The Oxygen Equilibrium of Denatured Hemoglobin and the Mechanism of Denaturation[*]

By Masaji KUBO

(Received April 5, 1956)

Hemoglobin is perhaps one of the most interesting compounds known to biochemists. The development of the physico-chemical methods, such as x-ray analysis[1)] and magneto-chemical investigation[2)], has led to a considerable increase of knowledge in regard to the structure of the molecule of this important compound. The problem of the structure could also be tackled with the aid of chemical equilibria involving hemoglobin molecules capable of undergoing a great variety of reactions with other molecules and ions. Since, however, the normal physiological function of hemolobin is the transport of oxygen, the combination thereof with hemoglobin has been studied in the most extensive way. From the analysis of the oxygen equilibrium curve, it has been concluded that the four hemes in a hemoglobin molecule are located at the corners of a square (or a rectangle) and that stabilizing interaction exists between the hemes along the sides of the square[3,4,5)]. The effect of pH on the oxygen equilibrium, i. e., Bohr effect, has clarified the existence of oxygen-linked acid groups[6)].

---

[*] The substance of this paper was presented in a lecture delivered before the annual meeting of the Chemical Society of Japan on April 4, 1956.

1) M.F. Perutz, *Nature*, **143**, 731 (1939); ibid., **149**, 491 (1942). J. Boyes-Watson and M.F. Perutz, ibid., **151**, 714 (1943). J. Boyes-Watson, E. Davidson and M.F. Perutz, *Proc. Roy. Soc. London*, A **191**, 83 (1947).

2) L. Pauling and C.D. Coryell, *Proc. Natl. Acad. Sci. U. S.*, **22**, 159, 210 (1936).

3) L. Pauling, *Proc. Natl. Acad. Sci. U. S.*, **21**, 186 (1936).

4) J. Wyman, *Adv. Protein Chem.*, **4**, 407 (1948).

5) D. Allen, K.F. Guthe and J. Wyman, *J. Biol. Chem.*, **187**, 393 (1950).

6) B. German and J. Wyman, *J. Biol. Chem.*, **117**, 533 (1937).

From the temperature dependence of the oxygen equilibrium⁷⁾, the heat of oxygenation has been evaluated.

The effect of denaturation has attracted little attention⁸⁾, probably because the oxygen equilibrium curve of denatured homoglobin can be less easily interpreted than that of a fresh preparation. Although denaturation is a property common to a large group of proteins, it is doubtful whether a general theory can be worked out which would be universally applicable to all proteins. In view of this point, any theory of denaturation for a particular protein, if the theory is definite and detailed, will lead to a better understanding of this important phenomenon. The present investigations has been undertaken in order to infer the mechanism of denaturation of hemoglobin from its oxygen equilibrium curve.

Experimental Method and Results

Experiments were carried out at the Biological Laboratories of Harvard University. The experimental procedure was essentially the same as that used by Wyman and his collaborators⁵⁾. Hemoglobin solutions were prepared from cow blood and were buffered with a phosphate mixture, pH being equal to 6.8. Slight denaturation took place while the preparations were left to stand in an ice-box for a few days or more. The degree of partial saturation with oxygen was determined at 20°C by the spectrophotometric method described in a previous paper⁹⁾.

The oxygen equilibrium curve depends upon the degree of denaturation. A typical example in the case of slight denaturation is shown in Fig. 1, in which Y, the degree of partial saturation with oxygen, is plotted against $\log p$, the logarithm of the partial pressure of oxygen in equilibrium with the hemoglobin solution. It will be seen that the curve is not exactly symmetric across the center at $Y=1/2$ and $\log p = \log p_0$, where p_0 denotes the partial pressure of oxygen corresponding to 50% saturation. Therefore the data cannot be fitted by any symmetric curve of whatever shape. If a symmetric curve is drawn through the center in accordance with Hill's empirical equation described below, both sets of data lie above the curve for large as well as small values of saturation. This feature of the curve is characteristic of the denatured hemoglobin irrespective of the degree of denaturation. The deviation from symmetry seems to lie within the fringe of experimental errors in the case of a fresh preparation, but increases with increasing degree of denaturation, until it becomes apparent and indubitable in view of the accuracy of measurements.

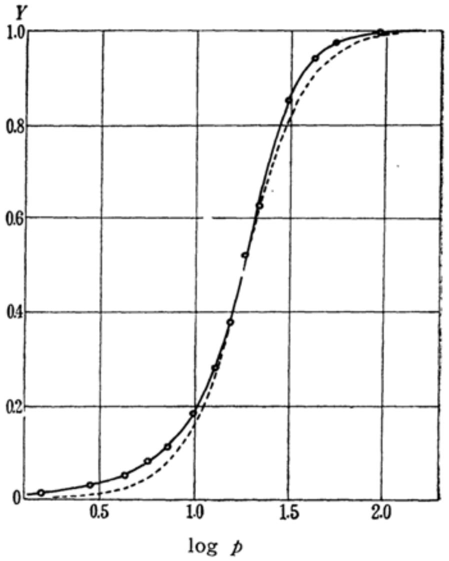

Fig. 1. The degree of partial saturation with oxygen of a slightly denatured cow hemoglobin plotted against the logarithm of the partial pressure of oxygen. The dotted curve depicts the symmetric equilibrium curve of Equation (2), which is applicable to a fresh preparation.

It should be mentioned in this connection that the same trend of asymmetry can also be seen in the oxygen equilibrium curves obtained by Forbes and Roughton¹⁰⁾ on sheep hemoglobin and by Courtice and Douglas¹¹⁾ on human hemoglobin. Both employed a gasometric method. Their data were considered to be most accurate before the advent of the spectrophotometric method, which shortened the time required for measurements and thus minimized the effect of denaturation.

Discussion

The oxygen equilibrium of a fresh preparation of hemoglobin accords, to a fair degree of accuracy, with Hill's empirical equation¹²⁾.

$$Y = Kp^n/(1+Kp^n), \tag{1}$$

where K and n are constants. The present writer¹³⁾ proposed an equation of the following form.

$$Y = \frac{1}{2} + \frac{1}{2}\tanh\left(\frac{n}{2}\ln\frac{p}{p_0}\right). \tag{2}$$

The relation between the two sets of constants is $K = p_0^{-n}$. Eq. (2) is mathematically equivalent to Hill's original equation but expresses the property of symmetry with greater clearness. The hyperbolic tangent curve has

---

7) J. Wyman, *J. Biol. Chem.*, **127**, 581 (1939).

8) A.E. Mirsky and L. Pauling, *Proc. Natl. Acad. Sci. U. S.*, **22**, 439 (1936).

9) M. Kubo, This Bulletin, **26**, 189 (1953).

10) W.H. Forbes and F.J.W. Roughton, *J. Physiol.*, **71**, 229 (1931).

11) F.C. Courtice and C.G. Douglas, *J. Physiol.*, **105**, 345 (1947).

12) A.V. Hill, *Biochem. J.*, **7**, 471 (1913).

13) M. Kubo, *Proc. Japan Acad.*, **29**, 156 (1953).

a center of symmetry, the existence of which has been confirmed very carefully by the experimental data of Wyman et al.[5]. From Eq. (2), the following relation can be derived.

$$\ln[Y/(1-Y)]=n(\ln p-\ln p_0). \qquad (3)$$

Therefore a plot of $\ln[Y/(1-Y)]$ against $\ln p$ yields a straight line, the inclination of which gives the value of n. In the case of denatured hemoglobin a similar plot gives a slightly curved line, which is convex towards the abscissa.

Obviously the experimental points can never be fitted by an empirical equation having two constants. One must resort to the more general expression for Y of the following form first proposed by Adair[14].

$$Y=\frac{L_1p+2L_2p^2+3L_3p^3+4L_4p^4}{4(1+L_1p+L_2p^2+L_3p^3+L_4p^4)}. \qquad (4)$$

The meaning of this equation becomes clear from the following considerations. At any given partial pressure of oxygen, hemoglobin will be present in five different forms distinguished by i, the number of molecules of oxygen combined with each molecule of hemoglobin. The different forms may be referred to by Hb, HbO_2, $Hb(O_2)_2$, $Hb(O)_3$, and $Hb(O_2)_4$, which are present in relative amounts, $1:L_1p:L_2p^2:L_3p^3:L_4p^4$. Obviously L_i is the equilibrium constant of the formation of $Hb(O_2)_i$ from Hb and i molecules of oxygen. Accordingly the equilibrium constant of oxygenation,

$$Hb(O_2)_{i-1}+O_2=Hb(O_2)_i,$$

is given by $K_i=L_i/L_{i-1}$. By definition, $L_0=1$.

Although Eq. (4) is general and theoretically sound, it is difficult to determine the four constants uniquely from a rather smooth curve, because the equation contains too many constants. Let the discussion be opened with a consideration of symmetry, which is realized, at least approximately, even in the case of slightly denatured hemoglobin.

If the relation (4) is plotted in a Y versus $\ln p$ diagram and if the origin of the coordinates is shifted to the center of symmetry at $Y=1/2$ and $\ln p=\ln p_0$ according to the relations,

$$y=Y-1/2,\ x=\ln p-\ln p_0,$$

one has

$$\begin{aligned}y&=\frac{-2-L_1p+L_3p^3+2L_4p^4}{4(1+L_1p+L_2p^2+L_3p^3+L_4p^4)}\\&=\frac{-2-L_1p_0e^x+L_3p_0^3e^{3x}+2L_4p_0^4e^{4x}}{4(1+L_1p_0e^x+L_2p_0^2e^{2x}+L_3p_0^3e^{3x}+L_4p_0^4e^{4x}}\\&=\frac{-2e^{-2x}-L_1p_0e^{-x}+L_3p_0^3e^x+2L_4p_0^4e^{2x}}{4(e^{-2x}+L_1p_0e^{-x}+L_2p_0^2+L_3p_0^3e^x+L_4p_0^4e^{2x})}.\end{aligned} \qquad (5)$$

The condition of symmetry implies that y is an odd function of x.

$$y=\frac{2L_4p_0^4e^{-2x}+L_3p_0^3e^{-x}-L_1p_0e^x-2e^{2x}}{4(L_4p_0^4e^{-2x}+L_3p_0^3e^{-x}+L_2p_0^2+L_1p^0e^x+e^{2x})}. \qquad (6)$$

The right hand sides of Eqs. (5) and (6) are identically equal to each other, irrespective of the value of x. Hence

$$1=L_4p_0^4,\ L_1p_0=L_3p_0^3,$$

or

$$\varDelta\equiv L_3^2-L_1^2L_4=0. \qquad (7)$$

Eq. (7) embodies the necessary and sufficient condition of symmetry of the equilibrium curve[4].

The same relation can be obtained in a different way, which is more instructive. The oxygenation of hemoglobin is analogous to acid-base titration in that the former represents progressive saturation of hemoglobin with oxygen while the latter is nothing but a process in which the negative charges of a base are successively neutralized with protons. The analogy is complete if one considers the reverse process of the dissociation of a polybasic acid, H_2R, and compares it with oxygenation in the following way:

$$R^{--}+2H^+=HR^-+H^+=H_2R,$$
$$Hb+4O_2=HbO_2+3O_2=\cdots=Hb(O_2)_4.$$

Just as pK_1 (or pK_2) of the polybasic acid is the value of pH at which the amounts of H_2R and HR^- (or HR^- and R^{--}) are equal to each other, one may define pO_i** as the value of $\ln p$ at which the amount of $Hb(O_2)_{i-1}$ is equal to that of $Hb(O_2)_i$. Since the equilibrium constant of oxygenation of $Hb(O_2)_{i-1}$ is $K_i=L_i/L_{i-1}$ as mentioned above, it follows that

$$pO_i=-\ln K_i=\ln(L_{i-1}/L_i). \qquad (8)$$

If pO_i increases with increasing i and the four values of pO_i are far apart from each other, the oxygen equilibrium curve would show seven inflection points at each of the pO_i's and also between the neighboring pO_i's. Then it would bear striking resemblance to a titration curve. If the pO_i's approach towards each other or if their order is reversed, a number of inflection points disappear leaving only one, as is actually found in the oxygen equilibrium curve of hemoglobin.

The condition of symmetry of the curve is that the middle point of pO_1 and pO_4 coincides with that of pO_2 and pO_3. In other words, pO_4-pO_3 is equal to pO_2-pO_1. This, in terms of the constants L in Eq. (8), is the following:

---

[14] G.S. Adair, *Proc. Roy. Soc. London*, A 109, 292 (1925); *J. Biol. Chem.*, 63, 529 (1925).

** This p should not be confused with p denoting the partial pressure of oxygen.

$$\frac{L_3}{L_4}\Big/\frac{L_2}{L_3}=\frac{L_1}{L_2}\Big/\frac{1}{L_1}\quad\text{or}\quad L_3^2=L_1^2L_4,\qquad(9)$$

which is identical with the relation (7). It will be seen that the constant L_2 has nothing to do with the symmetry property, although it affects the slope of the curve at the center, or at $Y=1/2$.

If, on the other hand, $L_3^2-L_1^2L_4$ is not equal to zero, the Y-$\ln p$ curve of Eq. (4) loses its symmetry. If the difference is positive, pO_4-pO_3 is greater than pO_2-pO_1 and the whole curve, except the point at $Y=1/2$, lies below the curve of Eq. (2) with the suitable choice of the constant n. Conversely if the difference is negative, the curve of Eq. (4) is above the symmetric curve of Eq. (2), as was actually found for denatured hemoglobin in the present experiment. How can the condition:

$$\varDelta\equiv L_3^2-L_1^2L_4<0\qquad(10)$$

be realized? One must look for the solution of this inequality.

Pauling[3] has given an analysis of the oxygen equilibrium curve obtained by Ferry and Green[15] on horse hemoglobin, assuming the four hemes to interact in pairs as if located at the corners of a square with interactions between adjacent hemes along the sides of the square but with no interactions along diagonals. His formula for the degree of oxygenation is as follows.

$$Y=\frac{kp+(2\alpha+1)k^2p^2+3\alpha^2k^3p^3+\alpha^4k^4p^4}{1+4kp+(4\alpha+2)k^2p^2+4\alpha^2k^3p^3+\alpha^4k^4p^4}.\qquad(11)$$

Here k is the equilibrium constant of oxygenation of a single heme unaffected by the reaction of its neighbors, that is to say, $RT\ln k$ is the decrease in free energy accompanying the combination of a heme with oxygen. Since there are four hemes in a hemoglobin molecule, $K_1=L_1=4k$. The constant α has the following significance. The equilibrium constant of the combination of two oxygen molecules with a hemoglobin molecule would be k^2 provided that no interaction exists between the hemes, but it is αk^2 if the two hemes are adjacent, because the equilibrium constant of the second heme is increased by the factor α as a result of the reaction of the first. In other words, $RT\ln\alpha$ is the additional free energy stabilizing two interacting oxygen-combining centers. The factors 2 and 4 in Eq. (11) result from the symmetry numbers. Pauling's model involves only two constants, and it is remarkable that with a suitable choice of these constants ($k=0.033$ and $\alpha=12$) it may be made to fit the data of the fresh preparation of hemoglobin apparently within the allowable limit of errors of observation.

From the comparison of Eq. (11) with Eq. (4), one has for Pauling's model,

$$L_1=4k,\ L_3=4\alpha^2k^3,\ L_4=\alpha^4k^4.\qquad(12)$$

Inasmuch as $\varDelta=0$, Pauling's formula satisfies the symmetry condition (7). Therefore his formula cannot be applied to denatured hemoglobin. When denaturation takes place, the oxygen equilibrium curve changes its shape. There are two conceivable causes bearing on this matter. One involves the effect of the change of the equilibrium constant k, the other the effect of the interaction constant α, on the form of the oxygen equilibrium curve. Therefore a number of conceivable cases will be subjected to critical examination to see what change in k or α leads to the required condition (10) of asymmetry actually found.

(1) Let it be assumed that k's, the equilibrium constants of the four hemes, are equivalent to each other, while the interactions between different pairs of hemes are not mutually equivalent. In other words, supposing interactions to be represented by distances, Pauling's model may be depicted by a square, while this more general model having different interaction constants, α_1, α_2, α_3, and α_4, between the successive pairs of oxygen-combining centers would be represented by a quadrilateral having unequal sides. A case in which one of the stabilizing interactions of Pauling's model is annihilated would be a special case of this general model. For this model, the equilibrium constants L_i are given by the following equations.

$$L_1=4k,\ L_3=(\alpha_1\alpha_2+\alpha_2\alpha_3+\alpha_3\alpha_4+\alpha_4\alpha_1)k^3,$$
$$L_4=\alpha_1\alpha_2\alpha_3\alpha_4k^4.\qquad(13)$$

Accordingly,

$$\begin{aligned}\varDelta&=[(\alpha_1\alpha_2+\alpha_2\alpha_3+\alpha_3\alpha_4+\alpha_4\alpha_1)^2-16\alpha_1\alpha_2\alpha_3\alpha_4]k^6\\&=[(\alpha_1\alpha_2+\alpha_2\alpha_3-\alpha_3\alpha_4-\alpha_4\alpha_1)^2\\&\qquad+4\alpha_2\alpha_4(\alpha_1-\alpha_3)^2]k^6.\end{aligned}\qquad(14)$$

The right hand side of Eq. (14) is either zero (when $\alpha_1=\alpha_3$ and $\alpha_2=\alpha_4$) or positive (otherwise). In the former case, Y-$\ln p$ curve would be symmetric. In the latter case, although the curve is asymmetric it would lie below the symmetric curve of Eq. (2). In any way, this model does not satisfy the required condition (10). It is worth mentioning that even if interactions are assumed to exist along diagonals, $\varDelta$ always turns out to be either positive or zero.

(2) Having seen that the changes in α do not lead to the required condition provided that k remains unchanged, one must inquire

---

[15] R. M. Ferry and A. A. Green, *J. Biol. Chem.*, 81, 175 (1929).

into the effect of change in k. The next case taken up is that in which each of the k's, the equilibrium constants of the four hemes, is slightly altered by an amount δk_i independently of others, while the interaction constant is tentatively assumed to be unchanged. The equilibrium constants L_i are given by

$$L_1=(k+\delta k_1)+\text{three similar terms},$$

$$L_3=\alpha^2(k+\delta k_1)(k+\delta k_2)(k+\delta k_3)+\text{three similar terms},$$

$$L_4=\alpha^4(k+\delta k_1)(k+\delta k_2)(k+\delta k_3)(k+\delta k_4).$$

Disregarding higher powers in δk, one has

$$\begin{aligned} L_1&=4k(1+\textstyle\sum\delta k_i/4k),\\ L_3&=4\alpha^2k^3(1+3\textstyle\sum\delta k_i/4k),\\ L_4&=\alpha^4k^4(1+\textstyle\sum\delta k_i/k). \end{aligned} \qquad (15)$$

Accordingly $\Delta=0$, which is the condition (7) of symmetry. That is to say, small changes in k do not affect the symmetry property of Pauling's curve in any appreciable manner.

(3) Pauling's model was modified in such a way that the equilibrium constant of one of the four hemes is increased (or decreased) to βk. Then the constants L_i are given by

$$L_1=k(\beta+3),\ L_3=\alpha^2k^3(3\beta+1),\ L_4=\alpha^4k^4\beta. \qquad (16)$$

Therefore

$$\Delta=\alpha^4k^6[(3\beta+1)^2-(\beta+3)^2\beta]=\alpha^4k^6(1-\beta)^3.$$

If $\beta>1$, this expression is negative, satisfying the required condition (10). At first sight, it may appear rather surprising that the oxygen affinity of a heme increases as a result of denaturation. It is, however, a remarkable fact that in almost every case in which hemes are separated, there appears to be an increase of oxygen affinity, i. e., the decrease of p_0. For example, the data of Altschul and Hogness[16] on hemoglobin in dilute solutions, where the molecules may be expected to dissociate, show a decrease in p_0. The same effect is seen in the results of Hill and Wolvekamp[17] on diluted hemoglobin and of Taylor and Hastings[18] on spilt hemoglobin. The latter authors found that p_0 is considerably less for horse hemoglobin split in urea solution than for native hemoglogin.

(4) If two of the four hemes have an increased equilibrium constant βk relative to the remaining two, one has

$$L_1=k(2\beta+2), L_3=\alpha^2k^3(2\beta^2+2\beta), L_4=\alpha^4k^4\beta^2. \qquad (17)$$

Hence

$$\Delta=\alpha^4k^6[(2\beta^2+2\beta)^2-(2\beta+2)^2\beta^2]=0.$$

Again a symmetric Y-$\ln p$ curve would result.

(5) Finally let the case be considered, in which one of the four oxygen-combining centers has lost its ability to combine with oxygen. In this case,

$$L_1=3k,\ L_2=(2\alpha+1)k^2,\ L_3=\alpha^2k^3,\ L_4=0. \qquad (18)$$

Since $L_4=0$, the condition of symmetry of the equilibrium curve must be altered, which in terms of the constants L_i, is

$$\frac{L_2}{L_3}\Big/\frac{L_1}{L_2}=\frac{L_1}{L_2}\Big/\frac{1}{L_1}\ \text{or}\ L_2^3-L_1^3L_3=0, \qquad (19)$$

rather than Eq. (9), and the condition of asymmetry in conformity with experiment is

$$L_2^3-L_1^3L_3<0,$$

in place of Eq. (10). Introducing the values given by (18) into the left hand side of this equation, one is led to

$$\begin{aligned} L_2^3-L_1^3L_3&=[(2\alpha+1)^3-3^3\alpha^2]k^6\\ &=(8\alpha+1)(\alpha-1)^2k^6. \end{aligned}$$

The right hand side of this equation is positive inasmuch as $\alpha>1$ for stabilizing interaction. Here again the required condition is not fulfilled. It is of course possible that the changes of molecules due of denaturation take place only partly, leaving unchanged molecules. However, a mixture of two kinds of such molecules, one giving a symmetric oxygen equilibrium curve, the other an asymmetric one having a trend contrary to experimental observations, would never yield a curve even in qualitative agreement with experiments.

So far a numbers of conceivable cases have been examined for the change of k as well as of α. Although they cover by no means all the possible combinations of changes in k and α, yet relatively simple changes appropriate for the first stage of denaturation seem to have been taken into account. It was found that the condition of asymmetry (10) is so severe that it can rule out almost all except case (3). Therefore one may conclude that the increase in the equilibrium constant of the combination with oxygen of one of the four hemes takes place in the first stage of the denaturation of hemoglobin.

Summary

The oxygen equilibrium curve of a slightly denatured preparation of hemoglobin solution was determined by the spectrophotometric method. The plot of Y, the degree of partial saturation with oxygen, against $\log p$, the logarithm of the partial pressure of oxygen, was not symmetric across the point at 50% saturation. The trend of asymmetry was

---

[16] A. M. Altschul and T. R. Hogness, *J. Biol. Chem.*, **139**, 315 (1939).

[17] R. Hill and H. R. Wolvekamp, *Proc. Roy. Soc. London*, **B107**, 286 (1930).

[18] J. F. Taylor and A. B. Hastings, *J. Biol. Chem.*, **144**, 1 (1942).

such that if the lower left portion of the curve was rotated through 180° about the point at $Y=1/2$, it lay below the upper right portion of the original curve. The condition of this trend of asymmetry was derived from the theory of chemical equilibrium. This condition was tested with various conceivable changes in k, the equilibrium constant of oxygenation of a single heme, as well as in α, the constant of interaction stabilizing the combination of each of two adjacent hemes with an oxygen molecule. It was found that the deviation from the equivalence of α, of whetever kind and degree, can never satisfy the required condition, provided that the k's of the four hemes remain equivalent to each other. A number of other conceivable cases were subjected to critical examination. The condition of asymmetry was so severe that it could rule out almost all except the case in which one of the four hemes increases its affinity towards an oxygen molecule compared with the other three. This conclusion is in conformity with the results on hemoglobin dissociated in a dilute solution or split in a urea solution.

Chemical Department, Faculty of Science
Nagoya University, Nagoya