 to 9 ($n = 2.8$); in contrast, the value of $n$ for the oxidation-reduction equilibrium rises from about 1.2 at pH 6 to about 2.5 at pH 9, which indicates that the free energy of interaction in the oxidation process depends strongly on pH (for a discussion see Reference 2). Nevertheless, in spite of this difference, treatments which essentially abolish the heme-heme interaction for the oxygenation process, such as digestion by carboxypeptidase, also do so for the oxidation process (4).

Treatment of hemoglobin with iodoacetamide or with disulfides (cystine and cysteamine) leaves the heme-heme interactions intact in the oxygen equilibrium, the value of $n$ being 2.7 (6). The value of $n$ for the oxidation-reduction equilibrium of the same modified hemoglobins is about 2 and in this case is roughly independent of pH in the range from $\sim 6$ to $\sim 9$ (see Fig. 4). Thus, at low pH values ($\sim 6$), the SH-treated hemoglobins have a value of $n$ which is higher than that of untreated hemoglobin; this is a remarkable finding, since in no other known instance does a chemical modification of the untreated hemoglobin; this is a remarkable finding, since in no other known instance does a chemical modification of the untreated hemoglobin produce an $n$ for the oxidation-reduction equilibrium rises from about 1.2 at pH 6 to about 2.5 at pH 9, which indicates that the free energy of interaction in the oxidation process depends strongly on pH (for a discussion see Reference 2). Nevertheless, in spite of this difference, treatments which essentially abolish the heme-heme interaction for the oxygenation process, such as digestion by carboxypeptidase, also do so for the oxidation process (4).

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The approximate invariance of $n$ with pH found by equilibrium potentiometric measurements in the case of these modified hemoglobins finds confirmation in the differential pH measurements. In this case, however, the change in pH coupled with the oxidation of the heme iron is not perfectly linear in $Y$, as would be expected for true invariance of $n$ with pH. This shows that differential pH measurements are a more sensitive tool under these experimental conditions for studying pH invariance of the equilibrium curves than direct measurements of the oxidation-reduction potentials themselves. It should be noted that, in cases in which the change of $n$ with pH is very small, it is not practical to try to calculate it from the differential titration data.

A complete analysis of the oxidation Bohr effect shows that there are considerable differences between the oxygen and oxidation Bohr effects. After allowing for the ionization of the water molecule coordinated with the iron atom in ferrihemoglobin, the $pK^*$ of which can be determined independently by spectrophotometry (13), it is profitable to compare the Bohr effect due to the other acidic groups, for the two processes. The comparison shows, as previously reported (1), that the oxidation Bohr effect is always larger than the oxygen Bohr effect. The excess has been called the "residual oxidation Bohr effect." It is quantitatively the same for normal and several modified hemoglobins and amounts to about 25 mv. Of the modified hemoglobins studied and reported in this paper its presence could only be verified in the case of IAA-Hb because of complications arising from the salt effects in the other modified hemoglobins.

The behavior of normal human hemoglobin in oxidation-reduction might be explained on the hypothesis that the $\alpha$ and $\beta$ chains have inherently different oxidation-reduction properties and contribute differently to the Bohr effect. In accordance with this, at any pH the shape of the equilibrium curve would be determined by the superposition of two effects: the heme-heme interactions, which tend to produce high values of $n$; and the intramolecular functional heterogeneity, which tends to decrease $n$. If chemical modifications of the $\alpha-SH$ groups alter specifically the oxidation-reduction properties of the $\beta$ chains, this might explain the fact that such modified hemoglobins have at acid pH a value of $n$ higher than that of normal hemoglobin. This hypothesis is being verified by measurements of the oxidation-reduction properties of the isolated $\alpha$ and $\beta$ chains of human hemoglobin with free and blocked sulphydryl groups.

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