SOLUBILITIES AND DIFFUSIVITIES OF OXYGEN IN HEMOLYZED HUMAN BLOOD SOLUTIONS

D. L. WISE *and* G. HOUGHTON

From the School of Engineering, PMC Colleges, Chester, Pennsylvania 19013, and the School of Engineering Science, Florida State University, Tallahassee, Florida 32306. Dr. Wise's present address is National Institutes of Health Research Fellow, Engineering Sciences Laboratory, Harvard University, Cambridge, Massachusetts 02138.

ABSTRACT By continuous absorption and by bubble collapse methods respectively, the solubilities and diffusion coefficients of oxygen in water and in dilute solutions of human hemoglobin (1.11, 2.22, and 4.44 wt%) have been determined at one atmosphere and 10°, 20°, 30°, 40°, 50°, and 60°C. Measured equilibrium constants, oxygen/hemoglobin ratios and isochoric heats of solution have been interpreted in terms of various mechanisms for oxygen-hemoglobin interaction. Oxygen diffusivities obtained experimentally for the hemolyzed blood solutions have been found to compare favorably with those predicted by a model of facilitated transport proposed by Houghton (1966). The diffusion measurements indicate that, while kinetic phenomena cannot be ignored, the over-all rate of exchange of oxygen with hemoglobin is not a controlling factor in facilitated diffusion. Anomalous equilibrium constants and temperature coefficients have been observed in the most dilute hemoglobin solution at the lowest temperatures.

INTRODUCTION

Using hemoglobin and myoglobin solutions respectively, Scholander (1960) and Wittenberg (1959) independently discovered that an oxygen-carrying protein can facilitate or enhance oxygen diffusion in aqueous solutions. Scholander (1960) found oxygen to diffuse up to eight times faster through hemolyzed human blood than through water when both were confined within the pores of a Millipore membrane with air on one side and a vacuum on the other, some enhancement being also found for nonhemolyzed or whole red blood cells. The existence of carrier-facilitated oxygen diffusion was subsequently confirmed by Hemmingsen and Scholander (1960), Hemmingsen (1962, 1963), Mochizuki and Forster (1962), Enns (1964), Wittenberg (1963, 1965, 1966), and Keller and Friedlander (1966), all measuring steady-state gas diffusion through liquid-saturated membranes. Oxygen transport rates have been found to be suppressed by solidifying the solution with agar gel (Scholander, 1960);

to be proportional to the carrier content at low concentrations but pass through a maximum at higher concentrations (Scholander, 1960; Wittenberg, 1966); to be anomalously reduced by an oxygen back-pressure on the membrane (Hemmingsen and Scholander, 1960; Enns 1964); to be decreased by increasing the protein molecular size (Wittenberg, 1966); and to be inversely proportional to the length of the diffusion path (Wittenberg, 1966). While Mochizuki and Forster (1964) report some enhanced diffusion of carbon monoxide through hemoglobin, Wittenberg (1966) could detect no such enhancement in similar experiments.

Scholander (1960) and Enns (1964) have proposed a collisional mechanism for facilitated diffusion in which oxygen molecules are passed on by contact collisions of neighboring hemoglobin and oxyhemoglobin molecules, which themselves merely oscillate about fixed positions or diffuse extremely slowly. Such collisional mechanisms have been eliminated by Wittenberg (1966) on the grounds that they predict facilitation to be independent of carrier molecule size, which is not found to be the case experimentally. Also, estimates of rotational and translational momentum contributions to diffusion have indicated (Wittenberg, 1966; Wyman, 1966) that molecular rotation effects will contribute negligibly to enhancement except perhaps when the hemoglobin molecules are closely packed inside red cells. From qualitative and quantitative studies of the existing experimental information Wittenberg (1966) and Wyman (1966) have unequivocally concluded that enhancement arises primarily from the translational diffusion of protein carrier molecules capable of holding a large number of bound oxygen molecules.

The experimental-theoretical deductions of Wittenberg (1966) and Wyman (1966) had been foreseen by Collins (1961), Wang (1963), LaForce and Fatt (1962, 1964), and Snell (1965), who had earlier devised mathematical models for enhancement based on the translational diffusion of oxygen carriers. The objective of these mathematical treatments was to define diffusion fluxes for bound and free oxygen that could be used to interpret the data from steady-state enhanced diffusion experiments. Although these earlier mathematical models usually assumed chemical equilibrium in the oxygen-hemoglobin system, Collins (1961) allowed for rate phenomena by modifying the effective solute concentration with an empirical factor. Snell (1965) has proposed to account for the oxygen-hemoglobin kinetics by numerical methods, the indications being that such rate phenomena may not be negligible. Recently (LaForce, 1966) a comparison of experimental steady-state fluxes with those calculated theoretically by a digital computer has demonstrated that kinetic effects are important in the carbon monoxide-oxygen-hemoglobin system, even though earlier studies (Fatt and LaForce, 1961; LaForce and Fatt, 1962) had indicated that equilibrium was reached virtually instantaneously in the oxygen-hemoglobin system. In this connection, various physicochemical models for the kinetics of hemoglobin-oxygen interaction have been discussed, in relation to existing kinetic and equilibrium data, by Rossi-Fanelli et al. (1964), Roughton (1965), and Wyman (1948, 1966).

The existing theories of facilitated diffusion, discussed above, have concentrated upon defining an effective diffusion flux that could be used to interpret the results from steady-state oxygen transport experiments. However, by starting from continuity rather than flux considerations, Houghton (1966) has formulated a general mathematical model for Brownian-enhanced mass transfer through dispersoids that is applicable to blood-like fluids under both steady and unsteady conditions. In addition to analytically accounting for kinetic or rate phenomena as perturbations on equilibrium theory, such considerations of unsteady-state diffusion have led to the conclusion that a solubility factor had been omitted from earlier steady-state definitions of the effective diffusivity. Thus, it is one purpose of the present communication to examine the validity of this model in comparison with measurements of the solubilities and diffusion coefficients of oxygen in hemoglobin solutions and water. The diffusion coefficients will be determined by timing the incremental collapse of small oxygen bubbles dissolving in the solvent (cf. Houghton et al., 1962; Wise and Houghton 1966) so that any influence on diffusion arising from the pores and membranes in earlier investigations will be absent from the present measurements.

THEORY

The system under consideration comprises a high molecular weight carrier molecule or particle dispersed in water containing a diffusing solute which interacts reversibly with the carrier. In the present experiments the carrier is human hemoglobin and the diffusing solute is dissolved oxygen. In what follows, the theory of Brownian-enhanced transport through dispersoids proposed by Houghton (1966) will be extended to the more complicated water-oxygen-hemoglobin system such that the model can be conveniently tested using experimental solubilities and diffusivities.

From the standpoint of general diffusion theory, the probability $P(\mathbf{r}, t)$ of finding a particle at a spatial point $\mathbf{r}$ at time t is given by (cf. Houghton, 1966):

$$\partial P/\partial t = D\nabla^2 P + Q \qquad (1)$$

where D is the diffusion coefficient and Q is the probable rate of creating or depleting the particulate species under consideration by such source-sink phenomena as chemical reactions. In molecular diffusion D would be the molecular diffusivity of a chemical species (cf. Kirkwood, 1946), whereas for the diffusion of colloidal particles D would become the Brownian diffusivity (cf. Einstein, 1905; Chandrasekhar, 1943). The physical assumptions and mathematical-stochastic methods (Chandrasekhar, 1943; Kirkwood, 1946) upon which diffusion equation 1 is founded confines its use to homogeneous systems and/or dilute solute systems. Fortunately for the present considerations, the carrier is homogeneously distributed and the solute is in dilute solution, so that equation 1 can be recast in different forms to rigorously represent both carrier and solute diffusion.

In the case of solute diffusion, $P(\mathbf{r}, t)$ will be proportional to X, the mole fraction

of unbound gas in the liquid phase, so that the transport equation for solute, corresponding to equation 1 is:

$$\partial X/\partial t = D_M \nabla^2 X - S \tag{2}$$

where D_M is the molecular diffusivity of unbound solute and S represents solute exchange between the carrier and solvent. For the sparingly soluble gases usually encountered in enhanced diffusion, X will be equivalent to the moles of dissolved gas per mole of water and D_M will be closely approximated by the aqueous diffusivity if the carrier concentration is not high. If the carrier is in high concentration, then equation 2 will still be valid, but D_M will then become a function of carrier concentration.

If M is the mole fraction of carrier species throughout the three-component mixture and Y is the moles of bound solute per mole of carrier, then the probability P of finding a carrier-bound oxygen molecule at any spatial point and time will be proportional to the product MY. Corresponding to equation 1 the transport of bound oxygen associated with the carrier will be governed by:

$$\partial(MY)/\partial t = D_C \nabla^2 (MY) + S \tag{3}$$

where D_C is the diffusivity of the carrier species. For dilute carrier solutions M will be the moles of carrier per mole of water. Since the hemoglobins are very large protein molecules of molecular weights 13,000–3,000,000, the carrier diffusivity can be estimated from the Einstein (1905) formula:

$$D_C = kT/6\pi a\eta \tag{4}$$

where a is the equivalent spherical radius of the particle and η is the solvent viscosity at an absolute temperature of T, the Boltzmann constant being k.

Equations 2 and 3 are coupled through the source-sink terms of magnitude S representing solute exchange between the solvent and carrier, opposite signs being required by mass conservation. In blood-like fluids, exchange is believed to involve oxygen-hemoglobin-oxyhemoglobin equilibria, the over-all reaction being:

$$Hb_4 + 4O_2 \underset{k_0}{\overset{k_0'}{\rightleftharpoons}} Hb_4(O_2)_4 \; ; \qquad K_4^0 = k_0'/k_0 \tag{5}$$

where k_0' and k_0 are velocity constants for the forward and reverse reactions and K_4^0 is the equilibrium constant. Following Adair's (1925) discovery that mammalian hemoglobin is a tetramer containing four atoms of iron, it has been proposed that the over-all reaction (equation 5) contains contributions from at least four consecutive chemical equilibria:

$$\begin{aligned} Hb_4 + O_2 &\rightleftharpoons Hb_4O_2, \qquad (K_1 = k_1'/k_1) \\ Hb_4O_2 + O_2 &\rightleftharpoons Hb_4O_4, \qquad (K_2 = k_2'/k_2) \\ Hb_4O_4 + O_2 &\rightleftharpoons Hb_4O_6, \qquad (K_3 = k_3'/k_3) \\ Hb_4O_6 + O_2 &\rightleftharpoons Hb_4O_8, \qquad (K_4 = k_4'/k_4) \end{aligned} \tag{6}$$

where $K_4^0 = K_1K_2K_3K_4$. Since at least eight unknown rate constants are involved in the reactions of equation 6 various attempts have been made to reduce the disposable constants to a number that could be determined by experiment (cf. Roughton, 1965). To base the exchange term S of equations 2 and 3 on such a plethora of consecutive reactions would lead to virtually insurmountable difficulties in their analytic solution, and even numerical solutions using a digital computer might be cumbersome (cf. Snell, 1965; LaForce, 1966).

However, the present measurements of oxygen solubility and diffusion in hemolyzed human blood solutions, to be described later, indicate that the experimental conditions are such that, except at the highest hemoglobin dilutions, the oxygen equilibrium is independent of hemoglobin concentration. Such hemoglobin independence implies that a simple equilibrium of the following form may be justified:

$$O_2(\text{unbound}) \underset{k_y}{\overset{k_x}{\rightleftharpoons}} O_2(\text{bound}); \qquad K = k_x/k_y\,. \tag{7}$$

For first order kinetics in both directions, equilibrium 7 leads to the rate equation:

$$\partial Y/\partial t = k_x X - k_y Y \tag{8}$$

where k_x and k_y are component rate constants of equilibrium equation 7. At equilibrium $\partial Y/\partial t = 0$, so that

$$Y = KX \tag{9}$$

where $K = k_x/k_y$ is the equilibrium constant. If the rate constants k_x and k_y are large then, following the perturbation method of Houghton (1964), the kinetic equation 8 can be rearranged into the form $(1/k_y)\partial Y/\partial t = KX - Y$, where $1/k_y$ is small and the exchange rate becomes a perturbation on the equilibrium state. Thus, $Y = KX$ when $k_y \to \infty$, so that a first approximation for large but finite k_y is:

$$Y = KX - (K/k_y)\partial X/\partial t. \tag{10}$$

It is now convenient to define a total solute mole fraction N, including bound and unbound solute, by introducing the solute balance:

$$N = X + MY. \tag{11}$$

By combining equations 2, 3, 10, and 11 the transport equation for total solute is

found to be:

$$\frac{\partial N}{\partial t} = \frac{D_M + D_C MK}{1 + MK} \nabla^2 N + \frac{(D_M - D_C)MK}{k_y(1 + MK)^2} \nabla^2 \frac{\partial N}{\partial t} \qquad (12)$$

The second term on the right-hand side of equation 12 represents the influence of a finite exchange rate between hemoglobin and dissolved oxygen, its effect being small since we have presumed large reaction rates and small deviations from equilibrium. Thus, introducing the difference approximation $\nabla^2(\partial N/\partial t) \approx \nabla^2(\Delta N/\Delta t)$ may not lead to large errors when the rate term is small, as the perturbation theory requires and experiment subsequently confirms. For an empty solute region interacting with a neighboring region containing saturated carrier particles, the time Δt for the solute concentration N to decrease to $1/e$ of its initial value will be of order of the relaxation time $1/k_y$, according to the linear kinetic equations 8 and 9. If the carrier region is almost completely scavenged of solute, we can write $\Delta N \approx N/e$ when $\Delta t \approx 1/k_y$, so that $\nabla^2(\partial N/\partial t) \approx (k_y/e)\nabla^2 N$, and equation 12 becomes:

$$\frac{\partial N}{\partial t} = \left[\frac{D_M + D_C MK}{1 + MK} + \frac{(D_M - D_C)MK}{e(1 + MK)^2}\right] \nabla^2 N \qquad (13)$$

where $e = 2.718$ is the base of natural logarithms. In equation 13 the small rate term of equation 12 has been approximated by neglecting the influence of the reverse reaction during unidirectional exchange.

Equation 13 takes the form of the parabolic diffusion equation and we can conclude that the total diffusion of bound and unbound solute can be represented by an effective diffusion coefficient D_{EK} composed of contributions arising from free solute and carrier diffusion as well as kinetic phenomena:

$$D_{EK} = \frac{D_M}{1 + MK} + \frac{D_C MK}{1 + MK} + \frac{(D_M - D_C)MK}{e(1 + MK)^2}. \qquad (14)$$

As required physically, $D_C \leq D_{EK} \leq D_M$ and $D_{EK} \approx D_M$ for $0 < K \ll 1$, while $D_{EK} \approx D_C$ for $K \gg 1$; facilitated diffusion occurs when $D_C MK > D_M$. One deduction from expression 14 that is not immediately obvious from the original assumptions of the Brownian model is that the rate effect will become a maximum when $MK = 1$, although zero rates in the limits $MK \to 0$ and $MK \to \infty$ can be expected from physical considerations.

If kinetic effects are ignored, then inspection of equations 1–14 reveals that, without altering the final results, the isotherm $Y = KX$ of equation 9 can be replaced by the somewhat more general, but still linear, form: $Y = KX + Y_0$, where Y_0 is a constant intercept. For instantaneous equilibrium, the diffusion equation corresponding to equation 13 would still be parabolic, $\partial N/\partial t = D_E \nabla^2 N$, but with

$$D_E = \frac{D_M + D_C MK}{1 + MK}. \qquad (15)$$

Thus, the equivalent diffusivity of equation 15 might apply to nonlinear isotherms if $K = (Y - Y_0)/X$ is taken to be the local average slope of the isotherm in the region of composition where most of the mass transfer occurs. In effect, such an approach would approximate a nonlinear isotherm by a linear line segment covering the region of interest.

The objective of the experiments which follow will be to examine the validity of the foregoing concepts and equations by measuring equilibrium constants K and diffusion coefficients D_L for oxygen in hemolyzed human blood at three hemoglobin concentrations.

EXPERIMENTAL

The solubilities of oxygen in aqueous hemoglobin solutions and water were determined by an adaptation of the technique of Morrison and Billett (1952). Oxygen diffusion coefficients were measured in the same solvents by following the solution rate of single oxygen bubbles using a modification of the methods described by Houghton et al. (1962) and Wise and Houghton (1966).

To prepare the hemoglobin solutions, a weighed amount of citrate-buffered and just outdated human red blood cells, as freshly released from sealed plastic containers, were frozen, thawed, and degassed in a 6 liter vacuum flask at 29 mm Hg for at least 45 min after which time the cells were completely hemolyzed as revealed by microscopic examination. The hemolyzed and degassed red cells were then mixed with a predetermined amount of degassed water to provide final blood solutions containing 5, 10, and 20% of original blood cells by weight. Degassed water was obtained by boiling deionized water for 60 min and cooling in a stoppered flask in the absence of air as discussed by Wise and Houghton (1966). The 10 and 20 wt% blood cell solutions were filtered through a thin layer of Dicalite filteraid to remove cell debris. Before use, all blood solutions were degassed for a further 30 min period at 29 mm Hg.

To analyze quantitatively for methemoglobin and hemoglobin, the optical densities of phosphate-buffered blood solutions were compared spectroscopically at a wavelength of 635 mμ after adding sodium cyanide and acetic acid and then at 540 mμ after adding potassium ferricyanide as described by Evelyn and Malloy (1938) and more recently by Eilers (1967). Based on 26 samples roughly equally distributed between solutions containing 5, 10, and 20 wt% packed cells, these hemolysates were found to average 1.11, 2.22, and 4.44 wt% hemoglobin respectively, corresponding to packed red cells containing 22.2 $\pm$ 1.8 wt% hemoglobin, the deviation being the average. By repeating light absorption measurements on the same samples, it was found that the reproducibility of the spectroscopic technique averaged 5.4% of the hemoglobin content, as compared with a deviation of 8.1% from the average (1.8% in 22.2 wt%) for the 26 samples. Therefore, most of the deviation resides in the spectroscopic technique itself rather than variations in the hemoglobin content between various samples of packed red cells. Independent corroboration of this comes from the solubility and diffusion measurements themselves, which were found not to be sensitive enough to detect variations in hemoglobin content between different batches of packed red cells. Thus, fresh samples of the hemoglobin solutions were made up as required. It is important to note that the final comparisons of theory with experiment are independent of a knowledge of the absolute hemoglobin content since these comparisons are ultimately based solely on the solubility and diffusion measurements for which the errors are about 3%. The analyses for methemoglobin showed that the hemoglobin converted to methemoglobin under the experi-

mental conditions was on the borderline of detectability by the spectral technique, which was about 1%. Conversions to methemoglobin of less than 1% of the hemoglobin are to be expected since degassed water was used in the experiments and the contact time of the hemoglobin with oxygen was always less than 40 min. Indeed, the dark brown color characteristic of significant conversion to methemoglobin was never observed in any of the blood solutions, either during solubility or diffusion measurements.

Solubilities. Oxygen solubilities in water and aqueous hemoglobin solutions were determined in the apparatus illustrated in Fig. 1 *a*, which permits a large volume of liquid to come into equilibrium with a small amount of gas a little at a time. Hemoglobin solution in the storage column was allowed to drip continuously through the Pyrex glass preheater or precooler column into the coils of the closed absorption column filled with pure oxygen at an accurately known pressure close to atmospheric. As gas was absorbed, the level of the liquid absorbent in the combined manometer-burette system (15 ml capacity) was continually raised to maintain atmospheric pressure. By plotting the volume of gas absorbed as a function of the weight of liquid collected, the solubility of the gas could be calculated from the slope of the resulting straight line, as discussed by Morrison and Billet (1952). Least squares regression methods using a digital computer showed that the experimental data fitted a straight line with a standard deviation of typically about 0.2%. As determined by inlet and outlet thermometers, the temperatures of the preheater or precooler and the absorption column could be adjusted and maintained at within 0.1°C of the temperatures 10°, 20°, 30°, 40°, 50°, and 60°C by water circulating from a thermostated bath.

Diffusion coefficients. The diffusivities of dissolved oxygen were determined by microscopically measuring the diameters D of small oxygen bubbles with time t as they dissolved in degassed hemoglobin solutions and water. The solvent was contained within an inner Acrylic plastic cell (55 × 6 × 5 mm), the upper wall of which was horizontal and served to confine the bubble as shown in Fig. 1 *b*. Thermostated water was circulated in the annular space between the inner cell and an outer jacket (70 × 20 × 11 mm), the temperature being adjusted and maintained to within 0.1°C of one of six different temperatures: 10°, 20°, 30°, 40°, 50°, and 60°C. The experimental technique was the same as that described by Wise and Houghton (1966) with the exception that the initial bubbles (0.2–0.6 mm diameter) in the

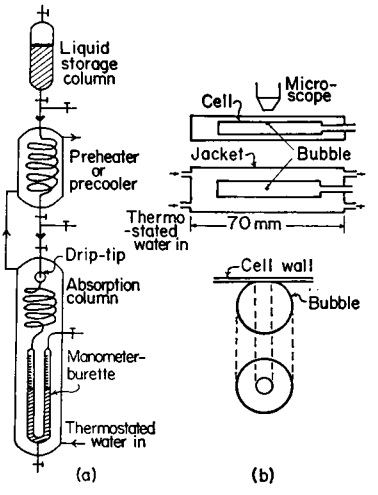

FIGURE 1 Equipment: (*a*) Solubility apparatus. (*b*) Bubble collapse apparatus showing side and top view sections to scale of the bubble absorption cell, with enlarged views of the bubble exhibiting the flattened inner ring arising from confinement by the horizontal cell wall.

present measurements were formed one at a time at the under surface of the cell wall (cf. Fig. 1 *b*) rather than in a group. It was found that single bubbles could be introduced under the cell wall by using an oxygen-filled hypodermic syringe with a fine needle that had been crimped near the point. The diffusion coefficient D_L was obtained from the collapse data by plotting D^2 vs. t, and determining the initial linear slope m in mm²/min from the equation:

$$D^2 = D_0^2 - mt \tag{16}$$

The diffusivity was then computed from the expression:

$$D_L = \frac{3.00\ m}{\alpha} \frac{273}{T} \frac{P}{P - p} \times 10^{-5}\ \text{cm}^2/\text{sec} \tag{17}$$

where α is the Bunsen absorption coefficient or gas solubility, P is the total pressure, and p the vapor pressure of the solvent at $T°K$, the temperature of the experiments. As discussed by Wise and Houghton (1966) the numerical geometry coefficient of 3.00 in equation 17 was

TABLE I

EXPERIMENTAL SOLUBILITIES AND DIFFUSION COEFFICIENTS OF OXYGEN IN AQUEOUS SOLUTIONS OF HUMAN HEMOGLOBIN

wt % packed red cells	Temp	α	S_0	Initial slope	$D_L \times 10^5$
	(°C)	(*ml/ml*)	(*ml/1000g*)	m(*mm²min⁻¹*)	(*cm²sec⁻¹*)
0 % (pure water)	10	0.0373	37.3	0.0234	1.82
	20	0.0309	30.9	0.0258	2.38
	30	0.0256	25.7	0.0272	2.97
	40	0.0231	23.3	0.0325	3.93
	50	0.0208	21.0	0.0315	4.31
	60	0.0192	19.5	0.0373	5.83
5 %	10	0.0461	46.1	0.0251	1.59
	20	0.0369	37.0	0.0265	2.05
	30	0.0300	30.1	0.0299	2.82
	40	0.0270	27.2	0.0332	3.46
	50	0.0257	26.0	0.0324	3.65
	60	0.0234	23.8	0.0385	5.03
10 %	10	0.0661	66.1	0.0254	1.12
	20	0.0499	49.9	0.0273	1.57
	30	0.0421	42.3	0.0304	2.04
	40	0.0358	36.1	0.0313	2.45
	50	0.0339	34.3	0.0346	2.94
	60	0.0292	29.7	0.0401	4.19
20 %	10	0.0925	92.6	0.0257	0.81
	20	0.0753	75.4	0.0286	1.09
	30	0.0608	61.1	0.0345	1.60
	40	0.0490	49.4	0.0347	1.98
	50	0.0401	40.6	0.0351	2.52
	60	0.0345	35.6	0.0406	3.60

determined by calibrating the cell with the oxygen-water system using a diffusivity of 2.60×10^{-5} $cm^2\ sec^{-1}$ at 25.0°C, determined to within ±1% by polarography (Kolthoff and Miller, 1941). The validity of the calibrations was checked against known diffusivities for other gases in water and against geometry factors estimated independently by theoretical methods. The oxygen bubbles were large enough, and their rates of collapse small enough, for surface tension and radial convection effects to be negligible compared with the experimental error (cf. Epstein and Plesset, 1950; Houghton et al., 1962; Cable, 1967).

The slope m used in equation 17 and reported in Table I is an average value based on at least 10 different bubbles at each temperature for the same gas-liquid system. By this means it was found that the slopes m for a single bubble could be reproduced with a standard deviation of 1.5% and a maximum of 3.5%. The improved precision obtained by initially forming a single bubble, rather than selecting a bubble from an initial group (cf. Wise and Houghton, 1966), can be attributed to the absence of interactions with neighboring bubbles.

RESULTS AND DISCUSSION

The experimentally determined solubilities and diffusivities of oxygen in water and in three aqueous hemoglobin solutions at one atmosphere pressure are summarized in Table I and Fig. 2. Semilogarithmic scales have been used in Fig. 2 to facilitate the direct plotting of S_0, D_L, Y, and K in terms of van't Hoff or Arrhenius-type relationships. Corresponding to aqueous blood solutions prepared from 5, 10, and 20 wt% of packed human red cells, the hemoglobin content averaged 1.11, 2.22, and 4.44 wt% as determined spectroscopically by the previously described methods of Evelyn and Malloy (1938) and Eilers (1967), the mole fractions M being 0.32×10^{-5}, 0.65×10^{-5}, and 1.30×10^{-5} respectively based on a molecular weight of 64,450 for Hb_4. The molecular weight of 64,450 for human hemoglobin is the most accurate presently available, being based on chemical analysis and amino-acid composition (Braunitzer et al., 1961; Hill et al., 1962).

The solubilities are reported in Table I as Bunsen absorption coefficients α,

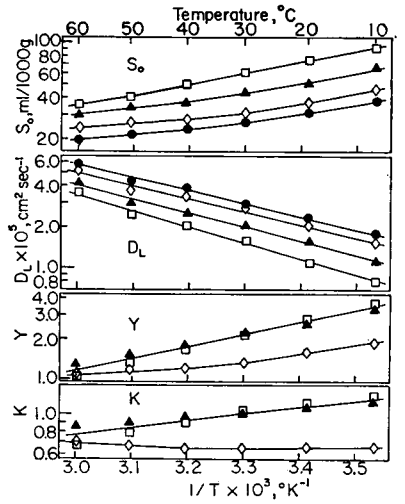

FIGURE 2 Solubilities S_0, diffusivities D_L, oxygen/hemoglobin ratios Y and equilibrium constants K for the water-oxygen-hemoglobin system, as a function of temperature: ● water; ◇ 5 wt % packed red cells; ▲ 10 wt %; □ 20 wt %.

representing the milliliters of oxygen (reduced to 760 mm Hg and 0°C) dissolved in one milliliter of solution at the temperature under consideration. Oxygen solubility increases proportionately with hemoglobin content but decreases slowly with increasing temperature, indicating that absorption is weakly exothermic. Another convenient method of reporting the solubilities of slightly soluble gases is in terms of the coefficient S_0, or milliliters of oxygen (reduced to 760 mm Hg and 0°C) dissolved in 1000 g of solution. The α and S_0 of Table I and Fig. 2 are related through the density of water, since the volume contribution from dissolved oxygen is negligible. To analytically interpolate the solubilities between adjacent temperatures $T°K$ the following solubility equation (cf. Valentiner, 1927) has been used, the corresponding constants A, B, and C being summarized in Table II:

$$\log_{10} S_0 = -A + \frac{B}{T} + C \log_{10} T. \qquad (18)$$

Equation 18 was programmed to fit the S_0 by a least squares linear regression method, the standard deviation of the fit being close to 1.0%. At 10–60°C the solubility data for oxygen in pure water were found to be 2.9% lower on average than those of Winkler (1891) and 2.4% lower than those of Morrison and Billett (1952). However, because of experimental difficulties in avoiding oversaturation and undersaturation, deviations averaging 3% are believed to be representative of those to be expected between different investigators, as discussed by Houghton et al. (1957), even though the solubility data for a single investigator may be highly reproducible. Duplication of solubility measurements has indicated that the data of Table I were reproducible to within 1.0%, the standard deviation being 0.6%.

Although the experimental precision is within 1%, and the absolute experimental errors average about 3% for solubility, the errors in determining the equilibrium constant $K = Y/X$ are somewhat larger since the calculation of this quantity depends upon taking differences between the solubilities of oxygen in water and those in the hemoglobin solutions, and upon a knowledge of the absolute hemoglobin content of the solutions. The equilibrium constants K of Fig. 2 and Table III have been

TABLE II

SOLUBILITY AND DIFFUSION CONSTANTS FOR THE WATER-OXYGEN-HEMOGLOBIN SYSTEM, BASED ON EQUATIONS 18 AND 20

wt % packed red cells	Valentiner equation (18)			Eyring equation (20)	
	A	B	C	$B \times 10^2$	ΔE
				($cm^2 sec^{-1}$)	($cal\ g\ mole^{-1}$)
0 %	11.5783	1044.40	3.8533	4.46 ± 0.17	4391 ± 20
5 %	19.497	1406.59	6.59453	2.49 ± 0.03	4124 ± 10
10 %	16.1187	1339.30	5.37665	5.89 ± 0.24	4815 ± 30
20 %	−0.9971	724.42	−0.64668	13.05 ± 0.25	5455 ± 10

TABLE III

THEORETICAL AND EXPERIMENTAL DIFFUSIVITIES OF OXYGEN IN SOLUTIONS CONTAINING 5, 10, AND 20 WT % OF PACKED RED CELLS, BASED ON EQUATIONS 14 AND 15

Mole fraction hemoglobin M	Temp (°C)	Y	$K \times 10^{-5}$	Diffusivity $\times 10^5$ (cm²sec⁻¹) D_M	D_C	$\frac{D_M}{1+MK}$	$\frac{D_C MK}{1+MK}$	$\frac{(D_M - D_C)MK}{e(1+MK)^2}$	Theor. D_E	Theor. D_{EK}	Exptl. D_L
	10	3.70	1.27	1.82	0.042	0.69	0.026	0.15	0.72	0.87	0.81
	20	2.83	1.14	2.37	0.057	0.96	0.034	0.21	0.99	1.20	1.09
1.30	30	2.18	1.01	2.97	0.074	1.29	0.042	0.26	1.33	1.59	1.60
$\times 10^{-5}$	40	1.69	0.89	3.93	0.094	1.82	0.050	0.35	1.87	2.22	1.98
	50	1.31	0.78	4.31	0.115	2.14	0.058	0.39	2.20	2.59	2.52
	60	1.02	0.67	5.83	0.139	3.12	0.065	0.52	3.19	3.71	3.60
	10	3.39	1.17	1.82	0.042	1.03	0.018	0.16	1.05	1.21	1.12
	20	2.70	1.09	2.37	0.057	1.39	0.024	0.21	1.41	1.62	1.57
0.65	30	2.20	1.02	2.97	0.074	1.79	0.029	0.26	1.82	2.08	2.04
$\times 10^{-5}$	40	1.83	0.96	3.93	0.094	2.42	0.036	0.33	2.46	2.79	2.45
	50	1.54	0.91	4.31	0.115	2.71	0.043	0.36	2.75	3.11	2.94
	60	1.32	0.87	5.83	0.139	3.72	0.050	0.48	3.77	4.25	4.19
0.32	50	1.11	0.66	4.31	0.115	3.56	0.020	0.22	3.58	3.80	3.65
$\times 10^{-5}$	60	1.04	0.68	5.83	0.139	4.79	0.025	0.30	4.82	5.12	5.03

computed from the solubilities of equation 18 using the constants A, B, and C of Table II. The scatter observed in the plot of log K vs. $1/T$ in Fig. 2 is due to the difference-magnification of experimental errors, these errors being greatest (about 20%) at the highest temperatures and lowest hemoglobin concentrations, where the solubilities are smallest. However, the solubility measurements are precise enough to detect the large differences in K that are shown between the 5 and 10 wt% solutions of packed red cells in Fig. 2 below 50°C. Also, within experimental precision, the equilibrium constants are found to be the same for the 10 and 20 wt% solutions over the entire temperature range of 10–60°C, there being also agreement with the 5 wt% solution of red cells at 50–60°C. Below 50°C the oxygen equilibrium constant K for the 5 wt% solution decreases slightly to remain approximately constant with decreasing temperature, whereas for the 10 and 20 wt% solutions of packed red cells, K increases slowly with temperature according to the following van't Hoff exponential:

$$K = 0.60 \times 10^4 \exp(1{,}700/RT). \quad (19)$$

The heat of interaction of dissolved oxygen with hemoglobin at the 10 and 20 wt% concentrations of packed red cells is therefore 1.70 ± 0.02 kcal g mole^{-1}. Within experimental precision we can conclude from Fig. 2 that the equilibrium constant K, as defined by equation 9, is independent of hemoglobin composition in the range

10–20 wt% red cells. Such hemoglobin-independence for K provides experimental evidence for the exchange concepts embodied in equations 8–12, when these are applied at the higher hemoglobin concentrations.

It is now interesting to conjecture on the cause of the anomalous behavior of K in the dilute, or 5 wt% red cell (1.11 wt% hemoglobin) solution; in this case, an increase in temperature produces only a slight increase in the equilibrium constant (cf. Fig. 2) in the range 20–60°C, indicating very weak endothermic, rather than noticeably exothermic, behavior as in the 10 and 20 wt% solutions. The anomaly appears to be detectable within the experimental precision of measuring the constants K. The possible existence of anomalous behavior for hemoglobin at high dilutions has been discussed by Roughton (1965) and Rossi-Fanelli et al. (1964), the latter investigators noting that anomalous effects seem to be facilitated by the formation of oxyhemoglobin. Roughton (1965) quotes from some unpublished work by Gilbert (1964)[1] who reportedly found that for 10 wt% hemoglobin solutions, 98% of human oxygenated hemoglobin is in tetramer form, whereas at 0.1 wt% aqueous hemoglobin there is 84% tetramer present, thus indicating an increase in polymer dissociation with dilution. Since the present measurements were made at high oxygen pressures (one atmosphere), the increased concentration of oxyhemoglobin might augment the dilution effect still further, as conjectured by Rossi-Fanelli et al. (1964). However, according to the results of Fig. 2, the effect of increased dilution can be offset by an increase in temperature since, at 50–60°C, all three hemoglobin solutions have about the same K within experimental error, thereby indicating that either tetramer dissociation decreases with increasing temperature or that some other phenomenon, such as hydration or colloidal dispersion, may be involved.

The oxygen/hemoglobin ratios Y of Fig. 2 and Table III were calculated from the solubilities S_0 of equation 18 using the constants A, B, and C of Table II. Within experimental error, the results show that the oxygen/hemoglobin ratio Y is smaller than 4.0, which is the stoichiometric number required for a tetramer formulation of hemoglobin. Also the ratio Y is independent of the hemoglobin concentration for 10 and 20 wt% red cells, while at 5 wt% the sharp drop in Y and its temperature insensitivity at the lower temperatures parallels similar anomalous behavior for $K = Y/X$. By lumping together all the intermediate forms of oxyhemoglobin in reactions (equation 6), we may define an over-all equilibrium constant $K^0 = 4K/(4 - Y)$ for reaction (equation 5) based on total unbound oxygen. This constant, because of the further difference-magnification of experimental errors, is obtained with even less accuracy than its component parameters K and Y of Table III, but its value ranges from about 3.5×10^5 at 20°C to 1.0×10^5 at 60°C for the 10 and 20 wt% red cell solutions, the over-all heat of reaction ΔH from a corresponding van't Hoff isochore being about -6 kcal g mole^{-1}. For comparison, over-all heats of reaction of oxygen with mammalian hemoglobin, as obtained calorimet-

[1] Gilbert, G. A. 1964. Unpublished data discussed by Roughton (1965).

rically (Roughton, 1935) and isochorically (Roughton, 1936; Hill and Wolvekamp, 1936; Dill, 1939;[2] Roughton et al., 1955; Enoki and Tyuma, 1964; Roughton, 1965), range from -6 to -20 kcal g mole^{-1} and are sensitive to oxygen pressure, pH, and dissolved solutes.

Before proceeding to evaluate the oxygen diffusion measurements, it is important to note that, within experimental error, the equilibrium constants K have been found to be independent of hemoglobin concentration for the solutions containing 10 and 20 wt% of packed red cells. In addition, at 50° and 60°C, the constants K for the 5 wt% solution closely approach those for the 10 and 20 wt% solutions. Such hemoglobin-invariance of the equilibrium constants K permits the use of the simple equilibrium (equation 7) and provides experimental justification for the intermediate equations and derivations leading to definitions 14 and 15 of the equivalent diffusivities for Brownian-enhanced transport.

The diffusion coefficients D_L of Table I and Fig. 2, as determined from equations 16 and 17 by microscopically measuring the collapse of oxygen bubbles, can be correlated by the Eyring (1936) equation:

$$D_L = B \exp(-\Delta E/RT). \tag{20}$$

The experimental diffusivities of Table I have been fitted to the Arrhenius-type expression (equation 20) by least squares linear regression methods using a digital computer, the pre-exponential constant B, and the activation energy ΔE, together with their standard deviations, being summarized in Table II. Diffusivities D_L for oxygen in pure water reported in Tables I and II are those of Wise and Houghton (1966), as previously determined by bubble collapse and corrected to the new solubilities of Table I using equation 17. With the constants of Table II, equation 20 fits the experimental diffusion coefficients of Table I with standard deviations of 2.5, 2.7, 2.8, and 4.3% respectively for pure water, 5, 10, and 20 wt% of packed red cells. It appears that the frequency factors B and activation energies ΔE increase with hemoglobin concentration for the 5, 10, and 20 wt% solutions, reflecting the increased amount of oxygen being transported by hemoglobin molecules than through the water. Paralleling the solubility behavior, the solution containing 5 wt% red cells is anomalous in exhibiting a reduced frequency factor and activation energy from those of pure water. The reduced frequency factor for the 5 wt% solution might indicate that dilution of hemoglobin increases its molecular size by hydration or agglomeration rather than the reverse phenomenon occurring, namely the dissociation of the tetramer, as mentioned earlier.

Regardless of changes in frequency factor and activation energy, the diffusion coefficients D_L of Table I and Fig. 2 decrease with increasing hemoglobin concentration, as predicted by expressions 14 and 15. Inspection of Table I also shows that

[2] Dill, D. B. 1939. Unpublished, quoted by Wyman (1948).

the rates of diffusion in all three hemoglobin solutions, as measured by the initial slope m of the bubble collapse curve, are generally higher (by up to 12%) than those for pure water at the same temperature. In this connection, it can be demonstrated that the slope m of the collapse curve for a spherical bubble in an infinite liquid is directly proportional to the radial diffusion flux $J = -D_L(\partial c/\partial r)_R$ of oxygen at the gas-liquid interface, where $R(t)$ is the instantaneous radius of the bubble and r the radial distance from the center. For a slowly dissolving bubble, the flux can be approximated (cf. Houghton et al., 1962) by its quasi-steady-state value $-(\partial c/\partial r)_R = c^*/R(t)$ provided that $R^{-1} \gg (\pi D_L t)^{1/2}$, where c^* is the saturation concentration of the gas in the liquid phase, which is initially oxygen-free in the present experiments. Since $c^* = \alpha/22{,}414$ for an ideal gas, we obtain $J = D_L\alpha/22{,}414\ R$. For a bubble dissolving against a plane impermeable wall, rather than in an infinite medium, it is feasible to introduce a constant geometric correction factor (cf. Houghton et al., 1962; Wise and Houghton, 1966). Thus, it follows from equation 17 that the product $D_L\alpha$ will be directly proportional to the slope m at constant temperature, with minor corrections (less than 1%) for changes in total pressure P, so that $J \propto D_L\alpha \propto m$.

According to Table I the oxygen flux enhancement, as measured by increases in slope $m \propto D_L\alpha \propto J$ above the slope for pure water, is about the same for all three hemoglobin solutions and represents a net balance between a decreased diffusivity D_L and an increased solubility α in the product $D_L\alpha$. For example, the experimentally measured diffusion coefficient D_L in the 20 wt% red cell solution is nearly one-half of that of pure water, but the solubility α is more than doubled, the over-all effect being a 10% flux enhancement. The enhancement or facilitation is clearly due to the increased amount of oxygen associated with the hemoglobin, rather than an increased diffusivity. In effect, D_L and α can be much more sensitive measures of the physicochemical processes accompanying flux enhancement than the flux itself.

Since D_L is a sensitive measure of the rate phenomena accompanying enhancement, and since dilute hemoglobin solutions have been used, equations 8–13, leading to the effective diffusivities of 14 and 15, should be valid for the solutions containing 2.22 and 4.44 wt% of hemoglobin, the equilibrium constant K of expression 9 having already been found to be independent of hemoglobin concentration. Furthermore, the use of dilute solutions in the present experiments renders it possible to obtain reliable estimates for the molecular diffusivity D_M of unbound oxygen as well as the diffusivity D_C for the hemoglobin carrier, since both these physical parameters are needed to predict the effective diffusivities D_{EK} and D_E from equations 14 and 15. The carrier diffusivity D_C of Table III was calculated from the Einstein equation 4, using a molecular diameter 2a of 75 A for hydrated hemoglobin as obtained by X-ray diffraction methods (cf. Perutz, 1948). At 20°C the value of 5.7×10^{-7} cm^2-sec^{-1} for D_C, predicted from equation 4, may by compared with the values of 5.5×10^{-7} by Rossi-Fanelli et al. (1958) and 6.0×10^{-7} by Gutter et al. (1956), both obtained from electrophoresis measurements. Older measurements of hemoglobin diffusion near concentration boundaries have yielded slightly higher diffusivities,

namely: 6.5×10^{-7} (Gralen, 1939), 6.9×10^{-7} (Lamm and Polson, 1936), and 6.3×10^{-7} cm^2sec^{-1} (Tiselius and Gross, 1934). Recently Keller and Friedlander (1966) have determined the concentration dependence of the diffusivity of methemoglobin at 25°C using a modified Stokes diaphragm diffusion cell; at a methemoglobin concentration of 5 g/100 ml the diffusivity was 7.5×10^{-7}, while at 30 g/100 ml it was 1.6×10^{-7} cm^2sec^{-1}. The values of D_M in Table III are those of Wise and Houghton (1966) for water as corrected to the present oxygen solubilities. Table III summarizes the three contributions to the effective diffusivity D_{EK}, namely the term $D_M/(1 + MK)$ arising from unbound oxygen, $D_CMK/(1 + MK)$ arising from oxygen bound to diffusing hemoglobin and the term $(D_M - D_C)\ MK/e\ (1 + MK)^2$ representing the kinetic correction for a finite rate of exchange between dissolved oxygen and hemoglobin. The equivalent diffusivity D_E of equation 15 sums the two diffusion contributions from bound and unbound oxygen but neglects the kinetic contribution that is included within the D_{EK} of equation 14.

Inspection of Tables I and III, as well as Fig. 2, shows that the oxygen diffusivity D_L for human hemoglobin solutions is lower than that for pure water, being 12, 33, and 48% lower, respectively, for the solutions containing 5, 10, and 20 wt% of packed red cells. Thus, enhancement arises through a proportionately greater solubility of oxygen in these solutions as the hemoglobin content increases. As discussed earlier, the theoretical diffusivities, D_{EK} and D_E, of Table III are based directly upon independent solubility and diffusion measurements, and therefore do not depend upon a knowledge of the absolute hemoglobin content of the hemolysates, as do the parameters Y, K, and K^0. In neglecting kinetic effects, equation 15 provides a theoretical $D_E = (D_M + D_CMK)/(1 + MK)$, which is uniformly 8.3% lower on average than the experimental diffusivities D_L for the solutions containing 10 and 20 wt% red cells of Table III; the theoretical D_E for the 5 wt% solution at 50° and 60°C are also lower by about 3.1% than the experimental D_L. The results at 10°–40°C for the 5 wt% packed cells are not reported in Table III since the equilibrium constant K has been demonstrated to be hemoglobin-dependent in this temperature range at this dilution. That the theoretical coefficients D_E of equation 15 are all lower than the corresponding experimental diffusivities D_L suggests that kinetic phenomena might account for the difference. Kinetic effects represent a positive contribution to the effective diffusivity D_{EK} of equation 14. A positive kinetic contribution is also to be expected from the physical standpoint since a finite rate of oxygen exchange would reduce the proportion of oxygen carried by the hemoglobin at diffusivity D_C and increase that diffusing through the water phase at the larger coefficient D_M. Incorporating the kinetic contribution, as in equation 14, produces a theoretical D_{EK} which is uniformly 5.4% higher on average than the experimental D_L, thus providing a theoretical rate correction amounting to 13.7% of the total D_{EK}. We recall that the kinetic correction, $(D_M - D_C)\ MK/e\ (1 + MK)^2$, arises from a finite difference approximation to the exchange term of equation 12 in which the oxygen of a region is depleted unidirectionally to $1/e$ of its original value in a time $\Delta t \approx 1/k_y$, the equilibrium concentration after depletion being negligibly small. However, the experimental results would suggest that the reverse reaction and the equilibrium concentration cannot be neglected, and that a better approximation

might be to take $\Delta t \approx 2/k_y$, when the theoretical D_{EK} and the experimental D_L would agree within an average deviation of 3%, the kinetic correction then being close to 7%. To obtain a simplified analytic formulation for the small rate correction, we have paid the price of physical approximation. If the amount of experimental information provided sufficient justification, a constant correction factor might be applied to the rate term only in the present model rather than empirically to all solute concentrations as proposed by Collins (1961). It is interesting to note from Table III that the kinetic component $(D_M - D_C)\ MK/e\ (1 + MK)^2$ for a certain temperature has the same magnitude for the blood solutions containing 10 and 20 wt% of packed red cells. Such insensitivity to mole fraction M of hemoglobin follows from the form of the kinetic term, since it has been demonstrated that when MK is unity, the kinetic effects will be a maximum. The present experiments encompass this maximum point ($MK = 1$) since the product MK is in the range 0.2–1.7 for the data of Table III. On the other hand, the carrier contribution $D_C MK/(1 + MK)$ to D_{EK} is more sensitive than the kinetic contribution in nearly doubling percentagewise on doubling the hemoglobin concentration.

In general, the quite remarkable agreement between the diffusivities predicted theoretically and those measured experimentally is of the magnitude of the experimental error; D_L can only be reproduced to within 3.5%, and the propogated errors involved in calculating equilibrium products MK from solubilities could lead to 5% variations in the D_E and D_{EK}.

This investigation was supported at the PMC Colleges by a research grant No. WP-000671 from the National Institutes of Health, U.S. Public Health Service. The assistance of Messrs. J. McCullough and P. Yatsuk in making solubility and diffusion measurements is gratefully acknowledged. We are indebted to the Fitzgerald-Mercy Hospital, Lansdowne, Pa. for the packed red cells.

Received for publication 5 June 1968.

REFERENCES

ADAIR, G. S. 1925. *J. Biol. Chem.* **63**:529.
BRAUNITZER, G., R. GEHRING-MÜLLER, N. HILSCHMANN, K. HILSE, and G. HOBOM. 1961. *Z. Physiol. Chem.* **325**:283.
CABLE, M. 1967. *Chem. Eng. Sci.* **22**:1393.
CHANDRASEKHAR, S. 1943. *Rev. Mod. Phys.* **15**:1.
COLLINS, R. E. 1961. *Bull. Math. Biophys.* **23**:223.
EILERS, R. J. 1967. *Amer. J. Clin. Pathol.* **47**:212.
EINSTEIN, A. 1905. *Ann. Physik.* **17**:549.
ENNS, T. 1964. *Proc. Nat. Acad. Sci. U.S.A.* **51**:247.
ENOKI, Y., and I. TYUMA. 1964. *J. Physiol. (Japan)* **14**:280.
EPSTEIN, P. S., and M. S. PLESSET. 1950. *J. Chem. Phys.* **18**:1505.
EVELYN, K. A., and H. T. MALLOY. 1938. *J. Biol. Chem.* **126**:655.
EYRING, H. 1936. *J. Chem. Phys.* **4**:283.
FATT, I., and R. C. LAFORCE. 1961. *Science.* **133**:219.
GRALÉN, N. 1939. *Biochem. J.* **33**:1907.
GUTTER, F. J., H. A. SOBER, and E. A. PETERSON. 1956. *Arch. Biochem. Biophys.* **62**:427.
HEMMINGSEN, E., and P. F. SCHOLANDER. 1960. *Science.* **132**:1379.
HEMMINGSEN, E. 1962. *Science.* **135**:733.
HEMMINGSEN, E. 1963. *Comp. Biochem. Physiol.* **10**:239.

HILL, R., and H. P. WOLVEKAMP. 1936. *Proc. Roy. Soc.* (*London*) **B120**:484.
HILL, R. J., W. KONIGSBERG, G. GUIDOTTI, and L. CRAIG. 1962. *J. Biol. Chem.* **81**:2272.
HOUGHTON, G., A. M. MCLEAN, and P. D. RITCHIE. 1957. *Chem. Eng. Sci.* **6**:132.
HOUGHTON, G., P. D. RITCHIE, and J. A. THOMSON. 1962. *Chem. Eng. Sci.* **17**:221.
HOUGHTON, G. 1964. *J. Chromatogr.* **15**:5.
HOUGHTON, G. 1966. *Chem. Eng. Sci.* **21**:469.
KIRKWOOD, J. G. 1946. *J. Chem. Phys.* **14**:180.
KELLER, K. H., and S. K. FRIEDLANDER. 1966. *J. Gen. Physiol.* **49**:663, 681.
KOLTHOFF, I. M., and C. S. MILLER. 1941. *J. Amer. Chem. Soc.* **63**:1013.
LAFORCE, R. C., and I. FATT. 1962. *Trans. Faraday Soc.* **58**:1451.
LAFORCE, R. C., and I. FATT. 1964. *Biopolym. Symp.* **1**:555.
LAFORCE, R. C. 1966. *Trans. Faraday Soc.* **62**:1458.
LAMM, O., AND POLSON, A. 1936. *Biochem. J.* **30**:528.
MOCHIZUKI, M., and R. E. FORSTER. 1962. *Science.* **138**:897.
MORRISON, T. J., and F. BILLETT. 1952. *J. Chem. Soc.* **1952**:3819.
PERUTZ, M. F. 1948. *Nature.* **161**:204.
ROSSI-FANELLI, A., E. ANTONINI, and A. CAPUTO. 1958. *Biochim. Biophys. Acta.* **30**:608.
ROSSI-FANELLI, A., E. ANTONINI, and A. CAPUTO. 1964. *Advan. Protein Chem.* **19**:74.
ROUGHTON, F. J. W. 1935. *Biochem. J.* **29**:2604.
ROUGHTON, F. J. W. 1936. *Biochem. J.* **30**:2117.
ROUGHTON, F. J. W. 1965. *J. Gen. Physiol.* **49**:105.
ROUGHTON, F. J. W., A. B. OTIS, and R. L. J. LYSTER. 1955. *Proc. Roy. Soc.* (*London*) **144**:29.
SCHOLANDER, P. F. 1960. *Science.* **131**:585.
SNELL, F. M. 1965. *J. Theor. Biol.* **8**:469.
TISELIUS, A., and D. GROSS, 1934. *Kolloid-Z.* **66**:12.
VALENTINER, S. 1929. *Z. Physik.* **13**:253.
WANG, J. H. 1963. *J. Theor. Biol.* **4**:175.
WINKLER, L. W. 1891. *Ber.* **24**:3607.
WISE, D. L., and G. HOUGHTON. 1966. *Chem. Eng. Sci.* **21**:999.
WITTENBERG, J. B. 1959. *Biol. Bull.* **117**:402.
WITTENBERG, J. B. 1963. *Nature.* **199**:816.
WITTENBERG, J. B. 1965. *J. Gen. Physiol.* **49**:57.
WITTENBERG, J. B. 1966. *J. Biol. Chem.* **241**:104.
WYMAN, J. 1948. *Advan. Protein Chem.* **4**:407.
WYMAN, J. 1966. *J. Biol. Chem.* **241**:115.