In the preceding paper of this series (1) we reopened the study of the oxidation-reduction potentials of normal hemoglobin, taking up the subject where it had been left by Taylor and Hastings (2) and by Havemann (3) nearly 20 years ago. These earlier investigators had studied horse and ox hemoglobin, respectively, and limited themselves to the dye titration method. Our work was on human hemoglobin, and in addition to the dye titration method we made use of the method of mixtures. (Both of these methods are explained below.) As regards the absolute values of $E^1_1$ and the Bohr effect, our results are in close agreement with the earlier observations on horse hemoglobin; on the other hand, as regards the shape of the equilibrium curves and the way in which the shape changes with $pH$, we obtained different results, which varied with the method. Our curves obtained by the method of mixtures are all symmetrical with values of $n$ as high as 2.0 at alkaline $pH$, although the value of $n$ depends somewhat on the way in which the ferrihemoglobin employed is made. In contrast, the curves obtained by the dye titration method are always more or less asymmetrical. In all cases $n$ drops towards 1 (about 1.2) at low $pH$. We believe that the method of mixtures, in which the possibility of the binding of dye by the protein is eliminated, is the more reliable.

In the present study we extend the measurements of oxidation-reduction potentials to derivatives of normal human hemoglobin obtained by digestion with carboxypeptidases $A$ and $B$. These are: HbCPA, in which the two COOH-terminal residues, tyrosine and histidine, occupying positions 145 and 146 of the $\beta$ chains, have been removed by the action of carboxypeptidase $A$; HbCPB, in which the COOH-terminal residue, arginine, occupying position 141 of the $\alpha$ chains, has been removed by carboxypeptidase $B$; and HbCPA + B, in which several residues of both the $\alpha$ and $\beta$ chains have been removed by joint action of the two carboxypeptidases. As regards molecular weight and spectral properties in the visible wave band, these derivatives are all like normal hemoglobin. Nevertheless, as may be recalled, in HbCPB the oxygen affinity at $pH$ 7 is increased about 10 times, the value of $n$ remains unchanged, and the Bohr effect is largely reduced; in HbCPA the oxygen affinity at $pH$ 7 is increased about 30 times, the value of $n$ drops to unity, and the Bohr effect is reduced about as in HbCPB; in HbCPA + B the oxygen affinity at $pH$ 7 is increased about 50 times, the Bohr effect is almost completely eliminated, and $n$ is reduced to 0 or even less than 1 (4). It is clearly of interest to see how this behavior is reflected in the equilibria with another ligand, the electron.

**EXPERIMENTAL PROCEDURE**

**Hemoglobin Preparation**

Human oxyhemoglobin was prepared from freshly drawn red blood cells by the ammonium sulfate method (5). In most cases ferrihemoglobin was obtained from oxyhemoglobin by the addition of stoichiometric amounts of $K_3Fe(CN)_4$; in a few others it was obtained by the treatment of red blood cells with sodium nitrite, as previously described in some detail (1). For the sake of convenience, we shall refer to the two kinds of ferrihemoglobin as $FeCy$ ferriHb and nitrite ferriHb.

Hemoglobin concentrations were determined spectrophotometrically on the basis of earlier work (5), with $E^1_{580}$ = 8.5 at 542 $\mu$m for HbO$_2$; $E^1_{580}$ = 7.6 at 555 $\mu$m for Hb; and $E^1_{580}$ = 5.8 at 505 $\mu$m for Hb$^+$ at $pH$ 6. The same extinction coefficients were used for the digested hemoglobin. Suits used for buffer solutions were analytical grade reagents and were not recrystallized.

**Digestion of Hemoglobin**

Most of the digestions were performed on oxyhemoglobin at $pH$ 7.5 to 8.0 in unbuffered solutions and at room temperature, with the use of carboxypeptidase $A$ from the Worthington Biochemical Corporation, or carboxypeptidase $B$ prepared from swine pancreas by the method of Folk et al. (6), or both. However, for some experiments involving the mixture method, a stock of nitrite-ferriHb was digested with carboxypeptidase $B$.
and the necessary amount of ferrohemoglobin was obtained from it by reduction with the enzymic system (7). In both cases the extent of the digestion was the same. This was established after trichloroacetic acid deproteinization, by the determination of the liberated amino acids by ion exchange chromatography, with a Spinco amino acid analyzer. In all cases but one it was at least 90% complete. At the end of the digestion period the enzymes were inactivated as recently described (8). From time to time the functional modifications following the digestion were controlled by measurements of the O₂ equilibrium by the method of Rossi-Fanelli and Antonini (9).

**Measurement of Oxidation-Reduction Potentials**

Oxidation-reduction potentials were read against a saturated calomel half-cell, with the use of a Leeds and Northrup type K₂ potentiometer in connection with a Rubicon galvanometer, and then referred to the normal hydrogen electrode, after the conventions of Clark (10). All the measurements were made in an atmosphere of purified argon, at constant pH and 30 ± 0.1° in phosphate and borate buffer (ionic strength 0.07 to 0.15). The Hb concentration was in the range 5 to 30 mg per ml.

The oxidation-reduction equilibrium curves were determined according to the procedures described in detail in the first paper of this series (1). Here we refer only very briefly to the two basically different methods employed.

**Dye Titration Method**—The dye titration method involves the addition of a reductant (reduced anthraquinone-β-sulfonate) to ferrohemoglobin, or an oxidant (potassium ferricyanide, K₃Fe(CN)₆) to ferrohemoglobin. This method has been more frequently used, in the form described in the first paper as la, in which the FeCy-ferriHb is progressively reduced with anthraquinone-β-sulfonate.

**Mixture Method**—The method of mixtures consists of the measurement of the potential of mixtures of ferro- and ferrihemoglobin in known proportions. It can be performed by using either FeCy-ferriHb or nitrite-ferriHb.

Toluylene blue and thionine were used as mediators (2), always in a very low molar ratio to the protein (2 to 3%).

**RESULTS**

**Reversibility**

Experimental evidence for the reversibility of the results obtained by the dye titration method is provided by Fig. 1, in which both the oxidative and reductive titrations of HbCPA at pH 7.45 are shown. These equilibrium curves were obtained by the dye titration procedure, the solution being reduced by titration with anthraquinone-β-sulfonate and then reoxidized by titration with K₃Fe(CN)₆. Similar results were obtained for the other modified hemoglobins.

**General Features of Oxidation-Reduction Equilibria at pH 7**

Fig. 2 shows the oxidation-reduction equilibrium curves of normal hemoglobin, HbCPA, HbCPB, and HbCPA + B, all measured by the dye titration method at about pH 7. The smooth curves are theoretical curves corresponding to n = 1. Without, at this point, going into complications involving shape, we call attention to the progressive decrease in Eₜ, which parallels the decrease in log pO₂ for the oxygen equilibrium, from normal hemoglobin to HbCPA to HbCPA + B. The total difference between normal hemoglobin and HbCPA + B amounts to about 100 mv at pH 7. It will be recalled that although the shape of curves depends on the method, the values of Eₜ are not visibly affected by the method.

**Bohr Effect**

Fig. 3 shows the oxidation Bohr effect for normal hemoglobin and the three digestion products. Some of the values plotted were obtained by the mixture method, others by the dye titration method. The progressive diminution of the Bohr effect from normal hemoglobin to HbCPA or HbCPB or HbCPA + B parallels that of the oxygen Bohr effect. Moreover, the oxidation Bohr effects for HbCPA and HbCPB are about the same.
It will be seen that at alkaline pH all four oxidation Bohr effect curves shown in Fig. 3 have a slope approaching 0.000 volt per pH unit. This limiting slope results from the dissociation of the water molecule occupying the sixth coordination position of the heme iron atom in ferrihemoglobin, something which does not occur in ferrohemoglobin. The reduction of the iron atom is thus accompanied by addition of 1 hydrogen ion at high pH (11). Experiments were made to see whether the dissociation constant of this water molecule was the same in all the forms. The degree of its dissociation was measured spectro-photometrically in buffers of known pH at an ionic strength of 0.1 and about 25°. The results are shown in Fig. 5, from which it is clear that the water molecule dissociates as a simple acid and that its pK' (8.00 ± 0.05) is the same in normal hemoglobin and the three digestion products.

**Shape of Equilibrium Curves**

The question of the shape and variation of shape of the oxidation-reduction equilibrium curves is confusing. As pointed out previously (1), the curves for normal hemoglobin obtained by the dye titration method are all asymmetrical and their shape is strongly dependent on pH; at alkaline pH the curves are much steeper than at acid pH, and the value of n corresponding to the midpoint of the curves drops from something like 2.0 at pH 8.5 to about 1.2 at pH 6. In contrast, the curves obtained by the mixture method are all symmetrical, and are again less...
steep at acid than at alkaline pH. However, the exact value of $n$ at alkaline pH, which may be as high as 2.6, varies with the method of preparing the ferrihemoglobin used in making up the mixture. (Nitrite-ferriHb gives the highest value of $n$.) It has been suggested that such differences are at least partly due to combination of the reducing or oxidizing agents with hemoglobin, and that the mixture method gives the most reliable results.

Most of the measurements on the digested hemoglobins were made by the dye titration method, but a few, chiefly those on HBCPB, were made by the mixture method. In all cases the curves, even those obtained by the dye titration method, are more or less symmetrical and $n$ tends to be lower than for normal hemoglobin. For HBCPA + B, $n$ is 0.8 to 0.9 at neutral pH (see Fig. 2) and, in the alkaline range, drops to lower values, indicating either heterogeneity of sites or negative interactions. For HBCPA, $n$ is everywhere close to 1 (0.9 to 1.2); for HBCPB it is slightly higher (1 to 1.6).

**DISCUSSION**

The most significant thing about the results of this study is the parallelism between the effects of digestion on the oxidation-reduction equilibrium and on the oxygen equilibrium, particularly as regards the values of $\log p_1$ and $E_1$. It will be recalled that at 30°, $E_1$ is the exact equivalent of 0.06 $\log p_1$. Table I lists values of $E_1$ and $\Delta E$ for normal hemoglobin and each of the three digestion products at two pH values; figures for horse myoglobin are included for comparison (12-14).

As will be seen from Fig. 3, the major part of the oxidation Bohr effects for all the hemoglobins arises from the dissociation of the water molecule coordinated with the heme iron. We have just shown that this dissociation is characterized by the same pK' (8.0) in each case. A good many years ago (15) it was suggested that, in the case of horse hemoglobin, if correction were made for the ionization of this group the residual Bohr effect would be about the same as the oxygen Bohr effect in which the water molecule does not come into play. In view of this we have corrected the Bohr effects for each of the human hemoglobins (normal, HBCPB, HBCPA, and HBCPA + B) in order to see how the residual Bohr effects compare with those for oxygen. The results are shown in Fig. 6. It is at once evident that in each case there is about the same difference between the

<table>
<thead>
<tr>
<th>TABLE I</th>
<th>Comparison of effects of digestion on oxygen and oxidation Bohr effects</th>
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<tbody>
<tr>
<td>pH 6</td>
<td>$E_1$</td>
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<tr>
<td>---------</td>
<td>-------</td>
</tr>
<tr>
<td>Hb</td>
<td>0.175</td>
</tr>
<tr>
<td>HBCPB</td>
<td>0.108</td>
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<td>HBCPA</td>
<td>0.079</td>
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<tr>
<td>HBCPA + B</td>
<td>0.040</td>
</tr>
<tr>
<td>Myoglobin (horse)</td>
<td>0.033</td>
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</tbody>
</table>

$* \Delta E$ is the difference between normal hemoglobin and its digestion products or horse myoglobin.

$\uparrow \Delta E'$ is the expected value based on observed differences in $\log p_1$ (4, 14, 16), taking account of the relation $E_1 - \log p_1 \times 2.303 RT/F$.

**Fig. 6.** Comparison of oxygen Bohr effect with oxidation Bohr effect corrected for ionization of the water molecule for normal and digested hemoglobins. Solid lines show oxygen Bohr effect from Antonini et al. (4, 16). Circles show $E_1 \times 16.65$. All results obtained at 30°.
two, the residual oxidation Bohr effect being always larger than the corresponding oxygen Bohr effect by about the same amount. Perhaps there is an oxidation-linked acid group (or groups), essentially the same in all four forms, which plays no role in the oxygen equilibrium. Although this group does not make a major contribution to the pH effect, it is nevertheless of significance. Its mean pK' value as between hemoglobin and ferrihemoglobin is in the neighborhood of 7. This strengthens a preliminary conclusion expressed in the preceding paper of this series. It is striking that in horse myoglobin, in which there is virtually no oxygen Bohr effect (14) and in which the pK' of the iron-linked water molecule is 8.80 (17), there is evidence for a similar supplementary oxidation-linked acid group, but one which comes into play about 1 pH unit more alkaline.

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