The Oxygen Equilibrium of Hemerythrin and Bohr Effect

By Masaji KUBO

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The transfer of oxygen from the external environment into the tissues of various animals is accomplished by respiratory pigments capable of combining with oxygen when its partial pressure is high and of giving it up when the pressure is low. There are four different types of the pigments, hemoglobin, chlorocruorin, hemerythrin and hemocyanin. The first three contain iron while hemocyanin is a copper compound. Surely hemoglobin has been studied in the most extensive way.(1) Among the iron-containing respiratory pigments, hemerythrin is interesting in view of the fact that although the common property as an oxygen transporter confers upon all these types of molecules their biological analogy, the chemical properties of hemerythrin are very markedly different from those of hemoglobin and chlorocruorin. Oxygen combines with hemoglobin(2) or chlorocruorin(3) in definite proportion of one molecule per one atom of iron, whereas the gas combines with hemerythrin(4) in the proportion of one molecule per three atoms of iron in the pigment molecule. Besides hemerythrin is not a heme derivative as are hemoglobin and chlorocruorin.(2)(5) The oxygen dissociation curve of hemerythrin from sipunculus under physiological conditions has already been studied by Florkin.(2) The present investigation was undertaken in order to examine more closely the oxygen equilibrium and Bohr effect, which together with magnetic studies to be reported later will reveal some features of interest concerning the function of iron atoms in the molecule.

Preparation of Hemerythrin Solution.—The coelomic fluid of a species of sipunculid worm called phascolosoma from the Marine Biological Laboratory in Woods Hole was centrifuged to throw down the dark red violet corpuscles. The sediment was washed several times with 2.5% sodium chloride solution to remove the plasma completely. It was made up to 60% of the original volume with distilled water and left to stand in an icebox for about two hours to lake the corpuscles. The stroma was removed by centrifuging and the dark red violet solution after filtration through a hard filter paper was ready for use. The solution contained a minute amount of sodium chloride originating from the washing solution. This does not affect the results at all. The exact control of the concentration was unnecessary in the present experiment. Still the difference between the optical densities of reduced and oxygenated solutions gives an accurate relative concentration for each solution. The simultaneous determination of optical density difference and iron content for one solution to be reported in the following paper can convert the relative value to the absolute concentration in gram atoms of iron per cc.

Apparatus.—The apparatus used was essentially the same as that used in the earlier experiments(1) on hemoglobin. The spectrophotometric method was employed for the determination of the degree of oxygenation of hemerythrin. As a constant light source a 6 V., 30 W. microscope lamp was used. It was run from two heavy duty accumulators connected in parallel. The light was filtered through an interferance filter backed by a blue masking filter to get monochromatic light of wavelength 4760 A. This wavelength was chosen as satisfying the basic requirement that it should be readily transmitted by both reduced and oxygenated hemerythrin solution, although much more readily by the former than the latter. The monochromatic light was then passed through an optical cell having a path length of 2.5 mm. for the light through the solution. Only 1 cc. of solution was enough to fill the cell. The cell was really a part of a tonometer made of glass, which could be evacuated and sealed by a rubber vaccine cap. The total volume enclosed in the vessel amounted to 292.5 cc. The light transmitted by the cell fell on a photomultiplier tube RCA 931. The current thus produced was passed through a suitably chosen high grade resistor and the resulting voltage drop was measured by means of a potentiometer with a Millivac vacuum tube millivoltmeter as a detector of the balancing point.

Experimental Procedure

The tonometer containing the oxygenated

(1) D. W. Allen, K. F. Guthe and J. Wyman, *J. Biol. Chem.*, **187**, 393 (1950).
(2) R. A. Peters, *J. Physiol.*, **44**, 131 (1912).
(3) H. M. Fox, *Proc. Roy. Soc. London*, B **115**, 368 (1934).
(4) M. Florkin, *Arch. intern. physiol.*, **36**, 247 (1933).
(5) R. Kobert, *Arch. ges. Physiol.*, **98**, 411 (1903) : G. F. Marrian, *Brit. J. Exptl. Biol.*, **4**, 357 (1927).

hemerythrin solution was evacuated and equilibrated three times with the atmosphere of 99.9% pure commercial nitrogen saturated with water, until the dark red violet solution changed into a pale milky white, almost colorless solution of reduced hemerythrin. The measurement of optical density of the medium was made putting the tonometer in the optical system. Then a desired amount of air was introduced into the tonometer through the vaccine cap by means of a syringe and hypodermic needle, calibrated as to the volume of delivery. The resulting oxygen pressure in the tonometer could be calculated from the volume of air introduced, that of the gas phase in the tonometer and the known composition of air, with due correction for the humidity of air. The correction was also made for the amount of oxygen taken up by the solution. It could be calculated from the volume of the solution, the concentration in gram atoms of iron per cc., and the percentage saturation of the hemerythrin with oxygen as given by the photometric measurements to be described in the following paragraph. This correction was usually small, amounting to less than a few per cent of the calculated pressure of oxygen. After the measurement of optical density of the solution at 20°C. for twelve different oxygen pressure was over, the last measurement was made on essentially 100 per cent oxygenated hemerythrin obtained by exposing the solution to atomospheric air. Because hemerythrin has high oxygen affinity, it was unneccessary to use pure oxygen at this point. The degree of saturation Y with oxygen of the hemerythrin solution was calculated as

$$Y=\frac{D-D_{\mathrm{red}}}{D_{\mathrm{ox}}-D_{\mathrm{red}}} \qquad (1)$$

where D's denote optical densities of the solution in equilibrium with oxygen of partial pressure p, completely deoxygenated and oxygenated solutions respectively. Since we were interested only in the difference of optical densities, it was convenient to use a neutral filter made of a piece of photographic film of appropriate darkness to reduce the value of I_0, the intensity of incident light, so as to make it match that of I, the intensity of light emerging from the cell. It should be noted that no knowledge of the extinction coefficients and the exact concentration was required for the calculation of Y values.

The prime requirement in this kind of experiment was accuracy as well as speed, since proteins are known to be subject to denaturation especially in solutions of pH values far apart from that of physiological condition. The agreement of individual data of I/I_0 was within 1%. The difference between the optical densities of reduced and oxygenated solutions amounted to about 1.3. Hence the probable error in the degree of saturation is estimated to be less than 1%. The crucial point seemed to lie not in physico-chemical measurement but rather in the biochemical problem of how to prepare definite specimens and how to minimize the effect of denaturation. The whole physico-chemical measurement comprizing twelve different values of oxygen pressure could be carried out in six hours. Thus a fresh sample could always be prepared on the day of measurement and used.

Results and Discussion

The first two experiments were made on unbuffered hemerythrin solutions of concentration 5.16 and 6.31×10^{-6} gram atoms of iron per cc. respectively. The pH value of the solution in oxygenated state as measured by a glass electrode was 6.25 pretty close to the isoelectric point 5.85.[6] The unbuffered solution was fairly stable and a solution left overnight in an icebox gave results practically identical with those of a fresher sample.

The degree of partial saturation Y with oxygen is plotted as a function of $\log p$, the logarithm of oxygen pressure in mm. Hg., in

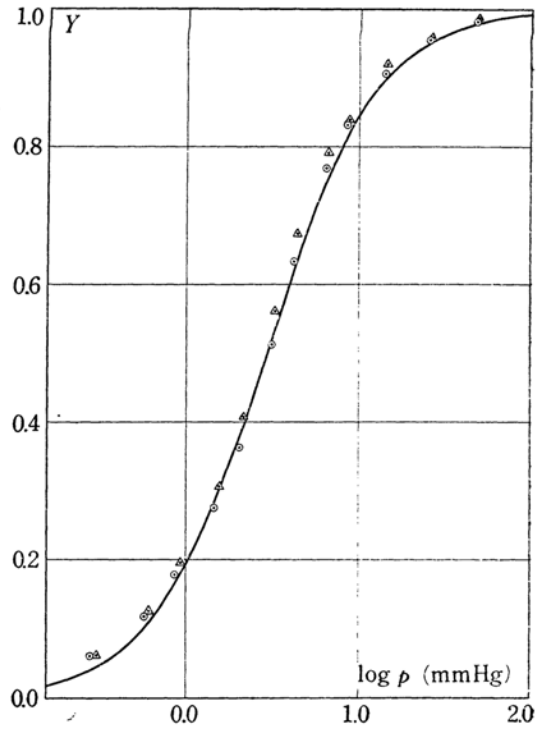

Fig. 1.—The degree of saturation with oxygen of hemerythrin as a function of the logarithm of the partial pressure of oxygen. 20°C., unbuffered, pH = 6.25. The circular points represent data on the solution of iron concentration 5.16×10^{-6} g. atom/cc., and the triangular points those of 6.31×10^{-6}. The full line depicts the empirical formula (2).

(5) J. Roche, *Ann. Rev. Biochem.*, **5**, 463 (1936).

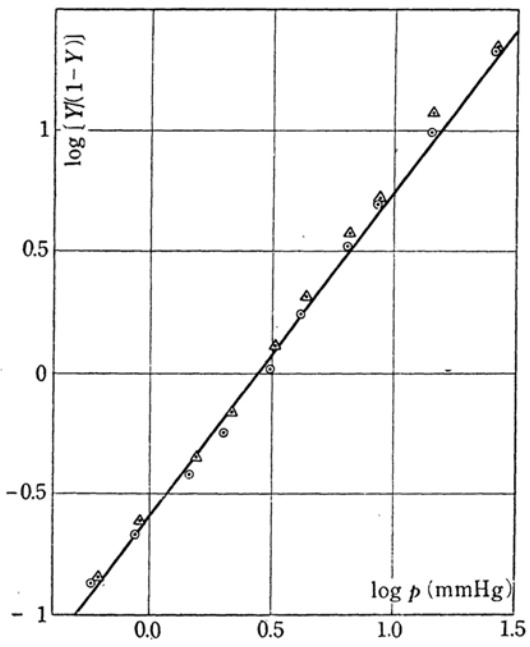

Fig. 2.—The dependence of $\log[y/(1-y)]$ upon $\log p$.

Fig. 1. The functional relation between Y and $\log p$ can be represented by the following empirical formula.

$$Y=\frac{1}{2}+\frac{1}{2}\tanh\left(2.303\frac{n}{2}\log\frac{p}{p_{1/2}}\right) \quad (2)$$

with $n=1.37\pm.05$ and $\log p_{1/2}=0.46\pm.02$. This is mathematically equivalent to the statement that $\log[Y/(1-Y)]$ is a linear function of $\log p$,

$$\log\frac{Y}{1-Y}=n(\log p-\log p_{1/2}) \quad (3)$$

as shown in Fig. 2. In fact the values of n and $\log p_{1/2}$ mentioned above were obtained in this way. From the equation (2) it is clear that $p_{1/2}$ is nothing but the partial pressure of oxygen in equilibrium with the solution in which the amount of oxygenated hemerythrin is just the same as that of the reduced form. The interpretation of the physical meaning of n in terms of the theory of linked groups is given in a paper by Wyman.[7] It will be found that the equation (3) can be written in an alternative way as follows.

$$\frac{Y}{1-Y}=\left(\frac{p}{p_{1/2}}\right)^n=Kp^n \quad (4)$$

or Hill's empirical equation:

$$Y=\frac{Kp^n}{1+Kp^n} \quad (5)$$

The constant K in the equation (4) may be interpreted, formally at least, as the equilibrium constant for the hypothetical reaction:

$$M+nX=MX_n \quad (6)$$

$n=1$ would mean that all the oxygen attaching centers in the molecule are mutually independent, whereas $n=2$ that two centers are linked together so that an oxygen molecule attached to one center induces another oxygen molecule to combine with the molecule at once. The value $n=1.37$ in this case suggests that the oxygen combining centers in hemerythrin molecule are not completely independent, but the stabilizing interaction is weak, weaker at least than in the case of hemoglobin, in which case n was found to be 2.8.[7] This conclusion will be important in the interpretation of magnetic susceptibility of hemerythrin.

$\log p_{1/2}=0.46$ gives $p_{1/2}=2.9$ mm. Hg. Florkin[8] studied the oxygen dissociation curve of hemerythrin from sipunculus, and found for $p_{1/2}$ the value of 8 mm. Hg. at 19°C. It is doubtful that the disagreement is really significant in view of the fact that these samples were extracted from different animals and that the conditions were slightly different.

Finally the curve of Fig. 1 has a very slight but unmistakable asymmetry which cannot be ascribed to experimental errors. Wyman and others[1] found that the similar asymmetry in the case of hemoglobin disappeared for a freshly prepared material. In the present case even freshly prepared solution gave slight asymmetry of the curve and a material left to stand overnight revealed no sign of increased asymmetry.

Bohr Effect.—Further studies were made on the effect of pH on the oxygen equilibrium of hemerythrin. The measurements were made on solutions at 20°C. prepared in the same way as before, buffered with mixtures given in Table 1 chosen to get desired pH values, which were actually measured by a glass electrode. The oxygen equilibrium curves obtained were very similar to that of the unbuffered solution, except the case for pH=4.4, in which case though the curve was symmetrical it was less steep. The values of n and $\log p_{1/2}$ are summarized in Table 2. In the case of pH=4.4, slight denaturation was

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

(8) M. Florkin, *C. R. Acad. Sci.*, **195**, 832 (1932).

Table 1

Concentration of Hemerythrin (g. atom Fe/cc.)	Buffer Solution	Buffer Concentration	*p*H Value
4.95×10^{-6}	Na_2HPO_4-citric acid	0.035 M	4.4
5.16, 6.31	Unbuffered	—	6.25
4.10	K_2HPO_4-KH_2PO_4	0.095	6.8
5.55	Borax-boric acid	0.022	8.8

Table 2

*p*H	*n*	$\log p_{1/2}$
4.4	1.11±.05	0.57±.02
6.26	1.37±.05	0.45±.02
6.8	1.35±.05	0.44±.02
8.8	1.27±.05	0.46±.02

clearly perceptible by the appearance of fine pale yellow suspensions, and at *p*H=2.7 or 10.0 denaturation was so rapid that measurement could not be made at all. If progressive denaturation takes place, the decrease of concentration decreases D_{ox} in the equation (1) and results in the overestimation of Y in the lower left branch of the curve in Fig. 1, while in the upper right branch the simultaneous decrease of D and D_{ox} gives rise to the underestimation of Y, with the consequent flattening of the sigmoid curve. Taking this into account, we can conclude that within the *p*H range of stable hemerythrin solution there is no dependence of oxygen equilibrium upon *p*H, i.e., Bohr effect is absent. So that no oxygen-linked acid groups are present in hemerythrin molecule, unlike the case of hemoglobin.

Summary

The oxygen equilibrium of hemerythrin and its Bohr effect were studied by spectrophotometric method. It was found that the oxygen combining centers in this molecule are not completely independent but the stabilizing effect is weak. The value of n in Hill's equation is equal to 1.37. The partial pressure of oxygen in equilibrium with the solution of 50% saturated hemerythrin is 2.9 mm. Hg. Within the *p*H range of stable hemerythrin solution, there is no dependence of oxygen equilibrium upon *p*H, hence no oxygen-linked acid groups are present in hemerythrin molecule.

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Chemical Department, Faculty of Science, Nagoya University, Nagoya