

# The Molecular Mechanism of Hemoglobin-facilitated Oxygen Diffusion\*

(Received for publication, August 9, 1965)

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## SUMMARY

Hemoglobin in solution augments the rate of steady state diffusion of oxygen. The flux of oxygen exceeds the diffusive flux by an amount, the facilitated flux, which is constant for a given hemoglobin concentration and is invariant with changing oxygen tension. The facilitated flux is inversely proportional to the length of the diffusion path. Facilitation decreases with increasing molecular size of the oxygen-binding proteins. From this it is concluded that facilitation results from random displacements of oxyhemoglobin molecules. Translational diffusion is considered to make a far larger contribution than rotatory diffusion. Facilitation is proportional to hemoglobin concentration in dilute solutions which approach ideal behavior and declines at greater protein concentration. It persists in very concentrated hemoglobin solutions (34%) in which the hemoglobin molecules are close packed. Facilitation requires no special property of hemoglobin other than reversible oxygen binding since hemerythrin which is not a heme protein facilitates oxygen diffusion. The reactions occurring during facilitated diffusion are shown to be combination and dissociation of hemoglobin and oxygen. Oxygen displacement or transfer reactions are excluded. The rates of chemical reactions may become rate-limiting. The rate-limiting step is a reaction of oxyhemoglobin. Comparison of the rates of facilitation by several hemoglobins suggests that the rate-limiting step is ligand dissociation. Facilitated carbon monoxide diffusion is minute compared to that of oxygen.

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Hemoglobin present in water solution augments the rate of steady state diffusion of oxygen through that solution (1, 2). The total flux of oxygen through a hemoglobin solution from a region of high to a region of very low oxygen tension may be considered to be the sum of two separate fluxes, which are additive. They are a simple diffusive flux which is independent of the presence of oxyhemoglobin and a hemoglobin-mediated flux which is responsible for the augmented rate of diffusion. The

\* Supported by Research Grants NSF G-6472 and G-20020 from the National Science Foundation.

† Research Career Program Awardee 1-H6-733 of the United States Public Health Service, National Heart Institute.

hemoglobin-mediated component of the flux is conveniently referred to as the "hemoglobin-facilitated diffusion of oxygen," or more simply, as "facilitated diffusion."

The experiments presented here lead to the conclusion that facilitation arises from the random displacement of oxyhemoglobin molecules. Several theoretical discussions based on this idea have been given (3-8). Wyman (8) has considered the phenomenon in detail. The physiological significance of facilitated diffusion has been treated elsewhere (8, 9).

The phenomenon of facilitated diffusion may best be understood by consideration of a typical experiment. In Fig. 1, the fluxes of oxygen and nitrogen through solutions of hemoglobin held in a Millipore filter are presented as functions of the gas partial pressures on one side of the filter, the partial pressures on the opposite side being maintained at zero, and the total pressure on each side being 1 atmosphere. The fluxes of oxygen and nitrogen through a solution of ferric hemoglobin, which does not bind oxygen (*open symbols*), and the flux of nitrogen through a solution of oxyhemoglobin (*solid triangles*) are seen to behave as purely diffusive fluxes which are proportional to the partial pressure differences of these gases across the solution, and are in the ratio of the diffusion constants of the two gases. The flux of oxygen through the oxyhemoglobin solution (*solid circles*) is governed by different rules. It exceeds the flux of oxygen through the ferric hemoglobin solution by an amount which is constant for a given hemoglobin concentration and is invariant with changing oxygen tension ( $p_{O_2} > 20$  mm of Hg). In this it contrasts with the diffusive flux which changes 20-fold in the range of oxygen tension presented, and at the lowest tension examined is only one-sixth of the augmented component of the flux. The excess oxygen flux brought about by the presence of oxyhemoglobin is the "hemoglobin-facilitated flux." The total oxygen flux may be considered to be the sum of two distinct phenomena: diffusion (indicated by the *light arrow* in Fig. 1) and facilitated diffusion (indicated by the *heavy arrow* in Fig. 1).

The constancy of the facilitated flux has the remarkable consequence that a slight back pressure (>20 mm of Hg) brings about a backward facilitated oxygen flux equal to the forward facilitated flux, and the net facilitated flux is abolished (10). The unidirectional fluxes persist and have been measured with the aid of  $^{18}O$  (11, 12).

For this reason, more than usual care was required to keep the backward flux of oxygen minimal, and indeed this consideration dominated the design of the experiment. Sensitivity was sacrificed and a vigorous stream of helium was directed at the

right hand face of the layer of hemoglobin solution to ensure rapid dilution of the diffusing gases and thus maintain very low partial pressures of these gases immediately adjacent to the Millipore filter surface.

#### EXPERIMENTAL PROCEDURE

**Diffusion Chamber**—The diffusion chamber, Fig. 2, was constructed of Plexiglas and consisted of two compartments between which the hemoglobin-loaded Millipore filter was clamped. The area of Millipore filter exposed to the gases was 11.5 cm<sup>2</sup>. The volume of the right hand chamber was 30 ml. The compartments were held together by bolts passing through a flange. A ring of grease between the flanges protected the Millipore membrane from the water bath. During the run, the chamber was submerged in a water bath maintained at  $20.3 \pm 0.05^\circ$ . Gas mixtures equilibrated with water at the temperature of the bath were passed through the left hand chamber at a rate of approximately 40 ml per min and were vented to atmosphere. Usually these mixtures consisted of varying proportions of oxygen and nitrogen; in some experiments the mixture contained helium or carbon monoxide. A stream of helium, 60 ml per min, equilibrated with water at the temperature of the bath, entered the right hand chamber and was directed toward the surface of the Millipore filter. The helium (ultra high purity, The Matheson Company, Inc.) is stated to contain less than 0.1 ppm of oxygen and 0.3 ppm of nitrogen and was drawn from the same tank as the carrier gas for the gas chromatograph.

The effluent helium stream from the right hand chamber was dried by passage over a small bed of Drierite (anhydrous calcium sulfate, W. A. Hammond Drierite Company). Drierite was shown to have very little "memory" for oxygen and nitrogen to which it has been exposed. The helium stream passed to a gas sampling valve, Fig. 2, by means of which 10-ml portions of the stream could be introduced into a gas chromatograph for analysis. The resistance in the effluent train was low, and

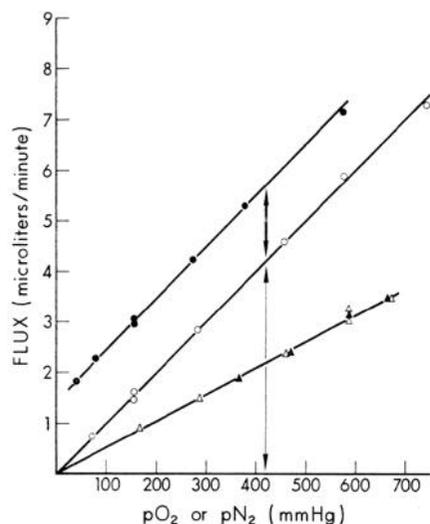


FIG. 1. Steady state diffusion of oxygen and nitrogen through a solution of oxyhemoglobin (solid symbols) compared to the diffusion through a solution of ferric hemoglobin (open symbols). Oxyhemoglobin 11.2 mm; ferric hemoglobin 11.8 mm. Circles, oxygen; triangles, nitrogen. The diffusive component of the oxygen flux is indicated by the light arrow. The hemoglobin-augmented component of the oxygen flux (facilitated flux) is indicated by the heavy arrow.

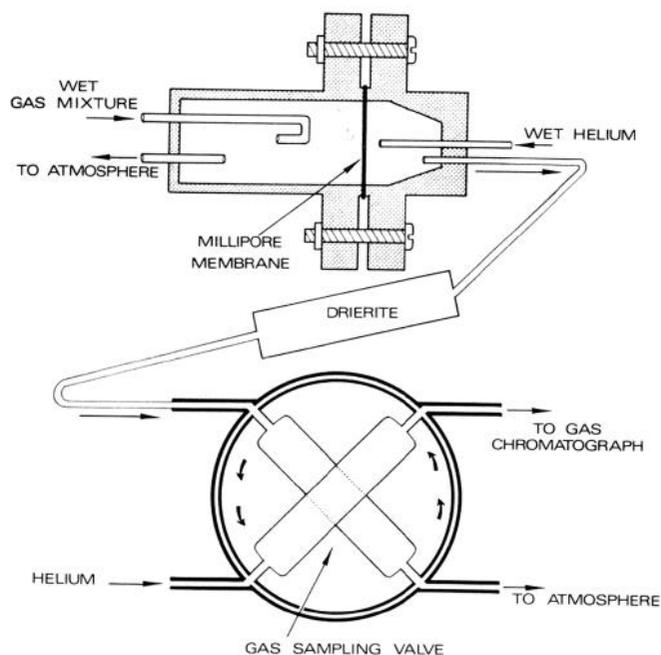


FIG. 2. Diagram of the diffusion chamber

the pressure in the right hand compartment of the diffusion chamber was only 1 to 2 mm of Hg above atmosphere.

Leakage or diffusion of air into the helium stream can be a serious problem. All of the tubing was of glass or metal except for short connections of butyl rubber (gases diffuse through butyl rubber one-tenth as fast as through other types).

**Gas Analysis**—Gases were analysed by gas chromatography. Very high sensitivity was required to give accurate determinations of the very low concentration of gases in the effluent stream. Optimal conditions were: volume introduced by the gas sampling valve, 10 ml; molecular sieve, type 5A (Linde Company) column, 6 feet by  $\frac{1}{4}$  inch outside diameter, operated at  $70^\circ$  (oxygen and nitrogen) or  $100^\circ$  (carbon monoxide-containing mixtures) and 40 ml per min of helium carrier gas flow; thermal conductivity detector. The area of the peaks was determined by a mechanical integrator (disc integrator, Disc Instruments).

The instrument was calibrated daily by generating electrolytically a known amount of oxygen into the helium stream of the diffusion chamber (13). The sensitivity to other gases was in proportion to their thermal conductivity. In practice, differences between the sensitivity to the three gases could be ignored.

**Millipore Filters**—Millipore filters (The Millipore Filter Corporation) are composed of cellulose esters and do not change in thickness when wetted. The manufacturer asserts that the fluid spaces or pores "penetrate . . . directly through the 150 micron depth of the filter with a minimum of cross linkage." Type HA, which was mainly used in this work, is specified to be  $150 \pm 10 \mu$  thick; the pore size is  $0.45 \pm 0.02 \mu$ , and the porosity or fluid-containing volume, 79% of the total volume. Through the courtesy of Dr. Richard A. Cotton, the Millipore Corporation prepared for the present work Millipore filters similar to type HA but of several thicknesses. Millipore filters  $25 \mu$  thick were type TH; the specifications are otherwise similar to type HA. The thicknesses, measured with a screw micrometer, are given in the legend to Fig. 3.

Filters were floated on a hemoglobin solution until wetted through and then submerged. Excess solution was allowed to drain from the filter in the diffusion chamber.

*Oxygen and Carbon Monoxide Equilibria*—Oxygen and carbon monoxide equilibria were determined spectrophotometrically essentially by the method of Allen, Guthe, and Wyman (14). The light path was 1 cm, the cuvette volume approximately 500 ml, the temperature, 20–21°, and the protein concentration about  $5 \times 10^{-5}$  M. All of the equilibria were measured in 0.1 M potassium phosphate buffer, pH 7.4. Spectra were traced from 650 to 500 m $\mu$ ; calculations were made from the sum of changes of extinction at the  $\alpha$  or  $\beta$  maxima, and the trough at about 560 m $\mu$ .

#### Protein Preparations

All of the hemoglobin solutions were prepared in 0.1 M potassium phosphate buffer, pH 7.4, and were concentrated by ultrafiltration in collodion bags, 5-m $\mu$  porosity (Schleicher and Schuell). When necessary, the solutions were further concentrated by the addition of dry Sephadex G-25 (Pharmacia Inc.), and they were separated from the Sephadex by centrifugal filtration through a fine screen. Tween 80 (lot number 594; a nonionic detergent of the Atlas Powder Company) was added to a final concentration of 0.1%, w/v. Human oxyhemoglobin concentration was determined spectrophotometrically with a molar extinction coefficient of  $15.7 \times 10^3$  at 577 m $\mu$ . Concentrations of other hemoglobins were referred to the pyridine hemochromogen with a molar extinction coefficient of  $32.0 \times 10^3$  at 557 m $\mu$ . All of the concentrations are expressed as moles of heme per liter (except hemerythrin which is expressed as moles of oxygen bound per liter).

*Human Hemoglobin A*—Washed erythrocytes were lysed by the addition of 10 volumes of water. After addition of buffer, the stroma were sedimented by high centrifugation, and the clarified solution dialyzed against buffer and concentrated. Ferric hemoglobin was prepared by oxidation with ferricyanide followed by exhaustive dialysis against buffer.

*Hemoglobin H*—Hemoglobin H was the generous gift of Dr. Helen Ranney (15).

*Hemoglobin-Haptoglobin Complex*—The complex between human hemoglobin A and human haptoglobin type 1-1 was prepared (16) by reaction of purified hemoglobin with haptoglobin purified by a minor modification of the method of Connell and Shaw (17). The preparation contained no detectable uncombined hemoglobin A.

*Oxymyoglobin*—Myoglobin was prepared from horse hearts by a modification of the method of Theorell (18) omitting the treatment with lead acetate. The solutions were maintained between pH 7.0 and 7.5 at all times. After two precipitations with ammonium sulfate, a fraction was obtained containing about 40% oxymyoglobin, 40% ferric myoglobin, and 20% hemoglobin. Final purification was achieved by starch granule electrophoresis in 0.05 M Tris-HCl, pH 8.0, which provided an adequate separation of oxymyoglobin from ferric myoglobin and hemoglobin. The solution of oxymyoglobin eluted from the starch block is stable toward air oxidation when stored at 0° as a concentrated solution.

*Earthworm Hemoglobin*—Earthworms (*Lumbricus terrestris*) (1 Kg) were minced finely with 1 liter of a solution of 0.1 M Tris-hydrochloride, pH 7.6, containing 0.001 M EDTA. The suspension was centrifuged, and the red supernatant solution was fractionated by the addition of solid ammonium sulfate.

During the fractionation, the solution was maintained between pH 7.0 and 7.3 by the addition of NH<sub>3</sub>. The fraction precipitating between 40 and 50% saturation with ammonium sulfate was retained, dissolved in 250 ml of 0.1 M Tris-HCl (pH 8.6), and again fractionated with ammonium sulfate. The fraction precipitating between 40 and 50% saturation was retained. Further purification was by starch granule electrophoresis in 0.05 M Tris-HCl, pH 8.0, for 16 hours.

The worms contained about  $1.2 \times 10^{-4}$  mole of hemoglobin heme per Kg. The recovery of purified hemoglobin was about  $0.6 \times 10^{-4}$  mole of heme per Kg of worm. In this preparation, the ratio of iron to heme was 1.14; the dry weight (dried over P<sub>2</sub>O<sub>5</sub> at 110°) per mole of heme was 28,000 g.

*Tubifex Hemoglobin*—*Tubifex* (1 Kg) was finely minced in 800 ml of a solution of 0.1 M Tris-HCl, pH 7.5, containing 0.001 M EDTA. The suspension was centrifuged at high speed to separate fat and the red supernatant solution was fractionated by the addition of solid ammonium sulfate; the solution was maintained between pH 7.0 and pH 7.5 by the addition of NH<sub>3</sub>. The fraction precipitating between 40 and 50% saturation with ammonium sulfate was retained and again fractionated by the addition of solid ammonium sulfate at the same pH. Final purification was by starch gel electrophoresis in 0.05 ionic strength sodium barbital, pH 8.6, for 16 hours.

The ratio of iron to heme in two preparations was 1.09 and 1.06. The dry weight in this preparation was 29,000 g per mole of heme.

*Ascaris Perienteric Fluid Hemoglobin*—This preparation is described elsewhere (19). Chromatography on DEAE-cellulose was not done.

*Succinyl Ascaris Perienteric Fluid Hemoglobin*—*Ascaris* perienteric fluid hemoglobin was allowed to react exhaustively with succinic anhydride (20). The preparation was judged by ultracentrifuge analysis to consist of approximately 85% of the dimers of the 40,600 molecular weight subunits.

*Ascaris Body Wall Hemoglobin*—This preparation will be described elsewhere.<sup>1</sup> Chromatography on DEAE-cellulose was not done.

*Hemerythrin*—A solution of hemerythrin was prepared by lysis of washed celomic corpuscles from the marine sipunculid worm *Phascolosoma (Golfingia gouldii)*. The oxygen-combining capacity of this solution estimated from the iron content ( $2.2 \times 10^{-2}$  g eq of iron per liter) was  $1.1 \times 10^{-2}$  mole per liter.

#### RESULTS

*Fluxes*—Fluxes are expressed as microliters (760 mm, 0°) diffusing per min. The hemoglobin-augmented component of the oxygen flux is taken as the value of the total oxygen flux extrapolated graphically to zero oxygen tension (at zero oxygen tension the diffusive component of the flux is of course zero). All of the values given are taken from plots similar to Fig. 1, with a minimum of four and usually six experimental points. This procedure has the advantage of being independent of the measurement of the nitrogen flux.

The diffusive flux of oxygen was measured in some experiments, e.g. Fig. 1. It may also be approximated from the nitrogen flux

$$\frac{\text{Flux O}_2}{\text{Flux N}_2} = \frac{\alpha_{\text{O}_2}}{\alpha_{\text{N}_2}} \times \frac{\sqrt{28}}{\sqrt{32}} = 1.88$$

<sup>1</sup> T. Okazaki, B. A. Wittenberg, R. W. Briehl, and J. B. Wittenberg, unpublished experiments.

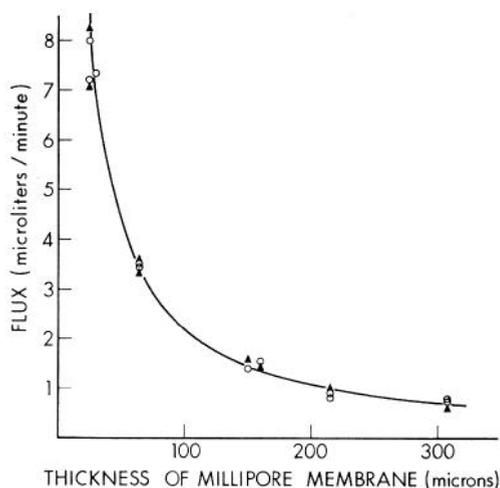


FIG. 3. Dependence of the gas fluxes through a hemoglobin solution on the length of the diffusion path. *Open circles*, hemoglobin-facilitated component of the oxygen flux (facilitated flux); *solid triangles*, nitrogen flux at 0.4 atmosphere of partial pressure difference; hemoglobin, 11 mM. The thicknesses of the Millipore filters used were 25, 64, 150, 220, and 300  $\mu$ . The *solid line* is calculated from the relation: flux = 220  $\times$  (1/d); where *d* is the thickness of the Millipore filter.

where  $\alpha$  is the coefficient for the solubility of each gas in water, and 32 and 28 are the molecular weights of the gases. In several of the figures, the expected diffusive component of the oxygen flux (1.88  $\times$  flux  $N_2$ ) is shown by a *dotted line*. If the hemoglobin-augmented component of the oxygen flux is indeed independent of the oxygen tension, the calculated diffusive oxygen flux should parallel the total oxygen flux. This relation was found frequently but not always. The deviations were small, apparently random, and not correlated with protein concentration or other parameters. They are tentatively ascribed to small changes in the water content of the hemoglobin solution during the course of the measurements.

The ratio of the diffusive fluxes of carbon monoxide and nitrogen may be approximated by a similar relation.

$$\frac{\text{Flux CO}}{\text{Flux } N_2} = \frac{\alpha_{CO}}{\alpha_{N_2}} \times \frac{\sqrt{28}}{\sqrt{32}} = 1.50$$

In each of the six experiments, this ratio was found to be 1.50 as expected.

The diffusive fluxes of nitrogen and oxygen through hemoglobin or ferric hemoglobin solutions were, respectively, about 0.06 and 0.13 ml of gas (0°, 760 mm) penetrating through 0.001-mm (1  $\mu$ ) thickness and 1-cm<sup>2</sup> surface per min when the pressure difference is 1 atmosphere. These are of the same magnitude as the diffusive fluxes through tissues measured by Krogh (21) and others (reviewed in Thews (22)).

*Dependence of Gas Fluxes on Length of Diffusion Path*—The length of the diffusion path was varied by using Millipore filters of different thickness. The augmented component of the oxygen flux was strictly inversely proportional to the length of the diffusion path<sup>2</sup> as may be seen in Fig. 3.

<sup>2</sup> Enns (23) states that Hemmingsen in unpublished experiments has also found the hemoglobin-facilitated flux to be inversely proportional to the diffusion path length. No details are given.

The relation

$$\text{Flux} = \frac{1}{\text{thickness}} \times \text{constant}$$

when expressed logarithmically

$$\text{Log flux} = \text{constant} - \text{log thickness}$$

permits the data to be plotted as a straight line. A plot of log flux against log Millipore thickness is presented in Fig. 4 and establishes that the data do indeed follow the expected relation. The diffusive flux of nitrogen is presented for comparison in Figs. 3 and 4.

*Relation between Hemoglobin-augmented Oxygen Flux and Protein Concentration*—This relation is presented in Fig. 5.

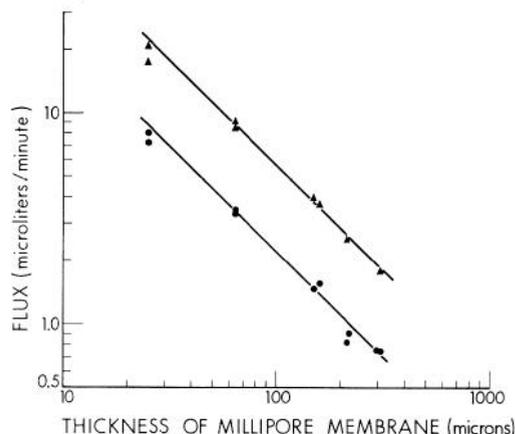


FIG. 4. Dependence of the gas fluxes through a hemoglobin solution on the length of the diffusion path. *Solid circles*, hemoglobin-facilitated component of the oxygen flux (facilitated flux); *solid triangles*, nitrogen flux per atmosphere. The *lines* are drawn with a slope of 1, expressing the assumption that the fluxes are inversely proportional to the path length.

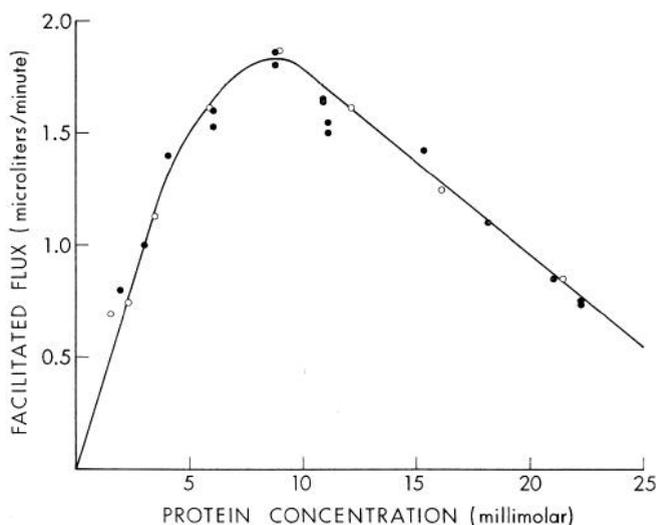


FIG. 5. Dependence of the hemoglobin-augmented component of the oxygen flux (facilitated flux) on protein concentration. *Solid circles*, hemoglobin; *open circles*, myoglobin; Millipore filter, type HA, 150  $\mu$  thick. All of the concentrations are expressed as millimoles of heme per liter.

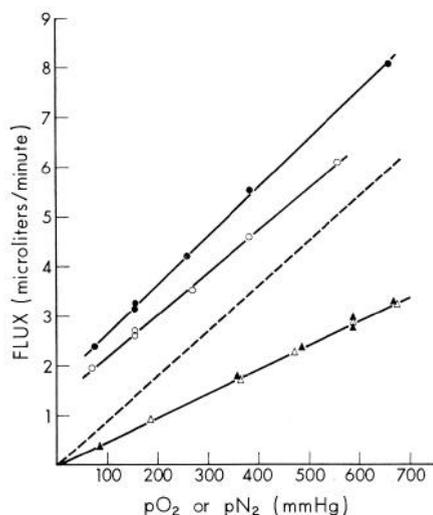
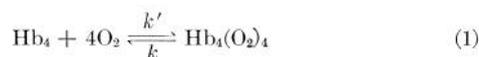


FIG. 6. Diffusion of oxygen and nitrogen through a solution of hemoglobin H (*open symbols*) compared to the diffusion through a solution of hemoglobin A (*solid symbols*) isolated from the same starch block. Hemoglobin H, 11.3 mM; hemoglobin A, 10.9 mM. *Circles*, oxygen; *triangles*, nitrogen. The *dashed line* is the calculated diffusive component of the oxygen flux.

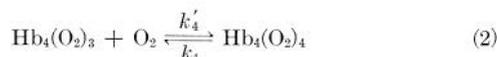
It will be noted that hemoglobin and myoglobin augment the oxygen flux to the same extent per mole of heme, not per mole protein.<sup>3</sup> The flux rises to a maximum at 9 mM heme. At concentrations greater than 9 mM, the flux decreases linearly with increasing heme concentration.

*Human Hemoglobin A*—This protein provides an appropriate standard with which to compare the several other proteins. The molecular weight is 64,450 and it is formed of 4 subunits of molecule weight about 16,100, each bearing 1 heme. The partial pressures for half-saturation of the preparation with oxygen and carbon monoxide were  $p_{1/2, O_2} = 4.5$ ;  $p_{1/2, CO} = 0.03$  mm of Hg.

The reaction of hemoglobin with oxygen and carbon monoxide may be described by rate constants ( $k'$ ,  $k$ ,  $l'$ ,  $l$ , see Table II) for the over-all reaction



or alternatively by rate constants ( $k'_4$ ,  $k_4$ ,  $l'_4$ ,  $l_4$ , see Table II) for the reactions of the "fourth" heme (24)



It is not clear which constants apply to the description of facilitated diffusion. The dissociation rate constant for the fourth heme,  $k_4$ , is independent of pH in contrast to  $k$  which decreases greatly with increasing pH (24). Heme-heme interactions are not found in myoglobin or hemoglobin H, and the rates are described by the constants for the over-all reaction.

At the optimal concentration, 9 mM, the augmented component of the oxygen flux through a standard Millipore filter, Fig. 1, was 1.9  $\mu$ l per min, or 0.025 ml per 1- $\mu$  thickness per cm<sup>2</sup> per min. Correcting for the fraction of the Millipore surface area occupied by the substance of Millipore filter (20%), this becomes 0.031 ml per 1- $\mu$  thickness per cm<sup>2</sup> per min.

<sup>3</sup> The diffusion coefficient of myoglobin is greater than that of hemoglobin, and the rate of oxygen dissociation is less. Possibly the opposing effects of these two rate-limiting parameters may balance each other.

Hemoglobin solutions less concentrated than about 7 mM behave as ideal solutions in the sense that the osmotic pressure is proportional to the concentration (25, 26). In this concentration range, the facilitated component of the oxygen flux, approximately proportional to the protein concentration, is 0.33  $\mu$ l of oxygen per min per 1.0 mm heme. In Krogh's units (correcting for the fraction of the Millipore surface area occupied by the substance of the Millipore), this becomes 0.0054 ml per 1- $\mu$  thickness per cm<sup>2</sup> per min per mm heme. Between 9 and 22 mM hemoglobin or myoglobin, the flux mediated per mm protein falls and is 0.034  $\mu$ l per min per mm protein at 22 mM protein.

*Hemoglobin H*—Hemoglobin H, in contrast to hemoglobin A ( $\alpha_2^A \beta_2^A$ ), consists of four  $\beta$  chains ( $\beta_4^A$ ) (15, 27). The molecular size is essentially similar to hemoglobin A; molecular weight, 63,480. The oxygen affinity is 20 to 40 times greater than that of hemoglobin A.  $p_{1/2}$  is 0.08 to 0.4 mm of Hg at 20° (15).<sup>4</sup> Heme-heme interactions are greatly reduced (15). The high oxygen affinity is largely accounted for by the large combination velocity constant (28). The kinetics of the reactions of hemoglobin H with gases is probably closely similar to those of carboxypeptidase-treated hemoglobins which are presented in Table II for comparison.

Hemoglobin H augments the diffusion of oxygen to nearly the same extent as hemoglobin A at the same concentration (see Fig. 6). The augmented flux with hemoglobin H was 1.3  $\mu$ l per min, that with hemoglobin A from the same blood sample was 1.6  $\mu$ l per min. The difference may either be real or may result from the well known instability of hemoglobin H.

*Hemoglobin-Haptoglobin Complex*—Haptoglobin type 1-1 is a glycoprotein with a molecular weight of 100,000 which combines stoichiometrically with hemoglobin to form a very stable complex which does not dissociate under any mild conditions (29). The molecular weight of the complex is approximately 170,000 (29). The partial pressure of oxygen required to half-saturate the complex is  $p_{1/2} = 0.30$  mm of Hg at 20° and pH 7.0 to pH 7.9 (16). The kinetics of reaction with oxygen has not been studied but it may well resemble hemoglobin H and carboxypeptidase-treated hemoglobin.

The hemoglobin-haptoglobin complex augments the diffusion of oxygen but to a very much less extent than does hemoglobin A. In two runs with type HA Millipore filters and heme concentrations of 2.76 mM and 4.80 mM, the augmented component of the oxygen flux was, respectively, 0.25 and 0.25  $\mu$ l per min. In a third run made with a 64- $\mu$  thick Millipore filter to increase the sensitivity of the measurements and heme concentration of 4.80 mM, the augmented component of the oxygen flux was 0.50  $\mu$ l per min. The hemoglobin-haptoglobin complex is known to be susceptible to air oxidation to the ferric state. Initially, the fraction of the complex in the ferrous oxygenated state was 90, 91, and 89% in the 3 runs, respectively. At the end of the runs, some oxidation had occurred; the fraction in the ferrous state was 84, 79, and 80%, respectively. The small augmented fluxes encountered demanded that special precautions be taken to prove that measured fluxes were real and not due to leakage in the apparatus. In these runs, the oxygen leak approached zero and did not exceed 0.04  $\mu$ l per min.

*Myoglobin*—The myoglobin molecule closely resembles the component subunits of the hemoglobin molecule. The molecular weight, 18,800, is one-fourth that of hemoglobin, and each mole-

<sup>4</sup> R. W. Briehl and H. M. Ranney, personal communication.

cule bears 1 heme. The oxygen affinity at 20° has been reported to be  $p_{\frac{1}{2}} = 0.65$  (30) and  $p_{\frac{1}{2}} = 0.70$  (31), and for this preparation was  $p_{\frac{1}{2}} = 0.56$  mm; an affinity 8 times that of hemoglobin under the same conditions. The carbon monoxide affinity is reported to be  $p_{\frac{1}{2}} = 0.03$  mm of Hg (30) and for this preparation was 0.03 mm of Hg. The high oxygen affinity of myoglobin is due mainly to the large oxygen combination velocity constant.

The augmented component of the oxygen flux through a myoglobin solution is of exactly the same magnitude as the augmented flux through a hemoglobin solution of the same concentration (expressed as moles of heme per liter, see Fig. 5).

Myoglobin is known to be readily oxidized by air. At the end of each experiment, from 10 to 25% of the myoglobin was found to be oxidized to the ferric state.

**Earthworm Hemoglobin**—The molecules of annelid hemoglobins are very large and are made of many subunits. Studies with the electron microscope (32–35) reveal a common plan for the structure of hemoglobins of a number of species (including *Lumbricus* and *Tubifex*). The molecular weight of earthworm hemoglobin is  $2.9 \times 10^6$  (36). We find the minimum molecular weight per heme to be about 28,000. Haughton, Kerkut, and Mundy (37) find the oxygen affinity to be  $p_{\frac{1}{2}} = 8$  mm at pH 7.3 and 20°. The oxygen dissociation curve was steeply sigmoid. We confirm their report and find  $p_{\frac{1}{2}} = 8.2$  mm of Hg, and  $n = 4$ . The rate of combination of oxygen with earthworm hemoglobin is similar to that found for mammalian hemoglobin (38). Since the oxygen affinities are similar, the rate of dissociation must also be of the same order as the rate of dissociation of oxygen from mammalian hemoglobin.

Earthworm hemoglobin, 4.3 mm heme, did not augment the diffusion of oxygen (Fig. 7).

**Tubifex Hemoglobin**—*Tubifex* hemoglobin is similar to earthworm hemoglobin except that the oxygen affinity is greater. Fox (39) reports  $p_{\frac{1}{2}} = 0.6$  mm; Scheler (40) reports  $p_{\frac{1}{2}} = 2.2$ ; we find  $p_{\frac{1}{2}} = 0.9$  mm of Hg;  $n = 1.3$ . The molecular weight is  $2.9 \times 10^6$  (41).

*Tubifex* hemoglobin, 1.5 mm heme, and 2.7 mm heme, did not augment the diffusion of oxygen (Fig. 7).

**Ascaris Perienteric Fluid Hemoglobin**—The hemoglobins of *Ascaris lumbricoides*, which have been investigated by Davenport (42) and brought to a high degree of purity by Wittenberg, Okazaki, and Wittenberg (19), are of particular interest because of the extreme slowness with which they give up oxygen. The half-time for the dissociation of oxygen from the perienteric fluid hemoglobin at 20° is 150 sec (42, 43), and the half-time for dissociation of carbon monoxide is 70 sec (43). The association velocity constants however are similar to those of mammalian hemoglobin (42, 43). The oxygen affinity is extraordinarily high;  $p_{\frac{1}{2}} = 0.001$  to 0.003 mm of Hg (43, 44). The molecular weight of the perienteric fluid hemoglobin is 320,000, and it is made of 8 subunits, each bearing 1 heme and of molecular weight 40,600 (20).

*Ascaris* perienteric fluid hemoglobin, 2.8 mm heme, did not augment the diffusion of oxygen.

**Succinyl Ascaris Perienteric Fluid Hemoglobin**—It was desirable to examine the diffusion of oxygen through a solution of hemoglobin of very high oxygen affinity and moderate molecular weight. Acylation of perienteric fluid hemoglobin with succinic anhydride gave a product with a molecular weight of 87,800, bearing 2 heme groups per molecule. The oxygen affinity was not studied.

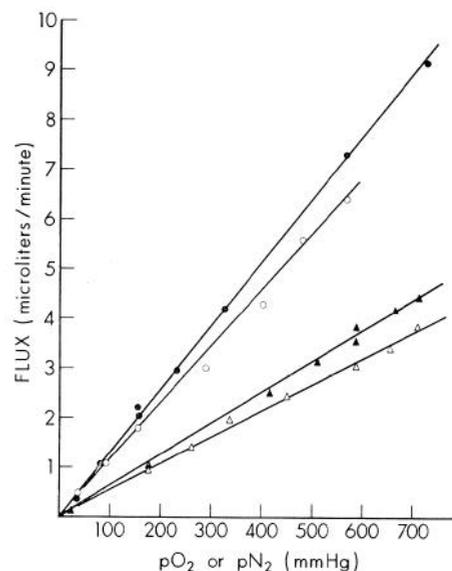


FIG. 7. Diffusion of oxygen and nitrogen through solutions of annelid hemoglobins. Earthworm hemoglobin, solid symbols; *Tubifex* hemoglobin, open symbols. Earthworm hemoglobin, 4.3 mm heme; *Tubifex* hemoglobin, 2.7 mm heme. Circles, oxygen; triangles, nitrogen.

Succinyl perienteric fluid hemoglobin, 3.1 mm heme, did not augment the diffusion of oxygen. The solution was viscous, precluding the study of more concentrated solutions.

**Ascaris Body Wall Hemoglobin**—The body wall hemoglobin of *Ascaris* shares with the perienteric fluid hemoglobin the unique property of dissociating oxygen very slowly. The half-time for the dissociation of oxygen at 20° is 70 sec (42, 43). The oxygen affinity calculated from the rate constants for oxygen combination and dissociation is  $p_{\frac{1}{2}} = 0.11$  mm of Hg (43). The molecular weight is 38,500.<sup>2</sup>

*Ascaris* body wall hemoglobin, 5.1 mm heme, did not augment the diffusion of oxygen through solutions held in standard Millipore filters, 150  $\mu$  thick, or through 64- $\mu$  thick Millipore filters.

**Hemerythrin**—Hemerythrin is an iron-containing protein of molecular weight of 107,000, composed of 8 subunits, each of molecular weight of 13,500 (45). Each subunit binds reversibly 1 oxygen molecule at a site which is thought to contain 2 ferrous iron atoms (46). The ratio of oxygen bound to the molecular weight of the protein is similar to that of hemoglobin and myoglobin. Kubo (47) reports the oxygen affinity as  $p_{\frac{1}{2}} = 2.9$  mm of Hg. We confirm his report and find for a concentrated solution of hemerythrin (4.4 mmoles of oxygen-binding capacity per liter);  $p_{\frac{1}{2}} = 2.2$  mm of Hg. Hemerythrin does not bind carbon monoxide.

Hemerythrin, 11 mmoles of oxygen-binding capacity per liter, augmented the diffusion of oxygen to a substantial extent (1.0  $\mu$ l per min, see Fig. 8 and Table I).

**Diffusion of Carbon Monoxide**—None of the hemoglobins examined augmented the diffusion of carbon monoxide. These included hemoglobin-A, 11 mm, 64- $\mu$  thick Millipore filter; hemoglobin H, 11 mm; *Ascaris* perienteric fluid hemoglobin, 2.8 mm; and *Ascaris* body wall hemoglobin, 2.9 mm and 5.1 mm, 64- $\mu$  thick Millipore filter. Representative experiments with hemoglobin and myoglobin are presented in Fig. 9.

Mochizuki and Forster (48) report that hemoglobin (6.5 mm,

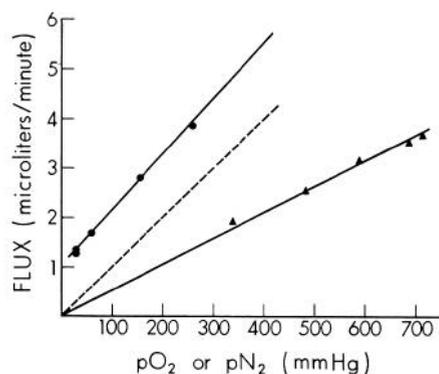


FIG. 8. Diffusion of oxygen and nitrogen through a solution of hemerythrin, 11 mmoles of oxygen-binding capacity per liter. *Solid circles*, oxygen; *solid triangles*, nitrogen. The *dashed line* is the calculated diffusive component of the oxygen flux.

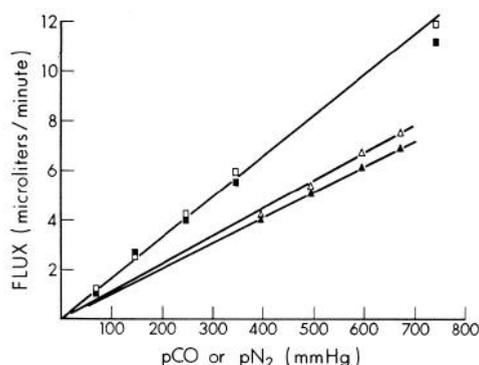


FIG. 9. Diffusion of carbon monoxide and nitrogen through solutions of hemoglobin and myoglobin. *Solid symbols*, hemoglobin; *open symbols*, myoglobin. Hemoglobin, 11 mM; myoglobin, 8.1 mM. *Squares*, carbon monoxide; *triangles*, nitrogen. Only a single line is drawn through the points for carbon monoxide since the two sets of data coincide.

37°, 150- $\mu$  thick Millipore filter) augments the flux of carbon monoxide to a limited extent, approximately 0.08  $\mu$ l per min.

Carbon monoxide abolishes the augmented flux of oxygen through hemoglobin solutions (1).

**Simultaneous Diffusion of Oxygen and Carbon Monoxide**—The diffusion of oxygen and carbon monoxide through a myoglobin solution, 7.2 mM, 64- $\mu$  thick Millipore filter, was measured under conditions where the myoglobin would be expected to be partitioned between oxygen and carbon monoxide in the proportion 60% oxymyoglobin, 40% carbon monoxide myoglobin. For this purpose, a mixture of oxygen and carbon monoxide, in which the ratio of oxygen to carbon monoxide was constant at 22:1, was mixed with varying proportions of nitrogen, and the resulting gas mixtures allowed to diffuse through a myoglobin-loaded Millipore filter. Separate portions of the myoglobin solution were equilibrated with each gas mixture, and the fraction of carbon monoxide myoglobin was estimated spectrophotometrically. This was 40% in each case. Initially, the myoglobin was 89% in the ferrous state; at the end of the run some oxidation had occurred and the fraction of ferrous myoglobin was 80%.

The diffusion of oxygen through this solution was augmented. The carbon monoxide flux was just above the detectable level as would be expected from simple diffusion alone at the very low partial pressures of carbon monoxide employed. The simul-

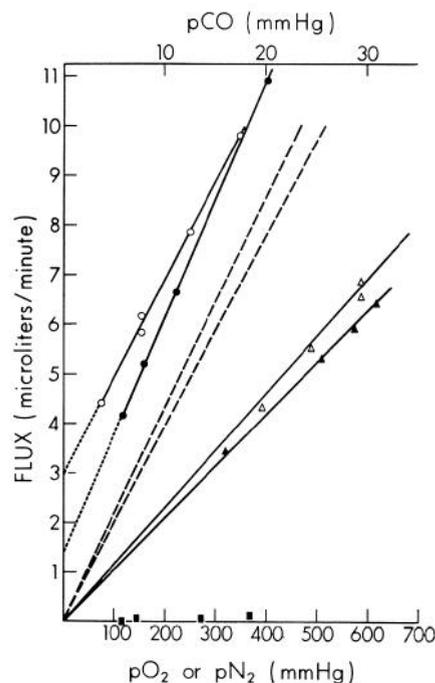


FIG. 10. Simultaneous diffusion of oxygen, carbon monoxide, and nitrogen through a solution of myoglobin (*solid symbols*) compared to the diffusion of oxygen and nitrogen through a solution of myoglobin in the absence of carbon monoxide measured in a separate experiment (*open symbols*). The ratio of oxygen to carbon monoxide in the gas mixture was constant (22:1), and it assumed that myoglobin in equilibrium with the gas would be 60% oxymyoglobin and 40% carbon monoxide myoglobin. Myoglobin, 7.2 mM; 64- $\mu$  thick Millipore filter. *Circles*, oxygen; *squares*, carbon monoxide; *triangles*, nitrogen. The *dashed lines* are the calculated diffusive components of the oxygen flux.

taneous diffusion of oxygen, therefore, did not bring about any substantial flux of carbon monoxide. The results of this experiment are given by the *solid symbols* in Fig. 10.

In a separate experiment, the diffusion of oxygen and nitrogen alone through a portion of the same myoglobin solution was determined (Fig. 10, *open symbols*). The augmented component of the oxygen flux, 2.95  $\mu$ l per min, was approximately twice the augmented flux in the presence of carbon monoxide myoglobin, 1.35  $\mu$ l per min.

Mochizuki and Forster (48) report that oxygen increases the very small augmented flux of carbon monoxide which they have detected. In these experiments, oxygen was present on both sides of the Millipore filter. They consider that the increase in carbon monoxide flux is probably not related to events in the bulk of the hemoglobin layer (*i.e.* facilitated diffusion), but results rather from the displacement by oxygen of carbon monoxide from hemoglobin at the surface. Stirring of the gas phase in their apparatus was necessarily limited. The oxygen probably served to suppress recombination of dissociated carbon monoxide with the hemoglobin at the Millipore surface.

#### DISCUSSION

The experiments were addressed to two questions. In what way does hemoglobin facilitate the movement of oxygen molecules? What chemical reactions are involved in this process? In addition, an effort was made to determine which parameters of the system could become rate-limiting.

It is at first essential to be certain that the rate-limiting step occurs in the body of the layer of hemoglobin solution and not at the boundaries. The flux was found to be inversely proportional to the length of the diffusion path, Figs. 3 and 4, and it is therefore limited by processes occurring in the body of the solution.

The inverse relation between flux and diffusion path length, characteristic of diffusion in many systems, implies that the concentration of a molecular species decreases with distance through the thickness of the layer of hemoglobin solution. The form of this profile has been discussed (23),<sup>5</sup> and treated fully by Wyman (8).

Two lines of evidence identify the diffusing species as oxyhemoglobin. First, the hemoglobin-augmented oxygen flux attains its maximal value at an oxygen partial pressure sufficient to saturate the hemoglobin on the side of the layer of solution exposed to oxygen (2)<sup>6</sup> and it is constant at greater partial pressure (2, 49) (Fig. 1). This behavior is expected if the hemoglobin-augmented flux depends on a gradient of oxyhemoglobin which reaches a maximum value when all of the hemoglobin on one side of the layer is converted to oxyhemoglobin. Second, Hemmingsen has demonstrated facilitated diffusion of <sup>18</sup>O through layers of hemoglobin (11) or myoglobin (12) solution exposed to oxygen ( $p_{O_2}$  greater than 20 mm) on both sides and thereby saturated with oxygen throughout. The gradients under the conditions of this experiment are: a gradient of <sup>18</sup>O-oxyhemoglobin diffusing into an environment of <sup>16</sup>O-oxyhemoglobin; and the converse, a gradient of <sup>16</sup>O-oxyhemoglobin diffusing into an environment of <sup>18</sup>O-oxyhemoglobin. Therefore, no large concentration of deoxyhemoglobin is required and oxyhemoglobin must be the diffusing species.

In general, two different types of carrier-mediated diffusion processes may be considered: "bucket brigade" or "stepping stone mechanisms" in which the oxygen-binding sites remain fixed in space and must be reasonably close for transfer to occur and, alternatively, mechanisms in which the binding sites or molecules bearing the binding sites must move. Bucket brigade mechanisms are well known and accelerate the movements, for instance, of ions in ion exchange membranes and soils, of protons in ice, and of electrons or holes in metals or in semiconductors. Scholander (2) has proposed a bucket brigade mechanism for hemoglobin-facilitated oxygen diffusion, and Enns (23), working in his laboratory, has recently reasserted this point of view.

<sup>5</sup> Enns (23) rests his argument on two premises: "... the enhanced transport due to hemoglobin is independent of oxyhemoglobin gradients and remains the same when there is no gradient at all." The first premise refers to the local gradient at particular points in the thickness of a layer of hemoglobin exposed to zero oxygen tension on one side. He believes the gradient through the layer to be nonlinear and reasons that since the flux is the same at all points in the layer, it is independent of the local gradient. This may be true, but it is not pertinent because at steady state the net rate of transport is a function of the concentrations at the boundaries only and is independent of the local gradients. Enns deduces his second premise from Hemmingsen's (11, 12) demonstration of the augmented flux of <sup>18</sup>O through a layer of hemoglobin solution fully saturated with oxygen. He states correctly that there is no oxyhemoglobin (all isotopic species) gradient in this situation, but errs in overlooking the gradient of <sup>18</sup>O-oxyhemoglobin. His deductions concerning the chemical reaction occurring, do not rest on firm premises. See also the previous discussion by Fatt and LaForce (5) concerning Hemmingsen's data.

<sup>6</sup> E. Hemmingsen, unpublished experiments (23).

TABLE I

Relation between facilitated oxygen flux and molecular size

The augmented oxygen fluxes are expressed as percentage of the augmented flux mediated by a hemoglobin solution of the same oxygen binding capacity.

Protein	Molecular weight	Minimum molecular weight	Diffusion coefficient	Augmented oxygen flux
			$cm^2/sec \times 10^7$	%
Earthworm hemoglobin	2,900,000	28,000	1.8 <sup>a</sup>	0
<i>Tubifex</i> hemoglobin	2,900,000	29,000	1.6 <sup>b</sup>	0
Haptoglobin-hemoglobin complex	170,000	43,000	3.4 <sup>c</sup>	18
Hemerythrin	107,000	13,500	6.1 <sup>d</sup>	60
Hemoglobin A	64,450	17,000	6.4 <sup>e</sup>	100
Hemoglobin H	63,480	16,000		80
Myoglobin	18,800	18,800	11.3 <sup>e</sup>	100

<sup>a</sup> Svedberg and Pederson (36).

<sup>b</sup> Scheler and Schneiderat (41); diffusion coefficients of related annelid hemoglobins are 1.90 and  $2.06 \times 10^{-7}$  cm<sup>2</sup> per sec (Antonini, Rossi-Fanelli, and Caputo (54) and Chew, Scutt, Oliver, and Lugg (55)).

<sup>c</sup> Guinand, Tonnelat, Boussier, and Jayle (56).

<sup>d</sup> Klotz and Keresztes-Nagy (45).

<sup>e</sup> Rossi-Fanelli, Antonini, and Caputo (57).

Diffusion mediated by moving carriers was firmly established by Osterhout (50), and it finds many examples in the work of Jacobs (51) and of Osterhout (52). A classic example is the diffusion of potassium ions, bound as potassium guaiacolate, through hydrophobic solutions containing guaiacol (53).

The trivial possibility that hemoglobin may augment the diffusion of oxygen merely by increasing the oxygen capacity of the solution may be rejected *a priori*, since the rate of diffusion is proportional to the gradient of the diffusing species, which is oxyhemoglobin and not total oxygen. One need not rely on deduction; this possibility is excluded by the results of experiments with annelid hemoglobins (Table I).

In order to establish whether movement of hemoglobin molecules is required to facilitate oxygen diffusion, the flux of oxygen through solutions of respiratory pigments of differing molecular size was measured (see Table I and Figs. 1 and 5 to 7). The three proteins of relatively low molecular weight, myoglobin, hemoglobin H, and hemoglobin A, facilitated diffusion to approximately the same extent. Two proteins of greater molecular size, hemerythrin and the haptoglobin-hemoglobin complex, augment diffusion to a lesser extent, and the two annelid hemoglobins of very large molecular weight either not at all or to a small extent within the limits of experimental error. Myoglobin, hemoglobin H, and the haptoglobin-hemoglobin complex probably react with oxygen at similar rates (Table II) and form a series within which comparisons are valid. Hemoglobin A and earthworm hemoglobin likewise react with oxygen at comparable rates (Table II). The decrease in facilitated diffusion with molecular size is greater than might be expected from the diffusion coefficients of the proteins (Table I). However, these coefficients are measured in dilute solution, and the hemoglobin solutions used to obtain the data of Table I, particularly the solution of haptoglobin-hemoglobin, were sufficiently concentrated to deviate substantially from ideality. Hemoglobin

TABLE II  
Rates of reaction and equilibria of some hemoglobins with ligands

The data are selected as close as possible to 20° and pH 7.0 or 7.4.

Protein	Equilibrium partial pressure for half-saturation with		Rate constants								References
	Oxygen	Carbon monoxide	Oxygen				Carbon monoxide				
			Combination		Dissociation		Combination		Dissociation		
			$k'$	$k'_1$	$k$	$k_1$	$l'$	$l'_1$	$l$	$l_1$	
mm Hg		$M^{-1} sec^{-1} \times 10^{-6}$		$sec^{-1}$		$M^{-1} sec^{-1} \times 10^{-6}$		$sec^{-1}$			
Sheep hemoglobin	14		2	20	30	30	0.12	3		0.04	24
Hemoglobin A	4-10	0.03	3	32	40	34	0.4	7		0.03	53
Hemoglobin H	0.08-0.4		30 <sup>a</sup>		<sup>b</sup>		8 <sup>a</sup>		<sup>b</sup>		15, 28
Myoglobin	0.7	0.03	14		11		0.5		0.017		31
Haptoglobin-hemoglobin	0.3										16
Hemoglobin											
Carboxypeptidase											
A-treated	0.3				16		9				58 <sup>c</sup>
Carboxypeptidase											
B-treated	1.0				23		5				58 <sup>c</sup>
Carboxypeptidase											
A- and B-treated	0.2				12		18				58 <sup>c</sup>
<i>Lumbricus</i>	8.2		2.3	12	30		0.3		<sup>b</sup>	<sup>b</sup>	38
<i>Tubifex</i>	0.9										
<i>Ascaris</i>											
Perienteric	0.002	0.1	1.5		0.0041		0.17		0.018		43, 44, 60
Body wall	0.11	0.13	1.2		0.23		0.12		0.039		42, 43, 60
Hemerythrin	2.2										47

<sup>a</sup> At 3°.

<sup>b</sup> Similar to hemoglobin A.

<sup>c</sup> Q. H. Gibson and E. Antonini, unpublished experiments quoted in Reference 59.

molecules therefore must move to facilitate the diffusion of oxygen.

The motions of hemoglobin molecules include both translational and rotatory components. An attempt was made to estimate the relative contributions of each to the facilitation of oxygen diffusion. Perutz (61) has shown that hemoglobin molecules in the red cell are arranged in a close packed lattice, in which the intermolecular distance, 75 Å, is the same as the effective diameter of the freely rotating molecules. Translational movement of the hemoglobin molecules at this concentration must be very limited, but they are in true solution and retain freedom of rotation.

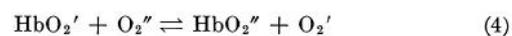
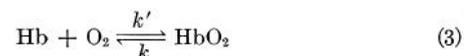
The concentration of hemoglobin in the red cell is 34% or 22 mM with respect to the heme groups. The corresponding concentration at which the molecules of myoglobin are close packed, 26 mM, may be estimated from the dimensions of the molecule, which for this purpose may be considered as a prolate ellipsoid with  $a = 22$  Å,  $b = 12$  Å.<sup>7</sup> Myoglobin and hemoglobin solutions of this concentration augment the diffusion of oxygen and to the same extent Fig. 5. The flux mediated per unit of heme concentration by these very concentrated solutions is about one-tenth the flux mediated per unit of heme in dilute solutions which approach ideality. The experiment might suggest that about 10% of the facilitated flux is brought about by molecular rotation.

This conclusion is contradicted sharply by diffusion theory (8) which indicates that molecular rotation contributes only a

negligible proportion of the facilitated flux. Perhaps the diffusion of protein molecules in concentrated solution is greater than anticipated. Resolution of this contradiction must await measurements of the self-diffusion of hemoglobin in concentrated solutions. In a very short diffusion path, such as may perhaps be found inside of cells, or in very concentrated solution, the contribution from molecular rotation may be relatively greater.

Reversible oxygen binding is a sufficient condition for facilitation and no special property of hemoglobin is required, since hemerythrin, which is not a heme protein, but which binds oxygen reversibly at a site which contains two ferrous iron atoms, facilitates oxygen diffusion (Fig. 8) (49).

Reactions of hemoglobin which might occur during facilitated diffusion include the well known combination and dissociation of oxygen (or carbon monoxide, Reaction 3) and ligand displacement in which an oxygen molecule displaces another from combination with hemoglobin (Reaction 4).

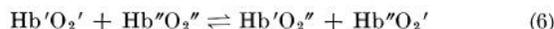


Ligand displacement reactions have never been discovered (24), although they would be difficult to detect if the rate were limited by the rate of ligand dissociation ( $k$  in Reaction 3). In the present context, the latter could not be distinguished from combination-dissociation reactions. Two reactions may be considered in which ligands are transferred from one hemoglobin molecule to another. These are

<sup>7</sup> J. C. Kendrew, personal communication.



and



Transfer of oxygen from oxy- to deoxyhemoglobin (Reaction 5) was suggested by Scholander (2) as one possible mechanism of facilitated diffusion. Exchange of oxygen molecules between oxyhemoglobin molecules (Reaction 6) is presumably the "molecular collision exchange" reaction invoked by Enns (23) to explain facilitated diffusion.<sup>5</sup> Ligand transfer between hemoglobin molecules (Reactions 5 and 6) requires that the molecules collide to react; the rate should be proportional to the square of the hemoglobin concentration. Actually the rate of facilitated diffusion is approximately proportional to the hemoglobin concentration at lower concentrations, and declines at higher concentrations where collision should be most frequent (Fig. 5). By exclusion, the reactions taking place during facilitated diffusion are solely combination and dissociation of oxygen and hemoglobin.

The rate limitation imposed by the chemical reactions is preferably described in terms of the individual rate constants for association and dissociation rather than by ligand affinities. Two proteins may have similar ligand affinities (*e.g.* hemoglobin H and *Ascaris* body wall hemoglobin) but facilitate diffusion to differing extents, or the converse may hold, hemoglobin H and hemoglobin A with very different oxygen affinities facilitate diffusion to nearly the same extent.

Hemmingsen's (11, 12) experiments with labeled oxygen probably restrict the rate-limiting step to reactions of oxyhemoglobin. He showed that the rate of facilitated diffusion of <sup>18</sup>O through a layer of hemoglobin saturated with oxygen throughout was equal to the rate of facilitated diffusion of oxygen through a similar layer of hemoglobin solution exposed to zero oxygen tension on one side. The two experiments were necessarily performed separately. To the extent that the rates actually were the same, the rate-limiting step must be the same in each case and must be a reaction of the only species present in appreciable concentration, oxyhemoglobin. Therefore, neither the ligand transfer to deoxyhemoglobin (Reaction 5), which is ruled out on other grounds, nor combination of deoxyhemoglobin with oxygen (Reaction 3, left to right) may be rate-limiting. In this experiment, the oxygen concentration everywhere in the layer of solution is large (more than  $3 \times 10^{-5}$  M) and the maximum permissible rate of combination of deoxyhemoglobin with oxygen is 5,000 to 100,000 times greater than the rate of oxygen dissociation. Oxygen dissociation must be rate-limiting.

*Ascaris* body wall hemoglobin and succinyl *Ascaris* perienteric fluid hemoglobin, although of appropriate molecular size, do not facilitate oxygen diffusion. This may be ascribed to the very small rate of oxygen dissociation.

The ligand dissociation rate may be changed by substituting for oxygen carbon monoxide which dissociates 300- to 1000-fold more slowly. The ligand combination rate is also decreased, but, particularly in the case of hemoglobin H, to a much lesser extent. None of the hemoglobins tested brought about a detectable facilitation of carbon monoxide diffusion. There is in fact a very small facilitation of carbon monoxide diffusion (48) which was detected by a more sensitive technique. The available data, Table III, indicate that large decreases in ligand dissociation rates abolish facilitated diffusion, but leave open the question of

TABLE III

Comparison of rate constants for dissociation of ligands with facilitation of diffusion

The augmented oxygen fluxes are expressed as percentage of the augmented flux mediated by a hemoglobin A solution of the same oxygen-binding capacity.

Protein	Dissociation rate constant		Augmented flux	
	Oxygen	Carbon monoxide	Oxygen	Carbon monoxide
	<i>sec</i> <sup>-1</sup>		%	
Hemoglobin A.....	34	0.03	100	<5
Hemoglobin H.....	<sup>a</sup>	<sup>a</sup>	80	0
Myoglobin.....	11	0.017	100	0
<i>Ascaris</i> body wall hemoglobin.....	0.04	0.2	0	0

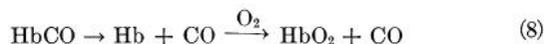
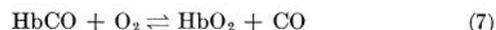
<sup>a</sup> Similar to hemoglobin A.

the effect of smaller changes. Wyman (8) has suggested possible alternative explanations of why facilitation is lacking in the case of carbon monoxide.

The available hemoglobins, Table II, do not provide a very great range of oxygen combination rate constants. However, the rate of combination is proportional to the oxygen concentration which is changed 20-fold during the course of each experiment, without any change in the facilitated oxygen flux. The profile of oxygen concentration through the thickness of the layer of solution changes nevertheless (8).<sup>6</sup>

Two experiments were designed to test the possibility that ligand diffusion might be accelerated by ligand displacement (Reaction 4) or ligand transfer (Reactions 5 and 6) even though these reactions were considered highly improbable. The possibility was considered that *Ascaris* body wall hemoglobin, which dissociates oxygen very slowly, might undergo relatively more rapid displacement or ligand transfer reactions. In fact, neither this protein nor succinyl *Ascaris* perienteric fluid hemoglobin facilitate oxygen diffusion.

Although the ligand displacement reaction (Reaction 7) is thought to occur in two steps (Reaction 8) of which the first is rate-limiting (24)



the possibility was considered that some form of bucket brigade mechanism might accelerate the diffusion of carbon monoxide when oxygen was diffusing simultaneously. In an experiment designed to test this possibility (Fig. 10), oxygen brought about no acceleration of carbon monoxide flux.

The major conclusions which emerge are as follows. Facilitation results from random displacements of the carrier molecules. Although these displacements are predominantly translational, the role of rotatory displacements (when the diffusion path is very short or the solution very concentrated) is left open. The chemical reactions taking place during facilitated diffusion are only combination and dissociation of oxygen and hemoglobin. Bucket brigade mechanisms presupposing ligand displacement or ligand transfer reactions are excluded. The rate of ligand dissociation may be rate-limiting although more experiments bearing on this point are to be desired. This suggests that a

mathematical description of the phenomenon should take into account not only the diffusion coefficient of hemoglobin but also the mean time during which hemoglobin and oxygen molecules remain in combination.<sup>8</sup>

*Acknowledgments*—Much of the writing was done during a stay in Rome and it is a pleasure to thank my hosts Drs. Antonini and Wyman for their hospitality, and to thank them and Dr. Noble for many discussions on this subject. I am grateful to Drs. Nagel and Ranney for their gifts of hemoglobins. It is a pleasure to acknowledge the skilled assistance of Miss Lidija Kucinskis.

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<sup>8</sup> Note—Since this paper was submitted, two related articles have appeared dealing with the profiles of oxygen tension and oxyhemoglobin concentration within a layer of hemoglobin solution through which oxygen is diffusing. These papers provide additional evidence that a gradient of oxyhemoglobin concentration is required for the facilitation of oxygen diffusion (Zilversmit, D. B., *Science*, **149**, 874 (1965); Scholander, P. F., *Science*, **149**, 876 (1965)).