Polarographic Studies on Oxygen Transport by Human Hemoglobin in Buffer Solutions

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Oxygen transport by human hemoglobin (Hb) to an electrode surface with hypo-oxygen partial pressure has been studied by the polarographic technique. The oxygen reduction current in Hb solution open to the atmosphere increased linearly with Hb concentration up to *ca.* 2 mM (1 M=1 mol dm^{-3}) heme. The current was ascribed to free and Hb bound oxygen. The increase in current with Hb concentration was attributed to the increasing oxygen supply to the electrode from oxygen bound Hb (oxyhemoglobin;oxyHb). Changes in temperature and pH largely affected the current, corresponding to the change in dissociation rate of oxygen from oxyHb. The relation between the current due to oxyHb and the apparent first order rate constant of the dissociation (k) measured by the stopped-flow method implies two different rate-determining steps of oxygen transport by Hb. When k exceeded *ca.* 60 s^{-1}, the limiting step altered from the dissociation of oxygen to the diffusion of Hb molecules. It is concluded that the oxygen transport by Hb on physiological conditions is controlled by the diffusion of Hb. The diffusion coefficient of Hb in 20 mM heme as found in erythrocytes was estimated to be 5.8×10^{-8} cm^2 s^{-1} at 25 °C and pH 7.4.

For better understanding of the oxygen transporting capacity of erythrocytes, it is important to clarify rate-determining factors of uptake or release of oxygen by erythrocytes. Hartridge and Roughton[1)] reported that the observed half-time of the oxygen uptake by erythrocytes was 40 times greater than that by free hemoglobin (Hb) in a rapid mixing continuous flow apparatus. Since then, investigators[2—4)] have suggested that the behavior of oxygen in Hb solution, particularly in concentrated solutions corresponding to those in erythrocytes, should be investigated. The mechanisms of the association of oxygen with Hb and also the dissociation, were extensively studied by static or kinetic methods.[5—8)] However, few quantitative studies have been carried out on the oxygen transport by Hb, further by erythrocytes to the region with hypo-oxygen partial pressure, *viz.* the transport phenomena involving diffusion process. Spectrophotometric methods which have been used for studying various problems of oxygen binding to Hb, do not seem very effective for the transport phenomena.

Scholander,[9)] and Wittenberg[10—12)] proposed the idea of "facilitated oxygen diffusion" for describing the oxygen transport by Hb in solution. They measured the flux of oxygen passing through a millipore filter holding Hb by gas-liquid chromatography and suggested that the rate-determining factor is the translational diffusion of Hb molecules. Wyman[13)] gave the theoretical explanation for the facilitated diffusion. However, the conditions for the clear demonstration of the diffusion-controlled oxygen transport have not so far been established.

We tried an electrochemical method for covering the shortcomings of the previous investigations. In our method, an electrode, to which an appropriate potential is applied, works as a model of biological surface on which oxygen is consumed immediately on its arrival, that is, the electrode generates a region with hypo-oxygen partial pressure. The diffusion of oxyhemoglobin (oxyHb) to the electrode surface may result in the dissociation of oxygen from oxyHb and the electrochemical reduction of the oxygen. The oxygen reduction current due to oxyHb (I_{Hb}) corresponds to the rate of oxygen transport by Hb to the electrode surface. Thus, by analyzing the current, it is possible to clarify the rate-determining factor of oxygen transport in Hb solution. The electrochemical method is also applicable to a relatively concentrated solution corresponding to that in erythrocytes, since the result obtained by the electrochemical method is not restricted by the concentration of Hb in the system. The optical measurements can not be applied to a solution of high concentration of Hb, without any modification of the conventional method.

In this study, we have applied the d.c.-polarographic method with a dropping mercury electrode to Hb solution. The rate-determining steps of oxygen transport to the electrode surface by Hb are discussed as regards the behavior of the reduction current of oxygen in Hb solution.

Experimental

Materials. Human Hb was prepared from fresh blood of donors. Erythrocytes were washed four times with 0.9% sodium chloride and hemolyzed by addition of an equal volume of distilled water and 0.4 volume of toluene and by vigorous shaking for 3 min. A clear Hb solution was obtained by centrifuging the mixture at 12000 rpm for 10 min. The resulting Hb solution was dialyzed for 20 h with renewal of a large volume of distilled water several times followed by centrifuging at 12000 rpm for 30 min. All the operations were carried out at 4 °C. The Hb concentration was measured with a Hitachi 323 recording spectrophotometer in term of the absorbance of oxyHb at 576 nm. Concentrated Hb solutions were obtained by centrifugal filtration through Amicon CF 25 membrane cones (Amicon Co., Lexington Mass.). Methemoglobin (metHb) was prepared by adding a slight excess of potassium ferricyanide to the dialyzed oxyHb and was again dialyzed for 20 h with renewal of a large volume of distilled water several times. Experiments were performed within a week from the completion of preparation. Reagents of analytical grade were used.

Polarographic Measurements. Polarograms were recorded with a Yanagimoto Polarograph Model PA 101. The dropping mercury electrode had the following characteristics: $m = 1.91$ mg s^{-1}, $t = 5.87$ s at open circuit in distilled water with 60 cm height of mercury reservoir. Saturated calomel electrode (SCE) was used as a reference electrode. All measurements were carried out in test solutions thermostated and under the oxygen partial pressure equilibrated with open atmosphere.

Kinetic Measurements. Over-all first order rate constants of oxygen dissociation from oxyHb were measured with a Yanagimoto SPU-1S stopped-flow spectrophotometer with a memoriscope. The light path of the cell was 10 mm. The dissociation of oxygen from oxyHb was forced by mixing Hb solution equilibrated with atmospheric oxygen with a sodium dithionite solution. The time course was followed by measuring absorbance at 576 nm. In most cases, the concentrations of Hb and dithionite after mixing were 50 μM (1 M = 1 mol dm^{-3}) heme and 30 mM, respectively. The dithionite solution was freshly prepared for each run of experiments.

Results and Discussion

Oxygen Reduction Current in Hb Solution. Oxygen gives two reduction waves at dropping mercury electrode in supporting electrolyte solutions, corresponding to the following equations.[14)]

$$O_2 + 2e + 2H^+ \longrightarrow H_2O_2 \qquad (1)$$

$$H_2O_2 + 2e + 2H^+ \longrightarrow 2H_2O \qquad (2)$$

The reduction current increased largely with addition of the Hb in electrolyte solution open to the atmosphere (Fig. 1). The increase of the current gives rise to a wave newly appearing between the 1st and 2nd waves. This may be due to the catalytic reduction of O_2 to H_2O involving the dismutation of H_2O_2 to O_2 and H_2O by some catalase or peroxidase action of Hb itself or a trace amount of a contaminant having a catalytic activity,[15,16)] at this potential, at which only the reduction (1) is expected electrochemically. The catalytic activities can not alter the value of the total reduction current of O_2 to H_2O (at -1.45 to -1.50 V *vs.* SCE), unless the total oxygen supply to the electrode changes. On addition of metHb which has no oxygen binding capacity, the total reduction current of oxygen was practically constant, though a catalytic phenomenon similar to that of Hb was observed. Thus, the increase of the total current (I) can be attributed to the reduction of oxygen transported by Hb, since the concentration of free oxygen in Hb solution should be constant, equilibrated with atmospheric oxygen. Considering the oxygen transport by Hb to the electrode with the above observations, we can utilize the potential at which the 2nd wave reaches its limiting current, *i.e.* from -1.45 to -1.50 V *vs.* SCE. The following description deals with the 4 electron transfer current of oxygen.

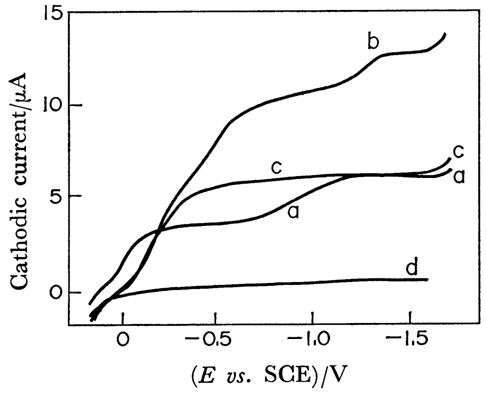

Fig. 1. Polarograms of oxygen reduction waves in electrolyte solutions open to the atmosphere.
a: Phosphate buffer (0.1 M, pH 7.4), b: a+Hb (2.3 mM heme), c: a+metHb (2.0 mM heme), d: b without oxygen. 25 °C.

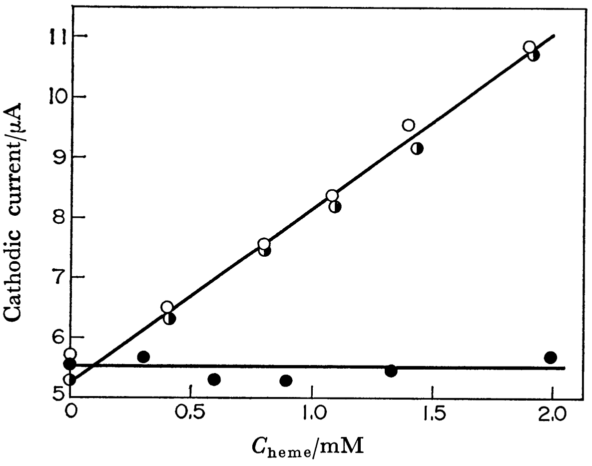

Fig. 2. Dependence of the reduction current of oxygen on the concentration of Hb without removing organic phosphates (○), stripped Hb (◐) and metHb (●) in phosphate buffer (0.1 M, pH 7.4) open to the atmosphere at 25 °C.

The dependence of I on the concentration of Hb is shown in Fig. 2. I is seen to increase linearly with Hb concentration. No appreciable difference between Hb prepared for the present study and stripped Hb prepared according to Benesch *et al.*,[17)] could be observed. Thus, the subsequent experiments were carried out without removing organic phosphates from the Hb preparation. Since the current in metHb solution was almost constant, the increase of I in Hb solution might be due to the increasing supply of oxygen to the electrode by Hb. It seems that a flux of oxygen in Hb solution consists of the contributions from both free and Hb bound oxygen.[9–13)] The current I might consist of the reduction currents of both:

$$I = I_{O_2} + I_{Hb}, \qquad (3)$$

where I_{O_2} is diffusion current of free oxygen, and I_{Hb} the oxygen reduction current due to oxyHb. From Ilkovic equation,[18,19)] we have

$$I_{O_2} = \kappa D_{O_2}^{1/2} C_{O_2}, \qquad (4)$$

where $\kappa = 607$ nm$^{2/3}$ $t^{1/6}$ with $n = 4$, D_{O_2} and C_{O_2} being the diffusion coefficient and the concentration of free oxygen in the solution equilibrated with the atmospheric oxygen, respectively. In relatively dilute Hb solution, D_{O_2} and I_{O_2} might be nearly equal to those in the medium without Hb ($*D_{O_2}$ and $*I_{O_2}$, respectively). They can be regarded as constant under given conditions. The increase of I is due to only the increase of I_{Hb}, being directly proportional to the concentration of Hb presented in heme (C_{heme}). Thus, I_{Hb} can be written as

$$I_{\mathrm{Hb}} = \alpha C_{\mathrm{heme}} \mathrm{Y}, \qquad (5)$$

where Y is fractional oxygen saturation of Hb. Under the present conditions we might put $\mathrm{Y}=1$, since Hb solutions are equilibrated with the atmospheric oxygen partial pressure. α is a factor characterized by the rate-determining step of oxygen transport by Hb.

We can postulate two steps for α, (a) the diffusion process of Hb molecules bound oxygen to the electrode and (b) the oxygen dissociation from oxyHb in the vicinity of the electrode with hypo-oxygen partial pressure. When (a) is predominant, α might be proportional to the diffusion coefficient of Hb (D_{Hb}) and I_{Hb} might be the diffusion-controlled current. In (b) where the rate constant of oxygen dissociation (k) is included in α, I_{Hb} might be the kinetic current.[20,21] It seems that the alteration of the rate-determining step is practically caused by change in the rate of dissociation, since the D_{Hb} value does not change a great deal with temperature of pH, as compared with the dissociation step.

For the sake of confirmation we examined the effect of temperature and pH on I_{Hb}, since the oxygen dissociation rate is strongly dependent on the above two factors,[22,23] in contrast to the rate of oxygen combination with Hb which is little affected.[22]

Effects of Temperature and pH on I_{Hb}. Figure 3 shows the linear relationship between I_{Hb} (Eq. 3) and C_{heme} at various temperatures open to the atmospheric oxygen. The slope of the curve, $I_{\mathrm{Hb}}/C_{\mathrm{heme}}$ *i.e.* α (Eq. 5) increases with temperature. The plot of α (slope of the curve, Fig. 3) against temperature indicates that α depends on the oxygen dissociation rate which changes with temperature (Fig. 4-a). The relation between the reciprocal temperature and logarithms of α obtained is shown in Fig. 4-b. When the rate-determining step of I_{Hb} is single, a straight line should be obtained. However, a bending curve with two different slopes was obtained indicating that I_{Hb} is controlled by at least two different steps. The apparent activation energy of each step was found to be 83.7 and 29.7 $\mathrm{KJ\,mol^{-1}}$ for the lower and higher temperature ranges, respectively. Since the value of the former is in line with that of oxygen dissociation reported by Gibson[23] and Dalziel and O'Brien,[24] and our Arrhenius plot of stopped-flow data also gave 79.1 $\mathrm{KJ\,mol^{-1}}$ as the activation energy, one of these steps might be oxygen dissociation, which turns to another step at the inflection point on the curve *i.e.* about 22 °C (at pH 7.4).

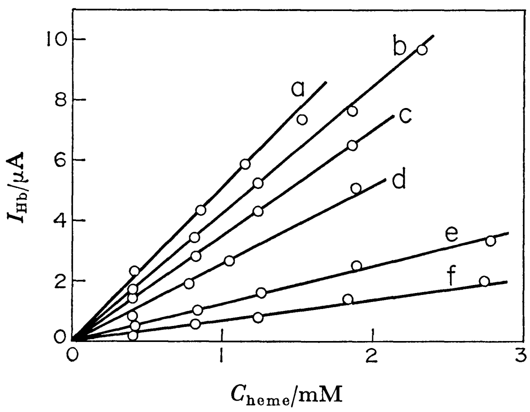

Fig. 3. Relation between I_{Hb} and relatively low concentration of Hb at various temperatures open to the atmosphere.

a: 40 °C, b: 35 °C, c: 30 °C, d: 25 °C, e: 15 °C, f: 12.5 °C. Other conditions are the same as in Fig. 2. I_{Hb} was obtained by subtracting $^*I_{\mathrm{O_2}}$ from I.

The change in pH also resulted in the variation in α (Fig. 5). The pattern of the variation is similar to that of the Bohr effect[25] which reflects the change in the dissociation rate of oxygen from oxyHb, and gives a symmetrical curve with $\mathrm{p}K'=7.0$, when k is plotted against pH.[24] The change in I_{Hb} with pH can be ascribed to that in the rate of dissociation of oxygen from oxyHb. In order to clarify the rate-determining step for I_{Hb}, effects of temperature and pH were examined on the basis of the dissociation rate of oxygen at various temperatures or pH.

Figure 6 shows the relation between α and k obtained by the stopped-flow method, carried out by

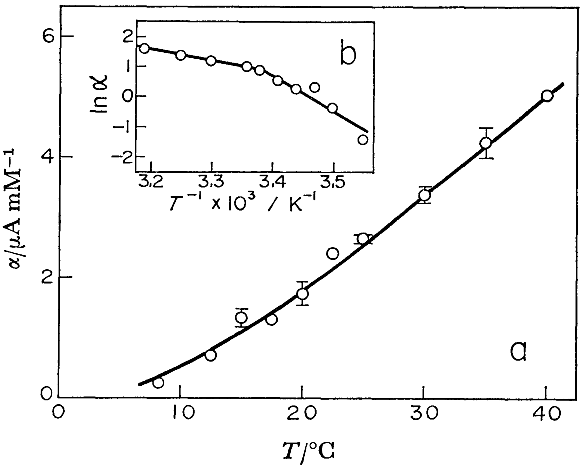

Fig. 4. Effect of temperature on α (a) and plots of logarithms of α against the reciprocal temperature (b).

α is $I_{\mathrm{Hb}}/C_{\mathrm{heme}}$ or $(I-{}^*I_{\mathrm{O_2}})/C_{\mathrm{heme}}$, and was obtained as the slope of the curve in Fig. 3.

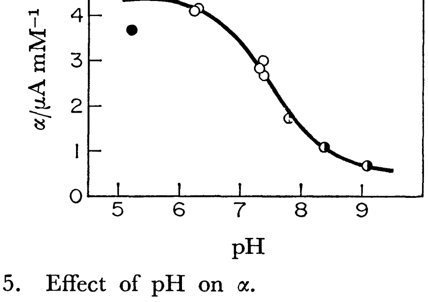

Fig. 5. Effect of pH on α.

○: 0.1 M phosphate buffer, ◑: 0.1 M tris-HCl buffer, ●: 0.1 M acetate buffer. Other conditions are the same as in Fig. 2.

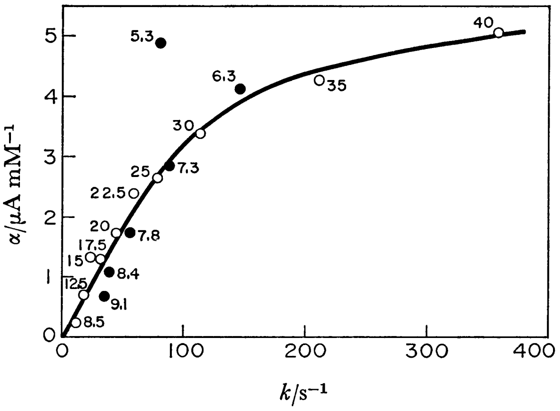

Fig. 6. Plot of α against apparent first order rate constant of oxygen dissociation from oxyHb (k). ○: Temperature effect at pH 7.4 (see Fig. 4-a), ●: pH effect at 25 °C (see Fig. 5). Numbers with experimental points are temperature (°C) (○) or pH (●) at which α and k were measured by polarography and by stopped-flow method, respectively.

TABLE 1. DIFFUSION COEFFICIENTS OF Hb(D_{Hb}) OBTAINED POLAROGRAPHICALLY AND APPARENT FRST ORDER RATE CONSTANTS OF OXYGEN DISSOCIATION FROM oxyHb (k) OBTAINED BY STOPPED-FLOW METHOD

k/s^{-1} (T/°C, pH)$^{a)}$	$D_{\mathrm{Hb}}/10^{-7}\,\mathrm{cm^2\,s^{-1}}$
78.4 (25, 7.4$^{b)}$)	2.8
81.6 (25, 5.3$^{c)}$)	9.4
114.0 (30, 7.4$^{b)}$)	4.4
148.4 (25, 6.3$^{b)}$)	7.0
209.1 (35, 7.4$^{b)}$)	6.2
357.8 (40, 7.4$^{b)}$)	7.6

a) Conditions for kinetic measurements. b) 0.1 M phosphate buffer. c) 0.1 M acetate buffer.
Values of k (Fig. 6) are given in increasing order, irrespective of experimental conditions. D_{Hb} is the value corrected for 25 °C, though each actual measurement was carried out at the temperature for the corresponding k measurement.

changing temperature at a fixed pH or changing pH at a constant temperature. A satisfactory coincidence could be obtained for the results of two series of different kinds of experiments. The curve obtained implies two rate-determining factors for oxygen transport by Hb to the electrode surface. At relatively small k values, where I_{Hb} is proportional to k, the rate-determining step is the dissociation of oxygen. When k value exceeds *ca.* 60 s^{-1}, where I_{Hb} is not appreciably affected by k, the dissociation is no longer rate-determining, and is replaced by the diffusion of Hb to the electrode. This can be supported by the following observation: current I is almost a diffusion current in the range beyond the k around 60 s^{-1}, while it obviously contains characteristics of kinetic current[20,21] up to the k value. This is in line with the idea of Wittenberg[11] and Wyman[13] who treated the oxygen transport by Hb in terms of translational diffusion of Hb and the oxygen dissociation from oxyHb. A slight increase in α with k in the diffusion-controlled region can be understood as due to the increase in D_{Hb} with temperature. The diffusion-controlled transport of oxygen should be confirmed by comparing the D_{Hb} value estimated from I_{Hb} with the published data.

In the diffusion-controlled region, D_{Hb} is involved in α. I_{Hb} can be expressed from Ilkovic equation,[18,19] by

$$I_{\mathrm{Hb}} = \kappa\, D_{\mathrm{Hb}}{}^{1/2} C_{\mathrm{heme}}. \tag{6}$$

$I_{\mathrm{O_2}}$ can not be measured directly, but it can be estimated by

$$I_{\mathrm{O_2}} = (D_{\mathrm{O_2}}/{*D_{\mathrm{O_2}}})^{1/2}\,{*I_{\mathrm{O_2}}}, \tag{7}$$

where $*I_{\mathrm{O_2}}$ is I measured in the medium without Hb, and is given by

$$*I_{\mathrm{O_2}} = \kappa\,{*D_{\mathrm{O_2}}}{}^{1/2} C_{\mathrm{O_2}}. \tag{8}$$

Combining Eqs. 3, 6, 7, and 8, we obtain

$$I/{*I_{\mathrm{O_2}}} = (D_{\mathrm{O_2}}/{*D_{\mathrm{O_2}}})^{1/2} + C_{\mathrm{O_2}}{}^{-1}(D_{\mathrm{Hb}}/{*D_{\mathrm{O_2}}})^{1/2} C_{\mathrm{heme}}. \tag{9}$$

The value D_{Hb} at a given concentration can be estimated by means of the equation, substituting $D_{\mathrm{O_2}}$ value for given Hb concentration and other measured values. In practice, the value D_{Hb} in relatively dilute concentration range was obtained using α obtained experimentally for diffusion-controlled region under the assumption of $D_{\mathrm{O_2}} = {*D_{\mathrm{O_2}}}$†. The value D_{Hb} obtained experimentally agrees with the reported values $6-7\times10^{-7}\,\mathrm{cm^2\,s^{-1}}$, irrespective of the increase of k with either temperature or with pH (Table 1). The result might support the validity of the assumption that the rate-determining step for I_{Hb} changes from the dissociation of oxygen to the diffusion of Hb with increase in k.

A relatively large deviation of the experimental point at pH 5.3 and 25 °C in acetate buffer from the curve drawn through other experimental points (Fig. 6) can be explained by the contribution of an apparent increase of D_{Hb} with the dissociation of tetramer to dimer or further monomer of Hb (Table 1), since such dissociation was observed more readily in acetate buffer than in other buffer at pH 5.3.[28,29]

Under the physiological conditions (pH 7.4, 37 °C), the oxygen transport by Hb is restricted by the diffusion rate of Hb molecules (Fig. 6). It is of interest to examine whether the relation (Fig. 6) is also applicable to a relatively concentrated solution of Hb. Figure 7 shows the relation between k and α at 18 mM heme of Hb concentration. A similar relation to that in Fig. 6 was obtained, the same k values being used,

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† Equation 9 is simplified as

$$I/{*I_{\mathrm{O_2}}} = 1 + C_{\mathrm{O_2}}{}^{-1}(D_{\mathrm{Hb}}/{*D_{\mathrm{O_2}}})^{1/2} C_{\mathrm{heme}}.$$

Further modification gives

$$C_{\mathrm{O_2}}(I-{*I_{\mathrm{O_2}}})/(C_{\mathrm{heme}}\,{*I_{\mathrm{O_2}}}) = (D_{\mathrm{Hb}}/{*D_{\mathrm{O_2}}})^{1/2}.$$

Since $(I-{*I_{\mathrm{O_2}}})/C_{\mathrm{heme}}$ is α (Fig. 4), we have

$$\alpha\, C_{\mathrm{O_2}}/{*I_{\mathrm{O_2}}} = (D_{\mathrm{Hb}}/{*D_{\mathrm{O_2}}})^{1/2}.$$

Thus

$$D_{\mathrm{Hb}} = {*D_{\mathrm{O_2}}}(\alpha\, C_{\mathrm{O_2}}/{*I_{\mathrm{O_2}}})^2.$$

The value $2.0\times10^{-5}\,\mathrm{cm^2\,s^{-1}}$ was used for $*D_{\mathrm{O_2}}$ at 25 °C in the present study, since similar values were reported for various electrolyte solutions at 20—25 °C.[26,27] The $C_{\mathrm{O_2}}$ value was taken from International Critical Tables. $*I_{\mathrm{O_2}}$ was obtained experimentally.

since the oxygen affinity of Hb is little affected by the variation of Hb concentration.[30] The observation enables us to consider that the behavior of oxygen in a relatively concentrated solution of Hb such as in erythrocytes is also restricted by the diffusion of Hb and not by the dissociation of oxygen from oxyHb.

In the following we examine the behavior of oxygen in concentrated solution of Hb corresponding to that in erythrocytes in term of D_{Hb}, since the value D_{Hb} in various concentrations can be calculated by Eq. 9, when I is diffusion-controlled.

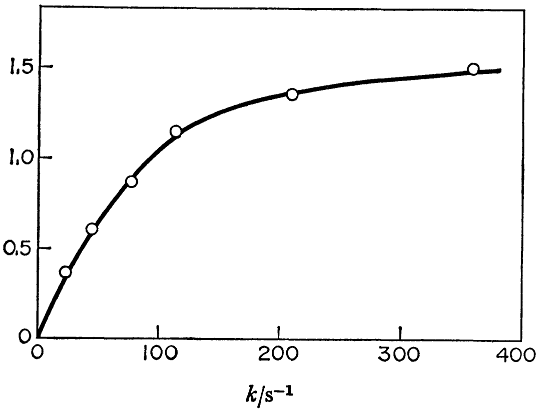

Fig. 7. Relation between apparent first order rate constant of oxygen dissociation from oxyHb (k) and α in a relatively concentrated Hb solution.
Hb concentration: 18 mM heme. α was obtained from the measurements at various temperatures at pH 7.4. The k values are the same as in Fig. 6. Other conditions are the same as in Fig. 2.

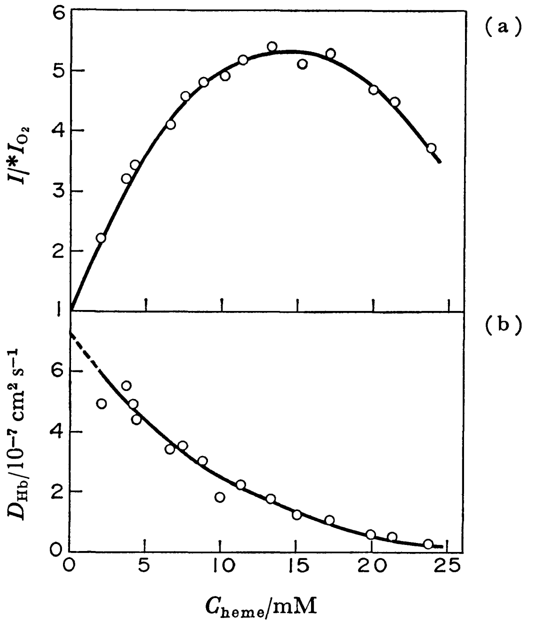

Fig. 8. Hb concentration dependence of $I/{*I_{\mathrm{O_2}}}$ (a), and diffusion coefficient of Hb (D_{Hb}) (b), in a relatively wide concentration range. D_{Hb} values were calculated by using Eq. 8 with $C_{\mathrm{O_2}}=0.26$ mM and ${*D_{\mathrm{O_2}}}=2.0\times10^{-5}\ \mathrm{cm^2\ s^{-1}}$. See the text for detail of estimation of D_{Hb}. Other conditions are the same as in Fig. 2.

D_{Hb} *in Concentrated Solution of Hb.* Figure 8-a shows the relation between $I/{*I_{\mathrm{O_2}}}$ and C_{heme} over a wide concentration range at pH 7.4 and 25 °C. The linear current increase with C_{heme} (Fig. 2 or 3) was observed only up to *ca.* 2 mM of C_{heme}, beyond which the current increase, whose maximum was around 14 mM, was lowered. This can not be attributed to the change in oxygen affinity of Hb, but to the decrease of D_{Hb} due to the increase of viscosity of the solution with C_{heme}. A similar observation has been reported by Wittenberg[11] by means of millipore filter method. However, no estimation was given on D_{Hb} in various concentration of Hb necessary for the demonstration of the diffusion-controlled oxygen transport. We can calculate D_{Hb} by substituting the $I/{*I_{\mathrm{O_2}}}$ value (Fig. 8-a) into Eq. 9, since we can expect diffusion current for oxygen reduction. In a relatively concentrated solution of Hb, $D_{\mathrm{O_2}}$ can not be identical with ${*D_{\mathrm{O_2}}}$. Hence, we employed the $D_{\mathrm{O_2}}$ value used by Roughton,[2] who estimated it in Hb solution, by comparison with D of nitrogen in a similar Hb solution. The relation between D_{Hb} obtained and C_{heme} in the present experiments is shown in Fig. 8-b. The increase of C_{heme} gave rise to a large decrease in D_{Hb}. The intercept on the ordinate of the curve passing through points experimentally obtained gave *ca.* $7\times10^{-7}\ \mathrm{cm^2\ s^{-1}}$, corresponding to the value known as D_{Hb} in dilute solution. In 20 mM Hb solution, *viz.*, equivalent concentration in erythrocytes, D_{Hb} is *ca.* $5.8\times10^{-8}\ \mathrm{cm^2\ s^{-1}}$. This is in agreement with $6.4\times10^{-8}\ \mathrm{cm^2\ s^{-1}}$ obtained by Kreuzer.[31] The fact that the D_{Hb} obtained is 300 times smaller than ${*D_{\mathrm{O_2}}}$, suggests that oxygen in erythrocytes can move only with about one-three hundredth mobility of oxygen outside erythrocytes.

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