pH Dependence of the Adair Constants of Human Hemoglobin

SUMMARY

In order to solve the problem of an apparent discrepancy between the pH variance of the shape of oxygen equilibrium curve and the linear relation between the number of released Bohr protons and the degree of ligation, precise oxygen equilibrium curves of human hemoglobin were determined at a number of pH values from 6.5 to 8.8. From the equilibrium data individual oxygen equilibrium constants for the four oxygenation steps (Adair constants), \( k_i \) (\( i = 1, 2, 3, 4 \)), were obtained and the number of Bohr protons (\( \Delta H_i^+ \)) released on the \( i \)th stage of oxygenation was estimated. The pH dependence of \( k_i \) was very small, while the other \( k \) strongly depended on pH over the pH range examined. As a consequence, the contribution of each step of oxygen binding to the alkaline Bohr effect was nonuniform: \( \Delta H_1^+ \) was very small as compared with \( \Delta H_2^+ \), \( \Delta H_3^+ \), and \( \Delta H_4^+ \). In spite of this, the calculation has shown that the fractional number of released protons is essentially proportional to fractional oxygen saturation because of cooperative effects in hemoglobin.

Thus, the present study indicates that the linear relationship between the fractional number of released protons and the degree of ligation, as obtained from titration experiments, is not necessarily incompatible with the pH variance of the shape of the oxygen equilibrium curve. The nonuniform pH dependence of the Adair constants implies that the two-state allosteric model of Monod, J., Wyman, J., and Changeux, J. P. (1965) J. Mol. Biol. 12, 88-118 is not adequate to describe the heterotropic effect caused by protons.

The oxygen Bohr effect, i.e., the shift of the oxygen equilibrium curve by pH changes, is one of the characteristic allosteric effects of hemoglobin. For many years it has generally been accepted that the shape of the oxygen equilibrium curve for mammalian hemoglobins, when oxygen saturation is plotted against logarithm of partial oxygen pressure, does not change over a wide pH variation (1-5). The pH invariance of the shape of the oxygen equilibrium curve implied that (a) the four oxygen binding sites of hemoglobin were all equivalent, i.e., the linkages of the four sites to the globin were all the same, (b) the number of released protons was the same for each successive oxygen binding, and (c) the amount of released proton was proportional to oxygen saturation. Results from titration experiments on horse hemoglobin (6) and human hemoglobin (7) were consistent with the three features above.

Recently Tyuma et al. (8) observed that the shape of the oxygen equilibrium curve for human hemoglobin evidently depends upon pH; the maximal slope of the Hill plot significantly decreases for a change from pH 7.4 to 9.1. These results appear to be contradictory to the above features since their data suggest that the amount of released proton is not proportional to oxygen saturation. Therefore, the problem is: to what extent the pH variance of the shape of the oxygen equilibrium curve can be reconciled with the results of the titration experiments. However, these data cannot be compared directly with the titration data since the change from pH 7.4 to 9.1 is too large to calculate the number of released protons.

In the present study, precise oxygen equilibrium curves of human hemoglobin were determined at values from pH 6.5 to 8.8, the Adair constants were obtained as a function of pH, and the number of released protons was estimated for each stage of oxygen binding. The analysis indicates that the pH variance of the shape of the equilibrium curve is not necessarily incompatible with the previous titration data.

EXPERIMENTAL PROCEDURE

Materials and Methods - Human red cells were washed four times with 0.9% sodium chloride and were hemolyzed by adding a 1.5 volume of distilled water and a 0.5 volume of toluene and by shaking vigorously for 5 min. A clear hemoglobin solution was obtained by centrifuging the above mixture (15,000 rpm, 20 min) after adjusting the sodium chloride concentration to 0.9%. The resulting hemoglobin solution was dialyzed overnight against a large volume of 0.05 M Tris-HCl buffer (pH 7.4) which contained 0.1 M sodium chloride. The dialyzed hemoglobin solution was freed of residual organic phosphates by passing through a Sephadex G-25 column (35 mm × 40 cm) equilibrated with the same buffer as used for the dialysis. Then the hemoglobin solution was dialyzed overnight against 0.1 M potassium phosphate buffers of different pH values. All of the above operations were carried out at 4°C. The concentration of the dialyzed hemoglobin solutions was determined by a method of Cooper and Cornwell (9) by use of a spectrophotometer.
was about 2.5 mm on a heme basis and was adjusted to 60 μm by dilution with appropriate phosphate buffers immediately before oxygen equilibrium experiments.

Oxygen Equilibrium Experiments—Oxygen equilibrium curves were determined by the automatic recording method of Imai et al. (9). The spectrophotometer, X-Y recorder, and oxygen electrode employed were a Cary model 118C, a Hewlett-Packard model 7800 AMR, and a Beckman Polarographic Oxygen Sensor (No. 30065), respectively. Experimental conditions were: heme concentration, 60 μm; in 0.1 m potassium phosphate buffer; 20° ± 0.1°. Oxygen saturation was calculated from the absorbance change at 500 nm. Methemoglobin contents of all the samples used were determined from the ratio of absorbance at 577 nm to absorbance at 560 nm. Methemoglobin contents of all the samples used were determined by the automatic recording method of Imai et al. (9). The spectrophotometer, X-Y recorder, and oxygen electrode were determined by the automatic recording method of Imai et al. (9). The spectrophotometer, X-Y recorder, and oxygen electrode were determined by the automatic recording method of Imai et al. (9).

Analysis—In this study, we assume that the α and β chains of hemoglobin are equivalent in function, i.e. oxygen randomly combines with these chains at any stage of oxygenation. The Adair constants, k₁, k₂, k₃, and k₄, were obtained by a least squares curve-fitting procedure on 20 experimental points of the oxygen equilibrium curve, as previously described (10). The median oxygen pressure (11), pₖ, and maximal slope of the Hill plot, nₘₐₓ, were calculated from the Adair constants (10). From the pH dependence of the Adair constants, the number of released Bohr protons was obtained for several pH conditions by using the following relation (6),

\[ \Delta H_{\text{ox}}/d \Delta H_{\text{ox}} = -d \log k_i/d \text{pH} \]

where \( \Delta H_{\text{ox}} \) is the number of Bohr protons released on full oxygenation of hemoglobin and

\[ \Delta H_{\text{ox}} = \Delta H_{\text{ox}} + \Delta H_{\text{ox}} + \Delta H_{\text{ox}} + \Delta H_{\text{ox}} + \Delta H_{\text{ox}} + \Delta H_{\text{ox}} \]

or

\[ \Delta H_{\text{ox}} = 4 d \log (1/p_{\text{ox}})/d \text{pH} \]

since \( 1/p_{\text{ox}} = \sqrt{v} k_{\alpha} k_{\beta} \). The fractional number of Bohr protons released during oxygenation from \( Y = 0 \) to \( Y = Y \), where \( Y \) is fractional saturation, was obtained by two methods. In one method, the fractional number, \( \Delta H_{\text{ox}} \), was calculated using the following equation

\[ \Delta H_{\text{ox}} = \Delta H_{\text{ox}} + \Delta H_{\text{ox}} + \Delta H_{\text{ox}} + \Delta H_{\text{ox}} + \Delta H_{\text{ox}} + \Delta H_{\text{ox}} \]

where \( F_i \) is fractional population of hemoglobin species combining \( i \) oxygen molecules at a given \( Y \). The other method was as follows. From Equation 1 of Ref. 10 it follows that

\[ \left( \frac{2 \ln p}{2 \text{pH}} \right) - \frac{a_i(Y-1) + a_i(Y-1) + a_i(Y-1) + a_i(Y-1) + a_i(Y-1)}{a_i(Y-1) + a_i(Y-1) + a_i(Y-1) + a_i(Y-1) + a_i(Y-1)} \]

(7)

where \( a_i \) is 0.434 d a/d pH. Since (d log p)/d (pH) dY expresses the number of Bohr protons bound to hemoglobin during oxygenation from \( Y \) to \( Y + dY \) (11), \( \Delta H_{\text{ox}} \) is given by

\[ \frac{\Delta H_{\text{ox}}}{\Delta H_{\text{ox}}} = \frac{1}{\Delta H_{\text{ox}}} \left( \frac{Y}{\Delta H_{\text{ox}}} \right) \frac{z \alpha}{dY} \]

where \( Z \) is the right-hand side of Equation 6 and

The least squares estimation of the Adair constants and other computations were made using a YDP-10 digital computer and a Calcomp plotter at the Medical School Computer Center, University of Pennsylvania.

RESULTS

Fig. 2 presents the Hill plots of oxygen binding by hemoglobin at various pH values. It is evident that the shape of the oxygen equilibrium curve is not fully saturated with oxygen even after flushing with pure oxygen. To obtain equilibrium curves of high precision, the saturation point was obtained by extrapolation of the AA versus 1/p plot, where AA and p are absorbance change and oxygen pressure, respectively (see Fig. 1). This procedure was useful to obtain accurate data at the top of curves since calculated oxygen saturation at the top strongly depends on the determination of the saturation point.

The parameters listed in Table I are plotted against pH in Fig. 3. As predicted from Fig. 2, the pH dependence of k₄ is much smaller than that of k₅. The pH dependences of k₂ and k₃ are greater than the pH dependence of k₁. Thus, the present study has shown that the Adair constants depend on pH in a nonuniform manner. This is another expression of the pH variance of the shape of oxygen equilibrium curve. The present result is in qualitative agreement with the earlier observation by Tyuma et al. (8) that in the presence of 2,3-diphosphoglycerate, k₅ is insensitive to a pH change from 7.4 to 9.1, whereas k₁, k₂, and k₃ are markedly increased on the pH change. As seen in Fig. 3, nₘₐₓ is also pH-dependent; it becomes smaller as the pH becomes higher than 7.4. This change of nₘₐₓ is significant, since experience with our automatic oxygenation instrument indicates that the S.E. for nₘₐₓ is smaller than 0.1. We are, however, not confident whether or not the nₘₐₓ significantly depends on pH below 7.4.

**Fig. 1.** Expanded recording of the top of a deoxygenation curve of hemoglobin, pH 7.4, 25°, in 0.1 m phosphate. The deoxygenation was started at p = 650 mm Hg followed by stepwise changes of sensitivity for the oxygen pressure scale. Figures attached to the right end of each curve are full scale readings of oxygen pressure. The upper and lower ends of the curve correspond to approximately 100% saturation and 90% saturation, respectively. Inset, the absorbance change is plotted against reciprocal values of oxygen pressure reading. The saturation point was obtained by extrapolation of this plot.
FIG. 2. Hill plots of oxygen binding by hemoglobin. Y, fractional oxygen saturation of hemoglobin; p, partial oxygen pressure in mm Hg. Heme concentration, 60 μM; 20°C, in 0.1 M phosphate buffer. Points were experimentally obtained. Curves were calculated using estimated values of the Adair constants which are listed in Table I. Some of the data are not presented in this figure in order to avoid overcrowding.

TABLE I

| pH | k₁ | k₂ | k₃ | k₄ | Pₘ | nₓₓₓₓₓ|x
|----|----|----|----|----|-----|------|
| 8.8 | 0.107 | 0.39 | 1.06 | 9.50 | 1.2 | 2.73 |
| 8.4 | 0.0811 | 0.41 | 0.66 | 9.62 | 1.5 | 2.75 |
| 8.0 | 0.0713 | 0.19 | 0.47 | 8.85 | 2.1 | 2.83 |
| 7.65 | 0.0498 | 0.10 | 0.27 | 7.85 | 3.1 | 2.91 |
| 7.4 | 0.0292 | 0.061 | 0.16 | 7.42 | 4.7 | 3.06 |
| 7.2 | 0.0188 | 0.047 | 0.069 | 8.88 | 5.9 | 2.99 |
| 7.0 | 0.0282 | 0.036 | 0.042 | 6.73 | 7.7 | 2.86 |
| 6.8 | 0.0219 | 0.027 | 0.038 | 4.98 | 9.7 | 2.88 |
| 6.7 | 0.0152 | 0.025 | 0.030 | 6.58 | 10.8 | 3.04 |
| 6.5 | 0.0138 | 0.010 | 0.020 | 7.03 | 12.5 | 3.00 |

From the pH-dependence plots for log kᵢ and log (1/Pₘ), the numbers of released Bohr protons were calculated using Equations 1 to 3, and the result is shown in Fig. 4. Small deviations between ΔHᵢ⁺ calculated from Equation 2 and ΔHᵢ⁺ calculated from Equation 3 result from errors in smoothing the pH-dependence plots of log kᵢ and log (1/Pₘ) and in reading slopes of the smoothed curves in Fig. 3, and this deviation is regarded as insignificant. The pH dependence of ΔHᵢ⁺ is in accordance with the earlier result of Antonini et al. which was obtained from oxygen equilibrium data under similar conditions. (See Fig. 7, curve 2 in Ref. 4. Note that ΔHᵢ⁺ is given per heme in that reference.) Fig. 4 clearly shows that the number of released protons is not uniform for each stage of oxygenation. At pH 7.4, for example, contributions of the first, second, and third oxygen bindings to the total proton release are approximately 18, 29, and 50%, respectively. This situation changes at other pH values. Over the pH range covered by the present experiments, the contribution of the fourth oxygen binding is very small, being only 3% of total proton release at pH 7.4. This means that almost all of the Bohr protons are released before the fourth stage of oxygenation.

In Fig. 5, the fractional number of released protons, ΔHᵢ⁺, which was calculated using Equation 7, is plotted as a function
of $Y$. Results obtained by calculation with Equation 4 are not presented in this figure since the calculations with Equation 4 gave exactly the same result. Plots for both pH 7.0 and pH 7.9 are fairly good straight lines, indicating that $\Delta H^+_{f}$ is essentially proportional to $Y$. Maximal deviation of the plots from the diagonal line was 0.8% at pH 7.0 and 2.4% at pH 7.9 in terms of the ordinate scale. Since no data on the linearity of $\Delta H^+_{f}$ versus oxygen saturation have been obtained from direct titration experiments, the present results were compared with the linearity data obtained from titration experiments using carboxyhemoglobin (7). This comparison would be meaningful since the Bohr effect is the same, or very nearly so, for oxygen and carbon monoxide (7). Fig. 5 includes experimental points which were obtained by Antonini et al. (7) under similar conditions. Although their experimental points slightly deviate from the present experiments, the present results were compared with the linearity versus oxygen saturation have been obtained from direct titration experiments on carboxyhemoglobin by Antonini et al. (7), $\Delta$, pH 7.9; 0, pH 7.9.

**DISCUSSION**

The present study has shown that the shape of the oxygen equilibrium curve of hemoglobin depends on pH over a wide range of pH values. This finding is due to the improvement of accuracy for determination of oxygen equilibrium curve particularly at the top and bottom of the curve. In most of the conventional oxygen equilibrium experiments using tonometers and manometers, the range of oxygen saturation covered by experiments has been limited to the middle ranges, in which the Hill plot is approximated by a straight line. It is hardly possible to recognize the pH variance of the shape of the equilibrium curve from those experiments, since the pH variance appears at the extremes of the equilibrium curve. Use of the Hill plot rather than $Y$ versus log $p$ plot is advantageous to distinguish the change of shape of the equilibrium curves since the top and bottom of the curves are expanded in the Hill plot.

The present study rules out the earlier idea of Wyman (2) and Rossi-Fanelli et al. (5) that the four hemes have the same oxygen-linked groups and oxygenation has the same effect on all of them. There is a profound difference among contributions of successive oxygen bindings to the release of the Bohr protons. The point is that even a linear relationship between proton release and ligand saturation, unless strictly linear, necessarily implies neither the pH invariance of the shape of the ligand binding curve nor the equivalence of proton binding sites. In fact, the present study has shown that even a virtually linear $\Delta H^+_{f}$ versus $Y$ plot can be reconciled with oxygen equilibrium data which shows the pH variance of the shape of the equilibrium curve and the nonequivalence of oxygen-linked groups. Thus, the present oxygen equilibrium data are not necessarily incompatible with the previous titration data of Antonini et al. (7). Why is it possible, then, that oxygen equilibrium data showing pH dependence of the shape of the equilibrium curve provide virtually linear relationships between proton release and oxygen saturation? This can be attributed reasonably to the cooperative oxygen binding by hemoglobin, i.e., to instability of the intermediate species which appear during oxygenation. In fact, for example, calculations with the estimated Adair constants indicate that the maximal percentage populations of HbO$_2$, Hb(O$_2$)$_2$, and Hb(O$_2$)$_3$, respectively, are 27, 10, and 8% at pH 7.0 and 21, 10, and 8% at pH 7.9, whereas the corresponding maximal populations for noncooperative hemoglobin are 42, 38, and 42%, respectively. Thus, facilitated successive oxygen bindings make the change of the population of Hb(O$_2$)$_3$ approximately proportional to oxygen saturation, producing a linear relation between proton release and oxygen saturation.

A major part of the alkaline Bohr effect is contributed by oxygen-linked ionization of the imidazole groups of the COOH-terminal histines of the $\beta$ chains and the $\alpha$-amino groups of the $\alpha$ chains (12-14). This is consistent with the present conclusion that the oxygen-linked groups are nonequivalent for each oxygen binding site. Kilmarin et al. (15) obtained pK values for these ionizing groups in both the oxy and deoxy forms and estimated contributions of these groups to the alkaline Bohr effect. At this time, it is impossible to assign these groups to particular stages of oxygenation on the basis of the present data, since the present analysis is too simple in the sense that the four oxygen binding sites are treated as equivalent in function. It is noteworthy, however, that the pH-dependence plots for $\Delta H^+_{f}$, $\Delta H^+_{f}$, and $\Delta H^+_{f}$ in Fig. 4 are similar to calculated pH-dependence plots for valine $1\alpha$, histidine 146$\beta$, and unknown alkaline Bohr groups, respectively, which are presented in Fig. 5 of Ref. 15. If we extend the Adair scheme (Equation 1 in Ref. 10) to a generalized case in which the $\alpha$ and $\beta$ chains are treated as non-equivalent, $k_l$ is equal to $(k_{n} + k_{b})/2$, where $k_{n}$ and $k_{b}$ are microscopic oxygen association constants for the $\alpha$ chains and $\beta$ chains, respectively, combining with the first oxygen molecule. If we assume that the first oxygen molecule combines with one of the $\alpha$ chains in preference to the $\beta$ chains (i.e., $k_n \gg k_b$), as predicted by Perutz (16), $k_l$ is approximated by $k_{n}/2$ and then the pH-dependence plot for $\Delta H^+_{f}$ will represent the contribution by the valine $1\alpha$, as suggested above. Similar consideration for the other oxygenation stages is not easy since the other $k$s are related to the microscopic oxygen association constants in more complicated manners.

As a result of the nonuniform pH dependence of the Adair constants, the mechanism of the cooperative oxygen binding seems to be dependent on pH values. Under acidic conditions,
there is no substantial enhancement of oxygen affinity during
the binding of the first three oxygen molecules, i.e. k₁ to k₃ but,
this is followed by a large increase in the Adair constant, k₄ to k₅
(see Fig. 3). Under alkaline conditions, the oxygen affinity
toward the individual hemes is successively increased. It may
follow that the conformational transition takes place in a con-
certed manner at lower pH values while in a sequential manner
at higher pH values.

The nonuniform pH dependence of the Adair constants throws
doubt on the validity of the two-state allosteric model proposed
by Monod et al. (17). Parameter estimation by the least squares
curve-fitting (10) with the present equilibrium data gave the
following values: $K_R = 8.90 \text{ mm Hg}^{-1}$, $K_T = 0.124 \text{ mm Hg}^{-1}$,
$c = 0.0140$, and $L = 1.5 \times 10^8$ at pH 8.8 and $K_R = 8.38 \text{ mm Hg}^{-1}$,
$K_T = 0.0145 \text{ mm Hg}^{-1}$, $c = 0.00173$, and $L = 1.2 \times 10^6$
at pH 6.5, where $K_R$ and $K_T$ are oxygen association constants
for the $R$ state and $T$ state, respectively, $c$ is the ratio $K_T:K_R$, and $L$ is the allosteric constant. Evidently, the value of $c$
strongly depends on pH as a result of the nonuniform pH de-
pendence of $K_R(\approx k_3)$ and $K_T(\approx k_4)$. This is contradictory to
the allosteric model since by definition $c$ must remain constant
with changes of concentration of the allosteric effector, proton.
Thus, the two-state allosteric model is no more adequate to
describe the heterotropic effect caused by proton than to describethe heterotropic effect caused by 2,3-diphosphoglycerate (10).
As recently pointed out (18), at least three affinity states would
be required to consistently describe both the homotropic and
eheterotropic effects which involve oxygen, proton, and anions.

REFERENCES
1. Ferry, R. M., and Green, A. A. (1929) J. Biol. Chem. 81,
175-203
2. Wyman, J. (1948) Adv. Protein Chem. 4, 493-531
3. Allen, D. W., Guthe, K. F., and Wyman, J. (1950) J. Biol.
Chem. 187, 303-410
4. Antonini, E., Wyman, J., Rossi-Fanelli, A., and Caputo, A.
(1962) J. Biol. Chem. 237, 2773-2777
5. Rossi-Fanelli, A., Antonini, E., and Caputo, A. (1964)
Adv. Protein Chem. 19, 73-222
6. German, B., and Wyman, J. (1967) J. Biol. Chem. 242,
533-550
7. Antonini, E., Wyman, J., Brunori, M., Bucci, E., Fronzi-
celli, C., and Rossi-Fanelli, A. (1969) J. Biol. Chem.
244, 2950-2957
8. Tyma, L., Kamigawara, Y., and Imai, K. (1973) Biochim.
Biophys. Acta 310, 317-320
9. Imai, K., Morimoto, H., Kotani, M., Watari, H., Hirata,
W., and Kurosaka, M. (1970) Biochim. Biophys. Acta 200,
189-196
10. Imai, K. (1973) Biochemistry 12, 708-808
11. Wyman, J. (1964) Adv. Protein Chem. 19, 223-286
12. Perutz, M. F., Muihead, H., Mazzeballa, L., Crowther,
R. A., Green, J., and Kilmartin, J. V. (1969) Nature 222,
1240-1243
13. Kilmartin, J. V., and Rossi-Bernardi, L. (1969) Nature
222, 1243-1246
14. Kilmartin, J. V., and Wootton, J. F. (1970) Nature 222,
766-767
15. Kilmartin, J. V., Breen, J. J., Roberts, G. C. K., and Ho,
G. (1973) Proc. Natl. Acad. Sci. U. S. A. 70, 1240-1249
16. Perutz, M. F. (1970) Nature 228, 720-729
17. Monod, J., Wyman, J., and Changeux, J. P. (1965) J. Mol.
Biol. 12, 58-118
18. Minton, A. P., and Imai, K. (1974) Proc. Natl. Acad. Sci.
U. S. A. 71, 1418-1421

Downloaded from http://www.jbc.org/ by guest on March 24, 2020
PH dependence of the Adair constants of human hemoglobin. Nonuniform contribution of successive oxygen bindings to the alkaline Bohr effect.
K Imai and T Yonetani

J. Biol. Chem. 1975, 250:2227-2231.

Access the most updated version of this article at http://www.jbc.org/content/250/6/2227

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/250/6/2227.full.html#ref-list-1