Steady-State, Hemoglobin-Facilitated O_2 Transport in Human Erythrocytes

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ABSTRACT We measured the rate of oxygen transport through thin (165 μ) films of packed erythrocytes (Hb concentration = 30 g/100 ml). Under optimal conditions steady-state O_2 diffusion was nearly three times that found when the hemoglobin was prevented from acting as a carrier molecule by carbon monoxide binding or high oxygen back pressure. After each experiment we measured hemolysis and found that it averaged less than 1 %. Hemolysis could not account for the facilitation, thus proving that facilitated transport of O_2 by hemoglobin can occur in red blood cells. The rate of facilitated transport was identical for Hb solutions of equal concentration to the cells. We interpret this to mean that under the conditions of our experiments the red cell membrane offers no detectable diffusion resistance to O_2 and that the mobility of Hb in intact red cells is the same as in concentrated Hb solution.

In their extensive studies on the diffusion and reaction of oxygen and other ligands in hemoglobin solutions and erythrocytes, Roughton and his collaborators suspected that the diffusion of hemoglobin might enhance the diffusion of molecules which react with hemoglobin. Nicolson and Roughton (1951) explicitly considered this problem. Klug, Kreuzer, and Roughton (1956) found that the oxygenation of layers of hemoglobin solutions of less than 30 g/100 ml hemoglobin concentration was too rapid to be accounted for by diffusion of molecular oxygen alone. They postulated that the diffusion of oxygen was augmented by oxyhemoglobin diffusion. Longmuir and Roughton (1952) concluded that in intact red cells (hemoglobin concentration greater than 30 g/100 ml) the diffusion of oxyhemoglobin is too small to contribute significantly to oxygen diffusion.

Hemoglobin-facilitated oxygen transport was studied more directly by Wittenberg (1959), who measured the penetration of oxygen through hemoglobin-containing agar membranes, and by Scholander (1960), who measured the rate of steady-state oxygen transport across Millipore filters saturated with hemoglobin solution. Both investigators found that hemoglobin en-

hanced oxygen transport and their results stimulated considerable interest in this phenomenon. Hemmingsen (1965) and Wittenberg (1966 *a*) have reviewed work in this field.

Scholander also measured the steady-state oxygen flux through red blood cells smeared on the underside of a Millipore filter and observed hemoglobin-facilitated oxygen transport. Roughton (1963) objected to Scholander's experiment on the grounds that the capillary forces in the Millipore filter might have lysed a substantial percentage of the cells, so that the observed facilitation might have occurred in the hemoglobin solution surrounding the red cells. Hemmingsen (1965) reported facilitated O_2 transport in red cells in two experiments in which only a small degree of hemolysis seemed to be present.

We have repeated Scholander's experiment and, indeed, found extensive hemolysis on Millipore filters. However, using fine stainless steel screen on which hemolysis averages less than 1 % we can still demonstrate considerable hemoglobin-facilitated O_2 transport in packed erythrocyte layers.

Roughton (1959) has long held that the membrane of the erythrocyte is an important barrier to the diffusion of oxygen. By comparing the kinetics of oxygen uptake by hemoglobin solutions and red cell suspensions, he calculated that roughly half the resistance to oxygen uptake is in the cell membrane. Kreuzer and Yahr (1960) using a spectrophotometer to follow the rate of O_2 uptake by thin (100–227 μ) layers of packed red cells and by concentrated hemoglobin solutions found no significant difference in one-third or one-half saturation times between these preparations and concluded that the oxygen diffusion resistance of the red cell membrane is not significant.

We have measured the rate of steady-state O_2 diffusion through thin films of concentrated hemoglobin solutions and, under equal conditions, found rates equal to those for intact red cells.

METHODS

Preparation of Erythrocyte and Hemoglobin Solution Films

We drew human blood from the brachial vein into a heparinized syringe, then immediately mixed four volumes of blood with one volume of acid-citrate-dextrose solution (Wintrobe, 1951). We refrigerated the blood and gently agitated it several times daily to disperse the sedimented red cells. Under these conditions we were able to maintain a supply of erythrocytes for several days' experiments without significant hemolysis. For each experiment we centrifuged 10 ml of blood for 30 min at 1500 *g*, carefully pipetted off the plasma and buffy coat, and poured the packed erythrocytes into a Petri dish.

To hold a film of packed erythrocytes we used a fine stainless steel wire screen (0.001 inch diameter wire, 400 mesh, 36% open area). We soaked a suitable piece of the screen (cemented to a brass ring) in the packed cells, tilted it to allow the excess to drain off, and immediately placed the screen in the Lucite apparatus shown in Fig. 1.

The cells remaining in the Petri dish were used for the determination of hemoglobin concentration. Fig. 2 is a photomicrograph of the erythrocyte film on the screen.

For experiments with hemoglobin solutions we lysed saline-washed packed cells by repeated freezing and thawing, then centrifuged the preparation at 1500 *g* for 45

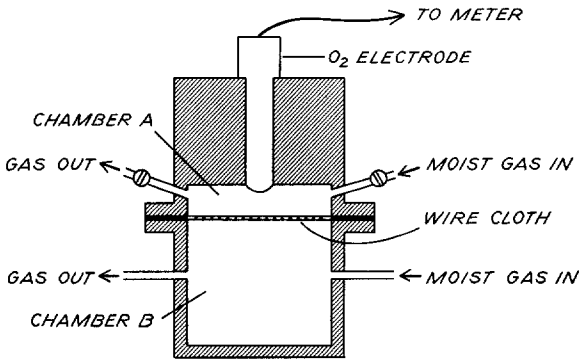

FIGURE 1. Diagram of Lucite chamber employed in these experiments. Inside diameter of chamber is 3.81 cm.

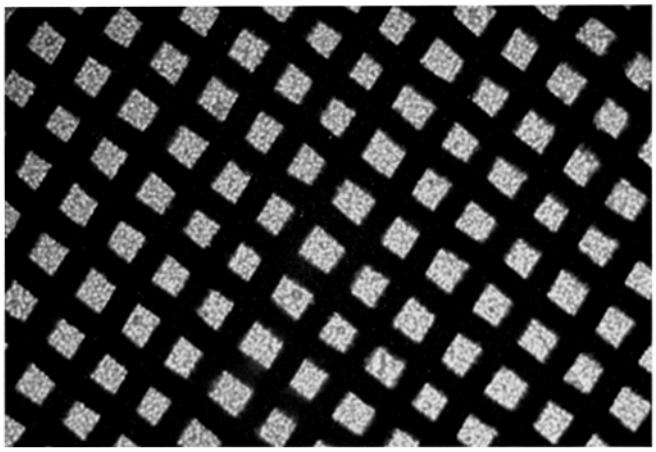

FIGURE 2. Photograph of the wire screen supporting a layer of packed erythrocytes. × 200.

min to remove the larger cell fragments, poured the supernatant concentrated hemoglobin solution into the Petri dish, and filled the wire screen with intact cells.

To estimate the amount of red cell stroma which remained in our hemoglobin solution preparations, we prepared 100 ml of packed red cells and 75 ml of hemoglobin solution (as described above) from the same blood sample. We extracted the lipids from these preparations in 2:1 chloroform-methanol by a modification of the method of Folch and Lees (1951). The hemoglobin solution yielded 0.82 mg lipid/ml, the packed red cells yielded 3.66 mg lipid/ml cells. We concluded that our method of preparation removed approximately 77% of the red cell stroma.

Arrangement of the Diffusion Experiments

The cylindrical diffusion apparatus (Fig. 1) consisted of a 2.28 ml chamber (A) and a 37 ml chamber (B) separated by the wire screen holding the film of blood. We flushed both chambers separately with humidified gases at room temperature (21–23°C). We monitored the oxygen partial pressure (P_{O_2}) in A continuously with a microcathode oxygen electrode (Beckman Instruments, Inc., Fullerton, Calif.) covered by a 1 mil Teflon membrane. The oxygen consumption of the electrode during the experiment was negligible. We read the electrode current on a Radiometer Gas Monitor (Radiometer Co., Copenhagen, model 27).

In each experiment we flushed A with gas of known P_{O_2} and B with a gas of lower P_{O_2} with or without added carbon monoxide. After 5 min A was closed off by stopcocks and the P_{O_2} read. The initial reading served as an electrode calibration since we knew the exact composition of the flushing gas having analyzed it in triplicate by the method of Scholander (1947). The amplifier sensitivity was adjusted so that the initial P_{O_2} in chamber A gave a full scale deflection on the meter. We also checked the zero calibration of the electrode before and after each day's experiments by using gas with no measurable oxygen.

As oxygen diffused into chamber B the P_{O_2} in A decreased. We recorded the P_{O_2} every 5 min for 30 min, then we flushed A again for 5 min with the same gas that we started with to recheck the electrode calibration. The electrode tended to drift slightly, but always in the same direction (toward decreasing sensitivity). The drift was very small over any 30 min period averaging about 1% of full scale.

We computed the average oxygen flux, J_{O_2}, in $\mu l/(\text{min} \times \text{cm}^2)$ from the average rate of change of P_{O_2} over the experimental period, the known chamber volume (2.28 ml), and the open area of the screen (4.0 cm^2).

Experimental Sequence

During the first experimental period we continued to flush chamber B with humidified 100% nitrogen. Following this we blocked any possible hemoglobin-facilitated transport of oxygen by flushing B with either 2% CO (Wittenberg, 1959) or 5.5% O_2 (Hemmingsen and Scholander, 1960). This period served as the control period during which only simple diffusion of molecular oxygen occurred through the blood film. In the third period we again flushed B with 100% nitrogen. Modifications of this basic sequence included periods with different oxygen tensions in chamber A or duplicate sequential runs to determine reproducibility.

Measurement of Hemoglobin Concentration and Degree of Hemolysis

We determined hemoglobin concentration on the screen from that in the Petri dish by the standard cyanmethemoglobin method (Crosby, Munn, and Furth, 1954).

To determine the degree of hemolysis in the packed cells on the screen we removed the screen from the apparatus and gently washed the cells from it using 8 ml of 0.9% NaCl solution. We centrifuged the washings for 30 min at 1500 *g*, reserved the supernatant, and hemolyzed the red cell button in a volume of distilled water equal to that

of the decanted supernatant. We determined the optical density at 422 mμ, an isosbestic point of hemoglobin and oxyhemoglobin. We calculated per cent hemolysis from the ratio of the supernatant optical density to that of the hemolyzed cell button.

Estimation of Film Thicknesses

The screen was constructed of 1 mil wire interwoven, so that its maximum thickness was 2 mil (51 μ). When dipped in packed red cells or hemoglobin solution and drained, the film, viewed through a dissecting microscope, appeared smooth and flat except for a narrow meniscus at the ring margin. Since the wires did not break the surface, the film must have been thicker than 51 μ.

From the measurements made to determine the percentage hemolysis in the packed red cell films, we calculated the total hemoglobin content of each film and the volume of red cell preparation required to provide this amount of hemoglobin. From this volume and the 11.1 cm^2 total area of the wire mesh, taking into account the volume occupied by the mesh itself, we computed an average packed red cell film thickness of 165 ± 9 (SDM) μ.

To estimate the thickness of the hemoglobin solution films, a series of 10 films was formed as described above, placed in the apparatus briefly, and the hemoglobin eluted from the wire mesh with 10 ml of 0.9% NaCl solution. We determined the hemoglobin concentration of the washings by the cyanmethemoglobin method and computed an average film thickness of 162 ± 6 μ.

The reproducibility of the experimental results indicates that the variation in film thickness was small. We attribute the variation in estimations of the thickness to the difficulty of washing from the wire mesh all the packed red cells or hemoglobin solution without disturbing the layer of excess material adhering to the supporting brass ring.

Determination of the Oxygen-Hemoglobin Equilibrium Curve

In order to check the applicability of the Severinghaus nomogram (Severinghaus, 1964) to the pH and temperature of our blood we mixed three samples of donor blood at room temperature with three different gas mixtures used in the study. We measured P_{O_2} with an oxygen electrode and pH with a glass electrode. We determined oxygen content and capacity by the method of Van Slyke and Neil. The three samples that we checked fitted the oxygen-hemoglobin equilibrium curve calculated from the Severinghaus nomogram for 21.5°C, pH 7.025.

RESULTS AND DISCUSSION

Oxygen Flux through Intact Erythrocytes

Figs. 3 and 4 show the P_{O_2} in chamber A in two experiments. When we used 2% CO to block possible facilitation during the second period, the rate of P_{O_2} decrease in the third period did not return completely to the value that it achieved during the first period. We attributed this to a failure to remove all of the CO from the film due to its slow dissociation from hemoglobin.

When we used 5.50 % O_2 to block possible facilitation during the second period, then the third experimental period closely duplicated the first one. The complete results of 13 experiments are given in Table I except that the data for the third period after CO blockade are deleted. Fig. 5 is a plot of the average oxygen flux vs. the average P_{O_2} difference between chambers A and B. Points on the lower curve are from periods when only diffusion of molecular oxygen could occur. Points on the upper curve are from periods

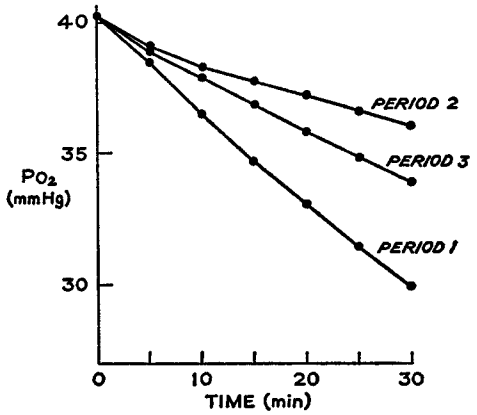

FIGURE 3. Oxygen partial pressure in chamber A vs. time for three consecutive 30 min periods in one experiment. Each period began with A at P_{O_2} = 40.2 mm Hg (remainder nitrogen). In periods 1 and 3, B was flushed with nitrogen. In period 2, B was flushed with 2% carbon monoxide in nitrogen. Incomplete recovery of the period 3 curve is attributed to failure to completely remove carbon monoxide from the film.

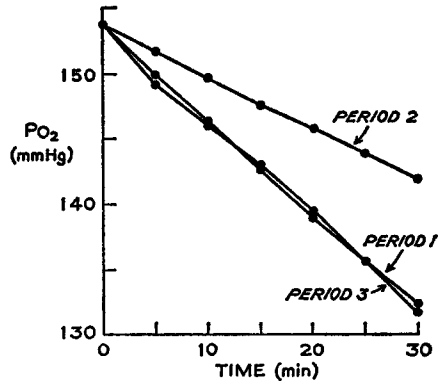

FIGURE 4. Oxygen partial pressure in chamber A vs. time for three consecutive 30 min periods in one experiment. Each period began with A at P_{O_2} = 154 mm Hg (remainder nitrogen). In periods 1 and 3, B was flushed with nitrogen. In period 2, B was flushed with 5.5% oxygen in nitrogen.

TABLE I

THE OXYGEN FLUX AT 22°C ACROSS A LAYER OF PACKED HUMAN ERYTHROCYTES SUPPORTED ON A STAINLESS STEEL SCREEN

Experiment No.	C_{Hbt}*	Hemolysis	Period	$P_{O_2,A}$‡	Gas in B	$\overline{\Delta P_{O_2}}$§	J_{O_2}‖
	g/100 ml	*%*		*mm Hg*		*mm Hg*	*μl/cm² × min*
1	28.5	1.0	1	9.56	N_2	8.61	0.042
			2	9.56	N_2	8.43	0.050
			3	9.56	2% CO	8.78	0.016
2	28.5	1.1	1	9.48	N_2	8.64	0.042
			2	9.48	N_2	8.72	0.040
			3	9.24	2% CO	8.85	0.025
3	29.5	0.8	1	9.48	N_2	8.70	0.041
			2	9.48	2% CO	8.88	0.021
4	30.0	1.0	1	40.2	N_2	35.0	0.267
			2	40.2	2% CO	38.1	0.109
5	30.0	1.5	1	40.1	N_2	34.9	0.270
			2	40.1	N_2	34.8	0.278
			3	40.1	2% CO	37.6	0.131
6	30.0	1.4	1	40.3	N_2	35.3	0.259
			2	40.3	N_2	35.2	0.265
			3	94.8	N_2	86.3	0.441
			4	94.8	5.5% O_2	51.3	0.162
			5	40.3	N_2	34.3	0.265
7	30.0	1.3	1	40.2	N_2	35.3	0.259
			2	94.4	N_2	87.3	0.370
			3	94.4	5.5% O_2	51.1	0.146
8	30.0	0.5	1	154	N_2	143	0.567
			2	154	5.5% O_2	108	0.302
			3	154	N_2	143	0.553
9	30.0	0.5	1	154	N_2	143	0.564
			2	154	5.5% O_2	108	0.310
			3	154	N_2	143	0.546
10	29.1	0.7	1	39.8	N_2	35.0	0.251
			2	93.8	N_2	85.3	0.446
			3	93.8	N_2	85.4	0.440
			4	39.8	N_2	34.4	0.284

* Hemoglobin concentration in the layer.

‡ Initial P_{O_2} in chamber A.

§ Average P_{O_2} difference across the layer.

‖ Average oxygen flux across the layer in μl/min per cm² open area of the screen. Note that since the erythrocyte layer is considerably thicker than the supporting screen, the effective area of the layer must be larger than the 4 cm² open area of the screen.

TABLE I—*Concluded*

Experiment No.	C_{Hbt}*	Hemolysis	Period	$P_{O_2 i_A}$‡	Gas in B	$\overline{\Delta P_{O_2}}$§	J_{O_2}‖
	g/100 ml	%		*mm Hg*		*mm Hg*	*μl/cm² × min*
11	29.1	0.4	1	40.2	N_2	35.2	0.254
			2	21.9	N_2	18.2	0.139
			3	21.0	N_2	18.4	0.139
			4	40.2	N_2	35.3	0.251
12	29.4	1.0	1	40.2	N_2	34.9	0.278
			2	153	N_2	142	0.600
			3	153	N_2	142	0.572
			4	85.0	N_2	77.0	0.416
			5	91.8	N_2	83.7	0.424
			6	40.2	N_2	35.7	0.255
13	29.4	0.7	1	40.2	N_2	35.5	0.249
			2	94.4	N_2	85.9	0.446
			3	94.4	N_2	86.4	0.418
			4	94.4	N_2	86.4	0.418
			5	153	N_2	142	0.559
			6	153	N_2	141	0.592
			7	153	2% CO	145	0.416
			8	153	2% CO	145	0.411

when both simple and facilitated diffusion could occur, that is, when chamber B contained 100 % nitrogen. It is clear that hemoglobin facilitation of oxygen diffusion is occurring.

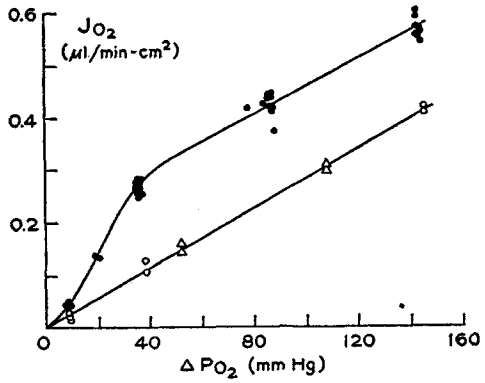

FIGURE 5. Oxygen flux in μl/min per cm² open area of the screen across the packed erythrocyte films. The filled circles represent periods in which the low oxygen side of the film was flushed with nitrogen. The open circles and triangles represent periods in which the low oxygen side was flushed with 2% carbon monoxide and 5.50% oxygen, respectively. Each symbol is the average for one 30 min experimental period. The curves were drawn by eye. The average temperature in these experiments was 21.7°C, the average barometric pressure 745.5 mm Hg.

The per cent hemolysis found at the end of each experiment is also shown in Table I. The range is from 0.4 to 1.5 %; the average being 0.9 %. This small degree of hemolysis could not possibly account for the observed enhancement. To construct the most unfavorable situation, let us suppose that

TABLE II

THE OXYGEN FLUX AT 21°C ACROSS CONCENTRATED HUMAN HEMOGLOBIN SOLUTION LAYERS

	C_{Hbt}	Period	$P_{O_2 i_A}$	$\overline{\Delta P_{O_2}}$	Gas in B	J_{O_2}
	g/100 ml		*mm Hg*	*mm Hg*		*μl/cm² × min*
1	29.8	1	153	142	N_2	0.580
		2	153	107	5.5% O_2	0.320
		3	153	142	N_2	0.565
2	30.0	1	154	143	N_2	0.549
		2	154	107	5.5% O_2	0.325
		3	154	55.0	13% O_2	0.181
3	29.6	1	40.2	35.3	N_2	0.254
		2	94.6	86.1	N_2	0.445
		3	94.6	86.1	N_2	0.445
		4	94.6	51.5	5.5% O_2	0.152
		5	94.6	51.4	5.5% O_2	0.156
4	29.6	1	40.2	35.3	N_2	0.254
		2	40.2	35.3	N_2	0.254
		3	40.2	35.1	N_2	0.264
		4	40.2	35.1	N_2	0.264
		5	94.4	86.5	N_2	0.414
		6	94.4	86.5	N_2	0.414
		7	94.4	51.3	5.5% O_2	0.154
		8	94.4	51.3	5.5% O_2	0.151
		9	94.4	89.4	2% CO	0.261
5	29.6	1	153	142	N_2	0.563
		2	153	142	N_2	0.577
		3	153	145	2% CO	0.422
		4	153	145	2% CO	0.422
		5	153	145	2% CO	0.418

For the meanings of symbols see footnotes to Table I.

hemoglobin in solution forms a diffusion pathway in parallel to that through the intact cells. To account for an enhancement of 2 in a film that contained free hemoglobin equal to 1 % of that in the cells, a 100-fold enhancement must occur in the hemoglobin solution. The largest enhancement of oxygen transport reported in hemoglobin solutions is only about 9 (Scholander, 1960).

The data in Fig. 5 show that both 2 % CO and 5.50 % O_2 block the facilitation equally. Under our experimental conditions 5.50 % O_2 in chamber B

(P_{O_2} = 40 mm Hg) would saturate more than 90 % of the hemoglobin in the film thus effectively blocking facilitation by that carrier molecule.

Oxygen Flux through Concentrated Hemoglobin Solutions

The results of our experiments on hemoglobin solutions are summarized in Table II and a graph of oxygen flux vs. the average P_{O_2} differences across the hemoglobin solution film appears in Fig. 6. The curves in Fig. 6 were traced from Fig. 5. The points on the upper curve are from periods in which nitrogen was used to flush chamber B; the points on the lower curve are from

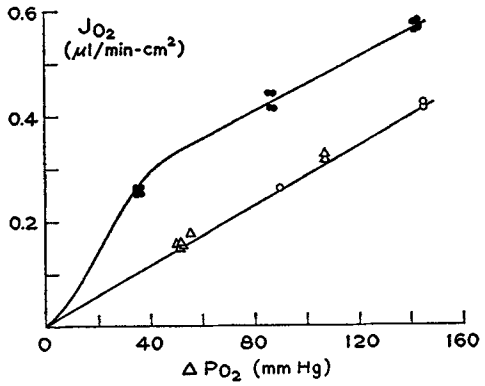

FIGURE 6. Oxygen flux in μl/min per cm² open area of the screen across the hemoglobin solution films. The filled circles represent periods in which the low oxygen side of the film was flushed with nitrogen. The open circles and triangles represent periods in which the low oxygen side was flushed with 2% CO and 5.50% O_2, respectively. Each symbol is the average for one 30 min experimental period. The average hemoglobin concentration was 29.7 g/100 ml, the average temperature 21.2°C. The curves were traced from Fig. 5.

periods in which facilitation of oxygen diffusion was blocked either by carbon monoxide or by oxygen back pressure.

The experimental points in Fig. 6 fit the curves that were determined in the experiments on packed red cell films. The enhancement of oxygen diffusion in the concentrated hemoglobin solutions cannot be distinguished from that observed in packed erythrocytes. The average hemoglobin concentration in the solutions studied was 29.7 g/100 ml, compared to 29.5 g/100 ml for the intact erythrocyte preparations.

CONCLUSIONS

We conclude that since the packed red cell and hemoglobin solution films were nearly the same thickness, the diffusivity of molecular oxygen and the extent of hemoglobin-facilitated oxygen transport are approximately the

same for the packed red cell and hemoglobin solution films. This indicates that under the conditions of our experiment (*a*) the *net* steady-state oxygen diffusion resistance of the red cell membrane is small relative to that of the red cell interior, and (*b*) the interior of the erythrocyte has no special structure or organization that affects the mobility of hemoglobin molecules. The latter conclusion is in agreement with Perutz (1948) who deduced from X-ray scattering data that hemoglobin molecules in red cells are quite closely packed, but are ". . . suspended in true solution."

The Mechanism of Hemoglobin-Facilitated Oxygen Transport

Collins (1961 *a*, *b*) and Fatt and LaForce (1961) offered a simple oxyhemoglobin diffusion theory to account for hemoglobin-facilitated oxygen transport across a layer of hemoglobin solution. They proposed that oxygen and hemoglobin are in chemical equilibrium at each point in the layer and that the facilitated transport is due to the diffusion of oxyhemoglobin down its concentration gradient. Wang (1961, 1963), Scholander (1965), Zilversmit (1965), andWyman (1966) have contributed to the further development of this theory. Wittenberg (1966 *b*) experimentally validated some of the assumptions of the theory.

Other explanations for hemoglobin-facilitated oxygen transport have been given. Moll (1962) proposed that the transport was mediated by the rotation of the hemoglobin molecules. Enns (1964) argued in favor of a collision-exchange transfer of oxygen between hemoglobin molecules. Snell (1965) offered a reaction velocity correction and Fox and Landahl (1965) proposed a pH gradient correction to the simple theory.

According to Fick's first law the diffusive flux of oxygen ($J^D_{O_2}$) across a layer of hemoglobin solution of thickness Δx in the steady state is

$$J^D_{O_2} = -D_{O_2} \frac{\Delta C_{O_2}}{\Delta x} \tag{1}$$

where D_{O_2} is the diffusion coefficient of oxygen in the layer and ΔC_{O_2} is the oxygen concentration difference across the layer.

According to the simple oxyhemoglobin diffusion theory the flux of oxygen ($J^T_{O_2}$) across the layer is equal to the sum of the diffusive flux and the hemoglobin-mediated flux and can be written

$$J^T_{O_2} = -D_{O_2} \frac{\Delta C_{O_2}}{\Delta x} - D_{HbO_2} \frac{\Delta C_{HbO_2}}{\Delta x} \tag{2}$$

where D_{HbO_2} is the diffusion coefficient of oxyhemoglobin in the layer and ΔC_{HbO_2} is the oxyheme concentration difference across the layer. Both concentration differences must be expressed as moles per volume.

Since the thickness and effective area[1] of our red cell films are not known very precisely, we chose to compare the predictions of Equation 2 with our results by considering the enchancement (E), which is defined by

$$E = \frac{J^T_{O_2}}{J^D_{O_2}} = 1 + \frac{D_{HbO_2}}{D_{O_2}} \frac{\Delta C_{HbO_2}}{\Delta C_{O_2}} \tag{3}$$

For reasons explained above, we can consider our packed erythrocyte films to be layers of hemoglobin solution of similar concentration. If we assume chemical equilibrium at the boundaries, $D_{O_2} = 0.9 \times 10^{-5}$ cm²/sec (Keller and Friedlander, 1966 *a*,*b*), and the solubility of oxygen to be the same as its solubility in water (Sendroy, Dillon, and Van Slyke, 1934), the value of

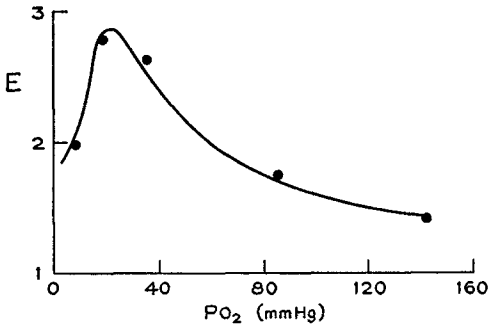

FIGURE 7. Enhancement vs. the oxygen pressure on the high oxygen side of the packed erythrocyte films, the P_{O_2} on the low oxygen side being zero. The points were calculated from the data of Fig. 5. The curve was calculated from Equation 3 taking the diffusion coefficient of oxygen to be 0.9×10^{-5} cm²/sec, the diffusion coefficient of oxyhemoglobin to be 0.54×10^{-7} cm²/sec, and the concentration of hemoglobin to be 30 g/100 ml.

D_{HbO_2} which best fits the data of Table I is 0.54×10^{-7} cm²/sec. Fig. 7 shows that with these assumptions Equation 3 fits our results well.

The diffusion coefficient of hemoglobin in 30 g/100 ml solution has not been determined. The measurements by Moll (1966) and Adams and Fatt (1967) of D_{Hb} in more concentrated solutions are not mutually consistent. Should the model of hemoglobin-facilitated oxygen transport outlined above be confirmed, 0.54×10^{-7} cm²/sec might be considered to be an estimate of D_{Hb} under our experimental conditions. For the present we should emphasize that the controversy over the mechanism of hemoglobin-facilitated oxygen transport cannot be completely settled until D_{Hb} and D_{O_2} are known accurately over a wide range of hemoglobin concentrations.

[1] Since the thickness of the red cell films is considerably greater than that of the supporting wire mesh, the effective area for diffusion is somewhat greater than the open area of the screen, but it is difficult to quantitate this effect.

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