

Studies on the Oxidation-Reduction Potentials of Heme Proteins

V. THE OXIDATION BOHR EFFECT IN NORMAL HUMAN HEMOGLOBIN AND HUMAN HEMOGLOBIN DIGESTED WITH CARBOXYPEPTIDASE A*

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In its narrower sense the term Bohr effect is used to describe the interaction between oxygen and proton as they combine with hemoglobin and other respiratory proteins. It may, however, be legitimately extended to include the interaction of proton with any ligand in any of these proteins. It is in this larger sense that we use the term in this paper to refer to the effects of pH on the oxidation-reduction potentials of hemoglobin.¹

We are concerned here with a typical heterotropic linkage phenomenon (5), which differs from the more usual ones only in the fact that one of the ligands is an electron. We cannot, of course, relate the activity of the electron to a measured concentration, as in the case of such more familiar ligands as oxygen or an isocyanide, but this really makes no difference. It has been pointed out that in dealing with oxidation-reduction phenomena the quantity EF/RT is the precise equivalent of $\ln y$, where y is the activity of any such familiar ligand as oxygen or carbon monoxide (6). This means that, passing to Briggsian logarithms, we may write

$$E_{\text{volta}} \sim 1.984 \times 10^{-4} \cdot T \cdot \log y \quad (1)$$

or

$$\log y \sim 0.5039 \times 10^4 \cdot E/T$$

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¹ We might indeed extend the definition even further, removing the condition that one of the ligands be proton, and apply the term Bohr effect to the interaction between any two ligands, *i.e.* to any linkage phenomenon; however, this would probably be going too far. It is to be recalled that the phenomenon originally described by Bohr, Hasselbalch, and Krogh (1) was simply the effect of carbon dioxide on the oxygen equilibrium of the blood. The converse effect, namely the influence of oxygenation on the amount of carbon dioxide taken up by the blood, was not discovered until 10 years later by Christiansen, Douglas, and Haldane (2), and even then the thermodynamic relationship between the two phenomena was not fully appreciated. Another few years were to elapse before the first essentially correct interpretation of the situation was given by Parsons (3) and then, in more complete form, by Henderson (4), who pointed out the reciprocal relationship between the two effects and showed that it was carbonic acid as a proton donor, rather than carbon dioxide as a gas, which was involved (4). As has been emphasized by Roughton (personal communication), the important part played by Krogh in the original discovery tends to be lost sight of owing to the fact that the phenomenon takes its name exclusively from the senior author of a joint paper. It should not, however, be forgotten

When two ligands are present, the basic linkage relation which governs the situation is

$$\left(\frac{\partial \ln y}{\partial \ln x}\right)_{\bar{Y}} = -\left(\frac{\partial \bar{X}}{\partial \bar{Y}}\right)_x = \left(\frac{\partial \bar{X}}{\partial \ln x}\right)_{\bar{Y}} \left(\frac{\partial \ln x}{\partial \bar{Y}}\right)_{\bar{X}} \quad (2)$$

where \bar{X} and \bar{Y} are the amounts of any two ligands X and Y bound per unit of macromolecule and x and y are their activities (see Reference 5 for this and following equations). In terms of the median ligand activity, y_m , as obtained from the ligand equilibrium curve of Y measured at constant x , this equation assumes the simpler form

$$\frac{d \ln y_m}{d \ln x} = -\Delta \bar{X} \quad (3)$$

where $\Delta \bar{X}$ is the difference in the amount of X bound per Y binding site between the molecules when fully saturated and when completely uncombined with Y . If the ligand equilibrium curve is symmetrical, y_m of course becomes the same as y_1 . If it is invariant in shape for changes of x , then $(\partial \bar{H}^+ / \partial \bar{Y})_x$ is constant, independent of \bar{Y} , and equal to $\Delta \bar{X}$. In either event, Equation 3 may be written as

$$\frac{d \log y_1}{d \log x} = -\Delta \bar{X} \quad (4)$$

In the case of oxygen, extensive studies on the mammalian hemoglobins show that the equilibrium curves are approximately, if not quite, symmetrical, and are invariant in shape between approximately pH 4.8 and 10 (7). This means that Equation 4 is applicable; if we identify X with proton and Y with oxygen it becomes

$$\frac{d \log p_1}{d \text{pH}} = \Delta \bar{H}^+ \quad (5)$$

where \bar{H}^+ is the amount of proton bound per heme. Justification for treating proton as an independent ligand, neglecting other ions associated with it, comes from the fact that values of $\log p_1$, measured as a function of pH in the presence of buffer, agree closely with those calculated by the equation from differential titration results on the oxy and deoxy forms of hemoglobin (8).² The invariance property of the curves implies, in accord-

² There are one or two exceptional cases in which there is a significant departure of the observed values of $\log p_1$ from those

ance with what has already been said, that at every pH the displacement of proton should be proportional to the amount of oxygen bound. That this requirement is satisfied has been shown by direct measurements in which, for technical reasons, carbon monoxide was substituted for oxygen (8); it provides additional justification for treating proton as an independent ligand as in Equation 4.

Recent studies on the oxidation-reduction potentials of human hemoglobin give a different picture (9, 10). Thus, the shape of the equilibrium curve changes greatly with pH, although slightly different results are obtained by using different methods.³ The results are best described in terms of the familiar parameter n , which for the oxidation-reduction case may be defined as

$$n = 2.303 \frac{RT}{F} \frac{d \log \left(\frac{\bar{Y}}{1 - \bar{Y}} \right)}{dE} \quad (6)$$

By the method of mixtures with ferrihemoglobin prepared by the nitrite method, n was found to increase from about 1.2 at pH 6 to about 2.5 at pH 9 (9). This of course implies that the binding of proton cannot be linearly related to the degree of oxidation.

Striking modifications in the characteristic features of the oxidation-reduction equilibrium of human hemoglobin were found to follow digestion of the protein by carboxypeptidases A and B. For example, digestion by carboxypeptidase A, which removes the two COOH-terminal residues of the β chains (Tyr₁₄₅ and His₁₄₆), reduces the value of E_1 at pH 7 from 0.150 volt for hemoglobin to approximately 0.066 volt for the digestion product,⁴ at the same time, the value of n becomes essentially 1 and is independent of pH. There is a general parallelism here between the effects of digestion on the oxygen and oxidation equilibria (10).

In view of these observations, it was decided to undertake a detailed study, similar to that already carried out for the oxygen Bohr effect, on the oxidation Bohr effect in both normal and carboxypeptidase A-digested hemoglobin. A previous study of this kind, involving differential titrations, had already been made on normal horse hemoglobin by Wyman and Ingalls over 20 years ago (6). This had shown the close phenomenological similarity between the oxygen and oxidation Bohr effects of that protein; the only clearly significant difference between the two observed at that time could be attributed to the dissociation of a water molecule coordinated with the heme ion and having a pK of approximately 8.0 in ferrihemoglobin. In the light of new and more extensive data, however, it now seems that the interpretation of Wyman and Ingalls was an oversimplification; the data

calculated by Equation 5 from differential titrations. These are cystine- and cystamine-treated human hemoglobin. In these cases there must be a significant differential binding of ions other than proton (E. Antonini, J. F. Taylor, M. Brunori, and J. Wyman, unpublished experiments). This situation does not arise in the present study.

³ For example, the method of mixtures (9) gives symmetrical curves between $\bar{Y} = 0.1$ and $\bar{Y} = 0.9$, whereas the dye titration method, with anthraquinone- β -sulfonate as reducing agent and potassium ferricyanide as oxidizing agent, gives asymmetrical curves the curvatures of which are greatest at the top. In all cases, however, the change of shape with pH is qualitatively the same.

⁴ The term E_1 (or $E_{0.50}$) is used in this paper to indicate the potential of the system when [ox] = [red]. The term E_m , in which m stands for median, is defined by Equation 1 if we set $E = E_m$ when $y = y_m$. The mean ligand activity, y_m , is defined in Reference 5, pages 238-241. Whenever the ligand equilibrium curve is symmetrical, E_m becomes the same as E_1 .

on normal and, especially, digested hemoglobins point to the presence of an additional oxidation-linked group with mean pK, as between deoxy- and ferrihemoglobin, near 7 (10). In view of all the facts, it is evident that a reinvestigation of the oxidation Bohr effect by differential measurements with the improved techniques now available is called for. In particular, in view of the change of shape of the equilibrium curves with pH, the non-linearity of the relation between oxidation of the heme iron and the gain or loss of protons presents a crucial problem. In this connection, a comparison of normal hemoglobin with carboxypeptidase A-digested hemoglobin, in which the value of $n = 1$ is independent of pH, is sure to be illuminating. The study of these phenomena is the main purpose of this work.

EXPERIMENTAL PROCEDURE

Hemoglobin—Human hemoglobin was prepared from freshly drawn red blood cells by the ammonium sulfate method (9). Hemoglobin concentrations were determined spectrophotometrically on the basis of published values for the extinction coefficients (11). Experimental precautions in dealing with hemoglobin and with the various reagents were taken as described in a previous paper (8).

Digested Hemoglobin (HbCPA⁵)—Digestion with carboxypeptidase A was carried out for us by Dr. R. Zito in accordance with the usual procedure (12). All experiments were performed on one preparation, in which it was shown by amino acid analysis that the removal of the last two COOH-terminal residues of the β chain, Tyr₁₄₅ and His₁₄₆, was 93% complete.

Spectrophotometric Measurements—These were made with a Beckman DK-1 recording spectrophotometer. For the stoichiometric study of the reaction between hemoglobin and ferricyanide, a spectrophotometric cell was adapted to make possible the performance of titrations in the absence of a gas phase and under anaerobic conditions. The volumes of the reagent were measured with an Agla micrometer syringe. Dilution effect was always less than 0.5%.

Differential Titrations—The manipulations involved were the same as in earlier studies (8). Deoxyhemoglobin was oxidized progressively by adding successive amounts of deoxygenated ferricyanide, and the pH change was recorded after each addition. (It may be noted that the transition from ferro- to ferricyanide involves no uptake or liberation of proton.) After 100% oxidation was reached, additional amounts of ferricyanide were added to make sure that no further pH changes occurred. The pH measurements were made at constant temperature, $25 \pm 0.1^\circ$, with a Radiometer model 4 pH meter sensitive to about 0.003 pH unit.

That neither ferricyanide nor the ferrocyanide formed as a product of the reaction has any significant buffering power was checked in blank titrations from pH 5 to pH 9.

RESULTS AND ANALYSIS

Stoichiometric Relationship between Heme Iron and Oxidant—To exclude the possibility of an effect other than the oxidation of the heme iron upon treatment of hemoglobin with ferricyanide under the conditions of these experiments, the stoichiometry of the oxidation reaction was checked by following it spectrophotometrically. Titrations of HbO₂, as well as Hb, with fer-

⁵ The abbreviation used is: HbCPA, normal hemoglobin which has been digested by carboxypeptidase A.

ricyanide were performed at pH 7.0 in 0.2 M phosphate buffer and at pH 9.2 in 2% borate, in both cases at 20°. Typical results on the oxidation of Hb are shown in Fig. 1, in which the change in optical density at $\lambda = 555 \text{ m}\mu$ is plotted against the amount of ferricyanide added. Similar results were obtained on HbO₂. In every case, a sharp end point corresponding to a ratio of 1 ± 0.05 between heme iron and ferricyanide was obtained.

pH Change Accompanying Oxidation in Normal Human Hemoglobin—Fig. 2 shows values of the total pH change accompanying oxidation, i.e. $(\Delta \text{pH})_t = \text{pH}_{\text{Hb}} - \text{pH}_{\text{Hb}^+}$ versus the pH of Hb at 25° and $\Gamma/2 = 0.2$. The core of these results was obtained by a stepwise oxidation of Hb with ferricyanide; in an unbuffered solution this produces a decrease of pH because of the deprotonation

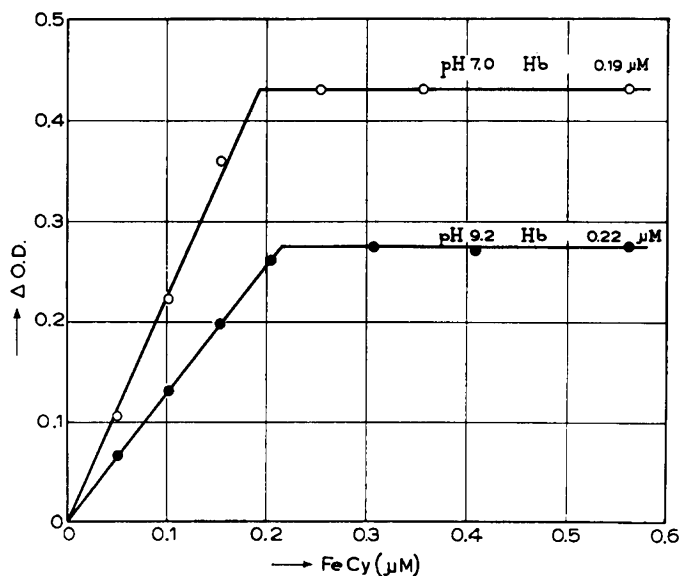


FIG. 1. Oxidation of deoxyhemoglobin (Hb) by ferricyanide (FeCy) at pH 7.0 in 0.2 M phosphate buffer and at pH 9.2 in 2% borate. Temperature was 20°. Ordinates give the change in optical density at $\lambda = 555 \text{ m}\mu$; abscissas give the amount of ferricyanide added in micromoles. The total hemoglobin in each experiment is given in heme equivalents.

of oxidation-linked ionizable groups. In every experiment the end point, as given by the pH measurements, corresponded, within 5%, with the stoichiometric ratio of 1 between heme iron and ferricyanide. In Fig. 3, the measured pH change, ΔpH , is plotted with respect to the amount of ferricyanide added at three pH values. Two main facts emerge: (a) the total pH change, $(\Delta \text{pH})_t$, realized in going from Hb to Hb⁺ is much larger at alkaline than at acid pH; (b) the pH change, ΔpH , realized at any stage in the oxidation process is essentially linear in fer-

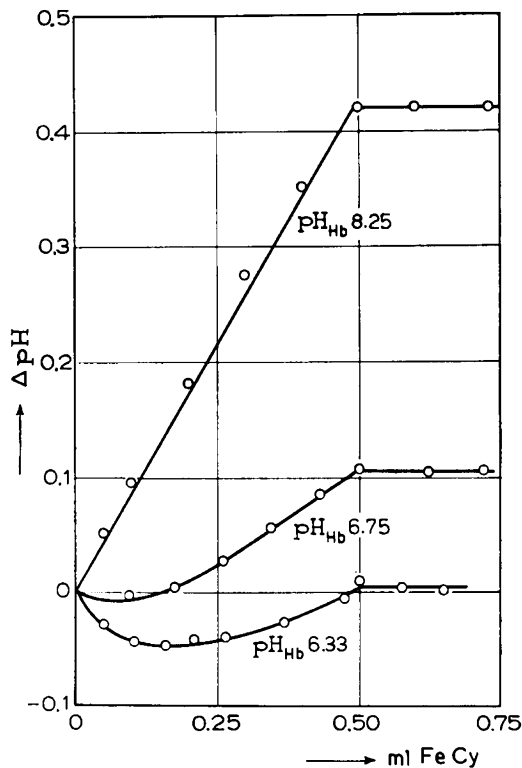


FIG. 3. Values of the pH changes measured during the oxidation of Hb versus the amount of ferricyanide (FeCy) in milliliters added at different pH_{Hb} values.

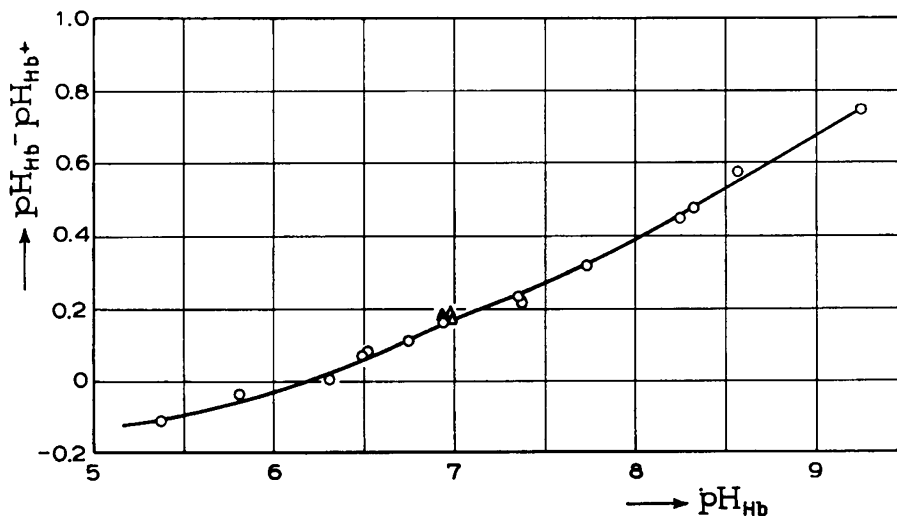


FIG. 2. Values of $(\Delta \text{pH})_t = \text{pH}_{\text{Hb}} - \text{pH}_{\text{Hb}^+}$ versus pH_{Hb} at 25° in 0.2 M NaCl. The notations are as follows: O, results obtained by stepwise oxidation of Hb by ferricyanide; Δ, results obtained in the presence of varying initial amounts of ferrocyanide; ▲, result obtained in the presence of toluylene blue (heme to dye ratio 100:3). Hemoglobin concentration was 15 to 20 mg per ml.

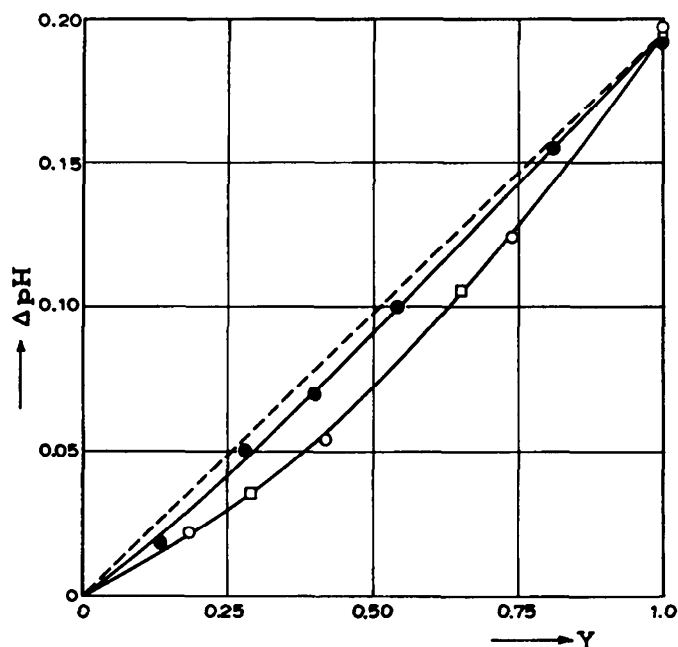


FIG. 4. Values of pH changes measured during the oxidation of Hb by ferricyanide versus the fractional oxidation \bar{Y} in the presence of different initial concentrations of ferrocyanide, namely: □, 0 eq per heme; ○, 1 eq per heme; ●, 4 eq per heme. Initial pH was 6.98. Hemoglobin concentration was 15 mg per ml.

TABLE I

Titration data, in terms of proton bound per heme, for human ferri- and deoxyhemoglobin

Measurements were made in approximately 0.20 M NaCl at 30°; protein concentration was about 0.5%.

pH	$\bar{H}^{+(\text{Hb})}$	$\bar{H}^{+(\text{Hb}^+)}$	$(\Delta\bar{H}^+)_t$
5.4	+3.33	+3.64	+0.31
5.6	+2.79	+3.04	+0.25
5.8	+2.36	+2.50	+0.14
6.0	+1.93	+2.00	+0.07
6.2	+1.48	+1.48	0
6.4	+1.03	+0.90	-0.13
6.6	+0.53	+0.26	-0.27
6.8	-0.01	-0.46	-0.45
7.0	-0.53	-1.19	-0.66
7.2	-1.14	-1.88	-0.74
7.4	-1.72	-2.56	-0.84
7.6	-2.27	-3.24	-0.97
7.8	-2.79	-3.83	-1.04
8.0	-3.28	-4.27	-0.98
8.2	-3.73	-4.65	-0.92
8.4	-4.06	-5.07	-1.01
8.6	-4.34	-5.40	-1.06
8.8	-4.63		
9.0	-4.93		

ricyanide added at pH 8.25, but shows a marked deviation from linearity at lower pH values. At very alkaline pH values a slight downward curvature was noted in some experiments.

Since one of the main objects of this work is to relate the values of the oxidation-reduction potential (E_4) measured in buffered solutions as a function of pH with the results of differential titrations, it is important to keep all incidental conditions as

nearly the same as possible in the two sets of measurements. In view of the fact that the oxidation-reduction potentials were always measured in the presence of electromotively active mediators (9), it was decided to try the effect of such mediators on the differential titrations.

An experiment performed at pH 7 in the presence of toluylene blue (molar ratio of Hb to dye, 100:3) showed that the mediator was without any effect either on the total pH difference between Hb and Hb⁺, or on the relation between ΔpH and fractional oxidation.

Oxidation of heme iron by ferricyanide occurs according to the equation



This formulation implies that during the process stoichiometric amounts of ferrocyanide are formed. Since it is known that ferrocyanide tends to combine very strongly with hemoglobin (13), it seemed important to examine the effect of ferrocyanide on the differential titrations. The results, given in Fig. 4, show that changing the initial concentration of ferrocyanide from 0 eq per heme to 1 eq per heme has no significant effect either on the total pH change (ΔpH), or on the relation between ΔpH and fractional oxidation; however, when the initial ferrocyanide concentration is increased to 4 eq per heme, the shape of the curve of ΔpH versus \bar{Y} does change significantly, although $(\Delta\text{pH})_t$ remains the same.

Analysis of Results—For many purposes, the significant quantity is $\Delta\bar{H}^+$ rather than ΔpH . The transition from one to the other involves a knowledge of the titration curves of hemoglobin at various degrees of oxidation for various values of \bar{Y} . In the present study this transition was achieved by essentially the same procedure as that used in the differential titrations involving oxygenation (8); however, the process was more complicated owing to the nonlinearity of the ΔpH - \bar{Y} relationship. As a first step, the titration curve of Hb⁺ was built up from that of Hb reported in an earlier study (14), together with the values of $(\Delta\text{pH})_t$ shown in Fig. 2. From this and the titration curve of Hb, values of $(\Delta\bar{H}^+)_t$ were read off. These are tabulated as a function of pH in Table I.

In order to obtain titration curves for intermediate values of \bar{Y} , the same procedure was followed, making use of the intermediate values of ΔpH . From these intermediate curves, values of $\Delta\bar{H}^+$ versus \bar{Y} at constant pH were obtained. They are shown in Fig. 5. This figure, which corresponds to Fig. 3, shows in a striking way the complexity of the situation, which is wholly different from that encountered in the case of the oxygen equilibrium. Only at high pH is the liberation of proton proportional to \bar{Y} .

In order to make use of Equation 2 to calculate values of E as a function of pH, it is necessary to know $(\partial\bar{H}^+/\partial\bar{Y})_{\text{pH}}$. This quantity is given, for any pH and any value of \bar{Y} , by the slope of such curves as those shown in Fig. 5. This was read, with aid of drawing table equipment, from each of the curves shown there for values of $\bar{Y} = 0.25, 0.50, \text{ and } 0.75$. The results so obtained, together with the values of $(\Delta\bar{H}^+)_t$ taken from Table I, are plotted against pH in Fig. 6. As follows from Equation 2, the integral

$$\int_{\text{pH}_1}^{\text{pH}_2} (\partial\bar{H}^+/\partial\bar{Y}) d \text{pH} \quad (8)$$

gives the change in E at constant \bar{Y} , to be expected between pH₁

and pH_2 , on the assumption that we may neglect the differential binding of all ions except proton. Similarly, the integral

$$\int_{\text{pH}_1}^{\text{pH}_2} (\Delta \bar{H}^+)_i d \text{pH} \quad (9)$$

gives the expected change in E_m (E median), based on the same assumption. Fig. 7 shows the extent of the agreement between the values of $E_{0.50}$ (or $E_{\frac{1}{2}}$) calculated in this way and the observed values. The agreement between observed and calculated values

of $E_{0.75}$ is equally good; that between observed and calculated values of $E_{0.25}$ is somewhat less so.

From the calculated changes of $E_{0.25}$, $E_{0.50}$, and $E_{0.75}$ with pH , we can of course derive the shape, as well as the position, of the oxidation-reduction curve at any pH , provided we know its shape and position at some *one* pH . Fig. 8 shows results obtained in this way by applying the calculated changes in the E values to the experimental curve measured at pH 7.1. The *smooth curves* and the *small points* are the observed values. The *large points* give the calculated or derived values of E at $\bar{Y} = 0.25, 0.50,$ and 0.75 . It will be seen that the agreement is reasonably good, except for one point, the calculated value of $E_{0.25}$ at pH 6.1. We have not attempted to compare values of E_m as observed and calculated, owing to uncertainties in assigning a precise value to E_m in the case of measurements made by the dye titration method, which gives asymmetrical curves. It is evident, however, from the fact shown in Fig. 6 that the curve for $(\Delta \bar{H}^+)_i$ lies below that for $(\partial \bar{H}^+ / \partial \bar{Y})_{\text{pH}}$ at $\bar{Y} = 0.50$, that the calculated curves cannot be symmetrical over the whole pH range. Since they are approximately symmetrical at high pH , where n is large (9), it can be deduced from Fig. 6 that at more acid pH E_m is less than $E_{0.50}$ and that departures from symmetry will be in the direction actually observed. Beyond this it is not practical to go.

Differential Titrations of Carboxypeptidase A-digested Hemoglobin (HbCPA)—It will be recalled that while for normal human hemoglobin the shape of the oxidation-reduction equilibrium curve changes with pH , for HbCPA the value of n is very near 1 and is insensitive to pH (10). This is clearly shown in Fig. 9, in which titration curves for normal hemoglobin and HbCPA at two pH values (6.4 and 8.3) and 30° are reported together for comparison. In view of this, the question of the proportionality between $\Delta \bar{H}^+$ and \bar{Y} in HbCPA acquires great interest. Results of differential titrations which answer this question are shown in Fig. 10. Unfortunately it is at present impossible to convert the

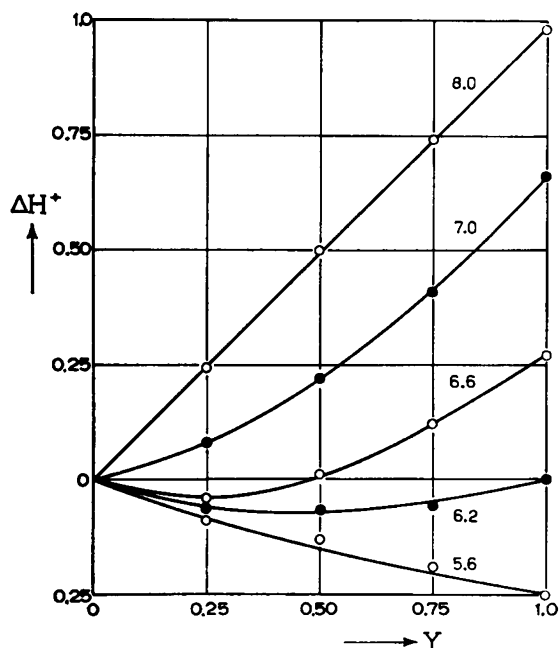


FIG. 5. Values of $\Delta \bar{H}^+$ (i.e. difference in proton bound between Hb and Hb^+) versus fractional oxidation (\bar{Y}) at different pH values.

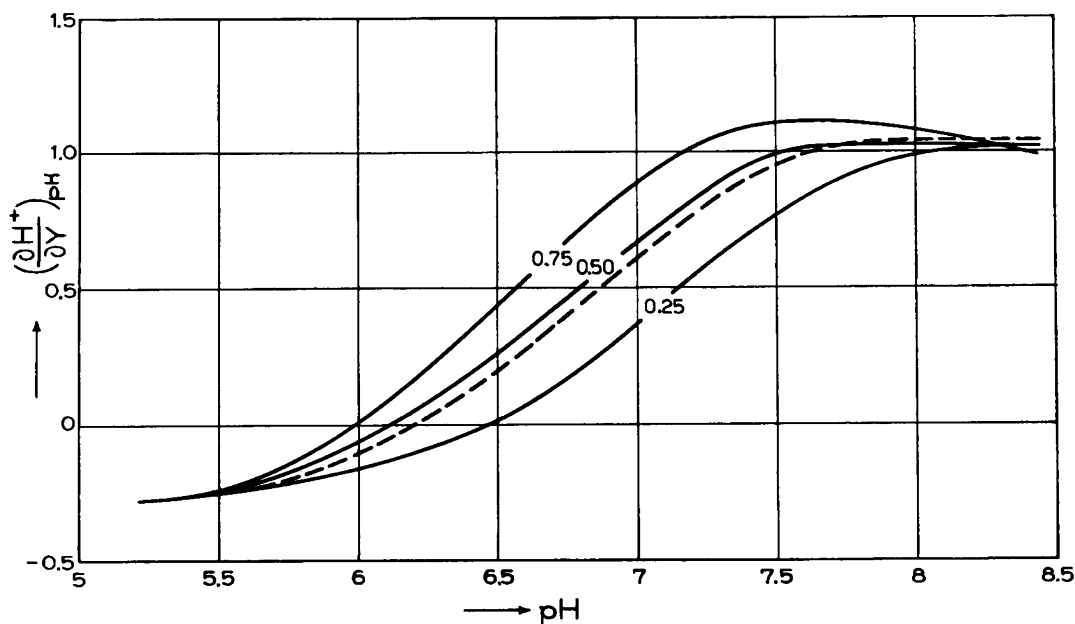


FIG. 6. Difference in proton bound between Hb and Hb^+ as a function of pH . Solid lines give $(\partial \bar{H}^+ / \partial \bar{Y})_{\text{pH}}$ as a function of pH for different values of \bar{Y} , i.e. 0.25, 0.50, and 0.75; dashed line gives the relation between $(\Delta \bar{H}^+)$, and pH as taken from Table I.

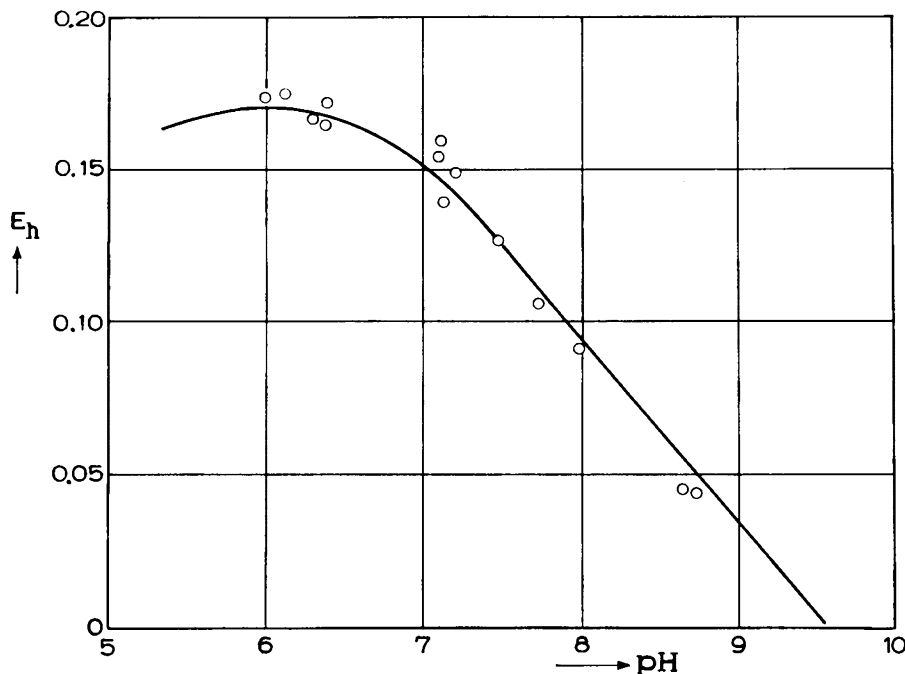


FIG. 7. Oxidation Bohr effect in normal human hemoglobin. Comparison of the directly observed values of $E_{0.50}$ (or E_4) (\circ) given by Antonini *et al.* (9), with values calculated from differential titration measurements (solid line). Ordinates give potential referred to the standard hydrogen electrode.

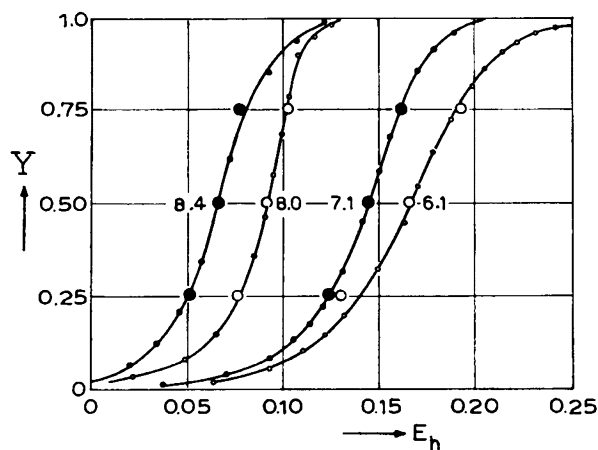


FIG. 8. Comparison of the observed and calculated shape and position of the oxidation-reduction equilibrium curve of normal hemoglobin at several pH values (6.1, 7.1, 8.0, and 8.4). Observed values, from Reference 9, are given by *small points*, through which smooth curves have been drawn; calculated values for $\bar{Y} = 0.25, 0.50,$ and 0.75 are given by *large solid circles* and *open circles*. Ordinates give fractional oxidation; abscissas give potential.

measured pH changes shown in this figure into values of $\Delta\bar{H}^+$ because acid-base titrations of HbCPA are still lacking. However, in the light of previous experience, we know that these two quantities are always very nearly proportional to one another. Consequently, we infer from the observed proportionality between ΔpH and \bar{Y} illustrated in Fig. 10 that $\Delta\bar{H}^+$ is also indeed proportional to \bar{Y} , as would be predicted from the invariance of the oxidation-reduction equilibrium curves. As in the case of the oxygen equilibrium, the total Bohr effect is much smaller than for normal hemoglobin. (See Fig. 3 of Reference 10.)

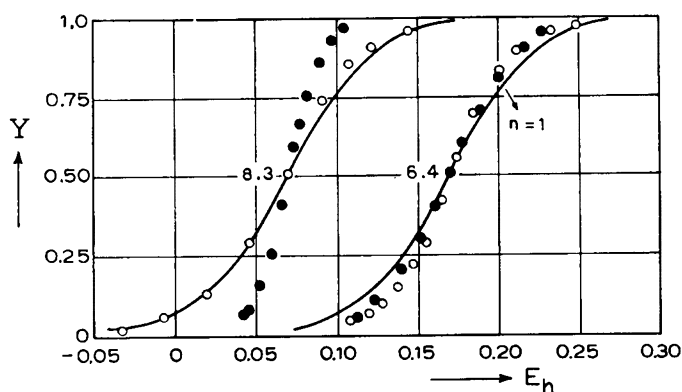


FIG. 9. Oxidation-reduction equilibrium curves for normal hemoglobin (\bullet) and carboxypeptidase A-digested hemoglobin (\circ) in phosphate buffer, $\Gamma/2 = 0.1$, at pH 6.4 and 8.3 and 30° . Hemoglobin concentration was 20 to 30 mg per ml. Solid line is calculated for a value of $n = 1$. Ordinates give fractional oxidation; abscissas give potential.

DISCUSSION

From a phenomenological point of view, the close agreement between the shape and position of the oxidation-reduction equilibrium curves, as directly measured and calculated from differential acid-base titrations, means that we may as a close approximation neglect differences of binding of all ions except proton, as between hemoglobin and ferrihemoglobin. Equation 2 is indeed applicable within the errors of measurements with \bar{X} identified with \bar{H}^+ . It provides adequately not only for the decrease of n with diminishing pH, but also for the increase of asymmetry associated with increasing pH, regardless of what may be the cause of this. It is now known (9) that this asymmetry,

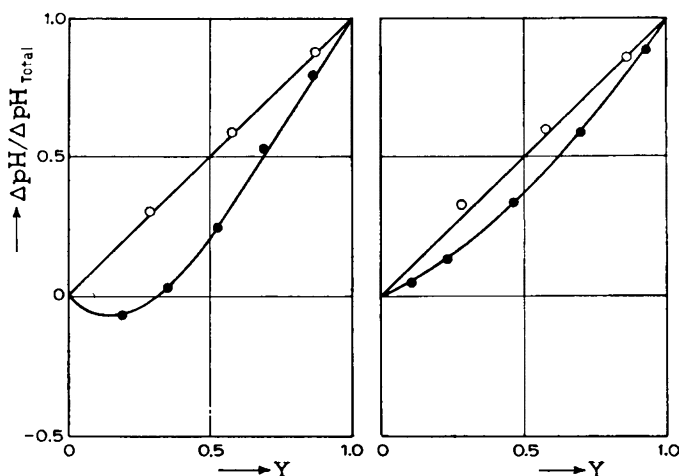


FIG. 10. Values of fractional pH change ($\Delta\text{pH}/\Delta\text{pH}_{\text{total}}$) versus fractional oxidation (\bar{Y}) for normal hemoglobin (●) and carboxypeptidase A-digested hemoglobin (○). Left-hand figure, pH = 6.65; right-hand figure, pH = 7.5.

which was first observed many years ago by Taylor and Hastings in their studies on horse hemoglobin (15), depends on the method used. The present results also establish the existence of a reverse oxidation Bohr effect at acid pH in human hemoglobin similar to that described many years ago by Wyman and Ingalls for horse hemoglobin (6). It is not possible at the present time to specify exactly all the groups responsible for the full oxidation Bohr effect. The alkaline part of it, in the range in which $dE_{0.5}/d\text{pH} = 0.060$, is unquestionably due to the ionization of the water molecule coordinated with the heme iron at position 6 in ferrihemoglobin (16). This is known to have a pK of 8.0 at 30°, and $\Gamma/2 = 0.10$. As to the rest, Wyman and Ingalls pointed out that in horse hemoglobin it could be roughly accounted for by the same set of acid groups subject to the same pK changes as the oxygen Bohr effect, but it now seems that some additional group or groups are required; however, we shall not go into this question here.

By far the most interesting aspect of the present study is the nonlinearity of the relation between proton binding and the degree of oxidation which prevails over most of the pH range, and explains, phenomenologically, the variance of shape of the oxidation-reduction equilibrium curves with pH. Between pH 9 and pH 6 the value of n drops greatly, approaching 1 at low pH. This corresponds to a decrease in the total free energy of interaction, as read from a Hill plot,⁶ from about 1300 cal to about 300 cal (9). At pH 8 or above (where n maintains a nearly constant value close to 2.5), oxidation leads to a liberation of proton which is very closely proportional to the extent of oxidation. This almost certainly represents the direct effect of positive charge developed by the iron atom and the associated change of bond type (17). At lower pH, on the other hand, as is brought out in Fig. 5, the curves become concave upward and finally develop a negative slope throughout the whole or a part of their course. It will be seen that at pH 6.6 or 6.2 the initial stage of the oxidation process results in an uptake of proton, whereas the subsequent stages lead to the expulsion of a proton. At pH 5.6 every stage of the process leads to proton expulsion, although this

⁶ For the definition of a Hill plot and descriptions of its uses, see Reference 5, pages 234–238 and 267–271.

is not linear. This behavior can hardly be explained by local effects involving the heme iron, such as applies at alkaline pH. The most plausible explanation is to suppose that the molecule passes through one or more intermediate stages or conformations as successive hemes are oxidized. At pH 6 the removal of an electron in the early stages of the oxidation process leads to an increase in the work of removing a proton, notwithstanding charge effects involved. The removal of subsequent electrons reverses this effect. At pH 5.6 the removal of an electron at every stage of the process increases the work of removing a proton.

It is a nice question, to which unfortunately no categorical answer can at present be given, whether, at pH 6.2, the protons liberated in the later stages of the oxidation process come from the same sites as those responsible for the initial uptake of protons or from different oxidation-linked sites. In the latter case, the net effect of the oxidation process would be a transfer to proton from one of the sites to another, possibly from one chain to another. Although the α and β chains have generally been assumed to behave alike, it is not at all impossible that they are inherently different in their functional properties and that these differences begin to show up when the stabilizing interactions are reduced or eliminated. It is significant that in certain natural and modified hemoglobins the same effect is observed even when the ligand is oxygen (18–20). The reason why it does not show up in normal hemoglobin A would then be that the α and β chains tend to “fire off” in pairs owing to the very strong stabilizing interactions between them.

SUMMARY

The oxidation Bohr effect has been studied by differential titration both in normal human hemoglobin and in human hemoglobin digested with carboxypeptidase A.

In the case of normal hemoglobin the release of proton ($\Delta\bar{H}^+$) accompanying oxidation is proportional to the fractional oxidation (\bar{Y}) only above pH 8; at more acid pH the release of proton is smaller during the early stages of the oxidation (*i.e.* the curve of ($\Delta\bar{H}^+$) versus \bar{Y} is concave upwards and its slope may even change sign). This is in agreement with the finding that the value of n for the oxidation-reduction equilibrium in normal human hemoglobin changes with pH, being as high as 2.5 at high pH and approaching 1 at pH 6.

On the other hand, in the case of the hemoglobin digested by carboxypeptidase A there is a linear relationship between the fractional $\Delta\bar{H}^+$ and the fractional oxidation \bar{Y} , which is what is expected from the fact that the value of n in equilibrium measurements is equal to 1 over the whole pH range.

These findings have implications regarding the possibly different contributions of the α and β chains to the oxidation Bohr effect.

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