Morphological and Physiological Factors Affecting Oxygen Uptake and Release by Red Blood Cells*

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The kinetics of oxygen uptake and release by human, salamander (Amphiuma means), and artificially constructed red cells were measured under a variety of physiological conditions using stopped-flow, rapid mixing techniques. The results were analyzed quantitatively using the generalized, three-dimensional disc model that was developed in two previous publications (Vandegriff, K. D., and Olson, J. S. (1984) Biophys. J. 45, 825-835 and Vandegriff, K. D., and Olson, J. S. (1984) J. Biol. Chem. 259, 12609-12618). The apparent rate of gas exchange is governed primarily by the oxygen flux at the red cell surface. In the case of uptake, this flux is roughly independent of intracellular chemical reaction parameters and inversely proportional to the thickness of the unstirred solvent layer which is adjacent to the red cell surface. For release experiments in the presence of high concentrations of sodium dithionite, the flux at the cell surface is inversely proportional to the oxygen affinity of the intracellular hemoglobin and roughly independent of the thickness of the external unstirred solvent layer. As a result, the effects of cell size, internal heme concentration, and pH are expressed differently in the two types of kinetic experiments. The rate of oxygen uptake depends on roughly the second power of the surface area to volume ratio of the erythrocyte, whereas the rate of release is much less dependent on the size and shape of the red cell. The half-time of oxygen uptake is directly proportional to intracellular heme concentration for cells of equivalent geometries; the half-time of oxygen release is linearly dependent on internal heme concentration but, at low heme concentrations, is determined primarily by the rate of oxygen dissociation from hemoglobin. The rate of cellular oxygenation is roughly independent of pH and internal 2,3-diphosphoglycerate concentration; in contrast, the rate of deoxygenation depends markedly on these conditions.

As the pH is lowered or the internal diphosphoglycerate concentration is raised, the overall oxygen affinity of the cell suspension decreases severalfold, and the rate of oxygen release increases by roughly the same extent.

Oxygen transport in vertebrate circulatory systems requires diffusion of \( O_2 \) into red blood cells and chemical combination.

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1 The abbreviations used are: DPG, 2,3-diphosphoglycerate; \((O_2)_{sat}\), the concentration of free oxygen required to half-saturate hemoglobin; \( S/V \), surface area to volume ratio.
the extent of the Bohr effect under the experimental conditions being examined. Using these approximations and assumptions, the appropriate sets of second-order differential equations describing diffusion and chemical reaction are integrated numerically in cylindrical coordinates, and the overall time course is computed. In the case of deoxygenation experiments, parameters describing the reaction of sodium dithionite with oxygen are taken into account after being measured independently (Vandegriff and Olson, 1984b).

The development of this theory has allowed a reassessment of the influence of various morphological and physiological parameters on the in vitro rates of O₂ exchange by red blood cells. In most cases, we carried out new experiments, compared the results with previous work, and analyzed all of the data quantitatively. Cell morphology was varied from a hemo-globin-containing, spherical liposome that was 0.2 μm in diameter to the red blood cell of the giant salamander (Amphiuma means) which is a flattened ellipsoid, ~50 μm in diameter and 5 μm thick. Intracellular hemoglobin was varied either by fractionating normal erythrocyte populations according to density or by removing some of the internal hemoglobin and resealing the cells. Oxygen-hemoglobin reaction parameters were varied by changing the pH of the cell suspension, altering the internal DPG concentration, modifying the internal hemoglobin with potassium cyanate, or adding inositol hexaphosphate to the cell cytoplasm.

MATERIALS AND METHODS²

RESULTS

Cell Size and Shape—The effects of two extremes in red cell size were examined by measuring the rates of oxygen uptake with Amphiuma erythrocytes and with small, spherical liposomes containing encapsulated human hemoglobin. Salamander red cells are large nucleated ovoids which are 60 × 40 μm on the face of the cell, about 6–7 μm thick in the middle, and 2–3 μm thick at the edges. In our calculations, this shape was approximated as a cylindrical disc, 50 μm in diameter and 5.5 μm thick, and compared to a human red cell which is 8 μm in diameter and 1.6 μm thick (Table I). The liposomes were prepared as unilamellar spheres, 0.2 μm in diameter, and contained 6 mM human hemoglobin on a per heme basis (see "Materials and Methods" and Gaber et al., 1983).

In air-equilibrated buffer, the small artificial red cells exhibited a half-time of O₂ uptake =0.004 s, and most of the reaction occurred within the 0.006 s dead-time of the stopped-flow apparatus. Under the same conditions, the half-time for oxygen uptake by the Amphiuma erythrocytes was 1.0 s, which is ~250 times greater than that for the same reaction with liposomes and ~10 times greater than that for human red blood cells. The slowness of the reaction for salamander cells made simulation of uptake time courses at low oxygen concentrations impractical due to excessive usage of computer time. The observed and simulated oxygen uptake time courses shown in Fig. 1A represent reactions in which deoxygenated cells were mixed with buffer equilibrated with 1 atm of pure O₂ (0.625 mM after mixing). Under these conditions, the uptake of O₂ by the artificial cells was too fast to be observed by mixing techniques, but the rate of uptake by human cells was still 10 times greater than that for salamander red cells.

A summary of the size, shape, and rate parameters for these three types of cells is given in Table I. The apparent bimolecular uptake rate, kₐ, was computed as In2/t₁/₂ divided by the concentration of free oxygen in the reaction mixture; t₁/₂ is the half-time of the observed or simulated time course. The rates of oxygen uptake for red cells from other species were taken from the work of Holland and Forster (1966). In all cases except the liposomes, the cell shapes were approximated as discs and the internal hemoglobin concentrations were assumed to be 18–20 mM heme (Holland and Forster, 1966). A plot of the apparent bimolecular rate of uptake versus the S/V of the various vertebrate erythrocytes is shown in Fig. 2. As pointed out by Jones (1979) there is a correlation between the S/V and the observed rate; the exact dependence appears to be parabolic.

When a simple disc model without unstirred layers is used to simulate the data in Fig. 2, the computed rates depend linearly on the S/V ratio, and the absolute values are roughly 5–8 times greater than those observed experimentally. A similar linear dependence would be observed if membrane resistance accounted for the difference between the experimental rates and those computed by simple packet models. The upward curvature in Fig. 2 is further proof that layers of unstirred solvent adjacent to the red cell surface limit the rate of oxygen uptake in rapid mixing experiments. In the simplest analysis, the rate of oxygen uptake is considered to be proportional to the oxygen flux at the red cell surface. This flux is given by:

\[ D \frac{d(C_O)}{dx} \text{ at surface} = \frac{D(C_s - C_l)}{d} \]  

(1)

where D is the oxygen diffusion constant; Cₛ, the oxygen concentration in the bulk aqueous phase; Cₐ, the oxygen concentration in the first cytoplasmic layer; and d, the thickness of the external unstirred layer. This equation was first proposed by Nernst (1904) to describe reactions at electrode surfaces and has been applied to O₂-red cell reactions by a variety of workers (Middleman, 1972; Kagawa and Mochizuki, 1982; Huxley and Kutchia, 1981, 1983). For spherical particles of the size and density of red cells, Harriot (1962) (see also Middleman, 1972; Friedlander, 1967) has shown experimentally that, in the absence of any significant convective mixing, d ≈ R where R is the radius of the sphere. Huxley and Kutchia (1983) used this type of analysis to interpret Holland and Forster’s (1986) data for mammalian red cells. Since the S/V ratio for spheres is proportional to 1/R, the rate of uptake by spherical cells in the absence of mixing is predicted to depend on the second power of the S/V ratio, one power for internal diffusion and another for external diffusion through the unstirred layer. Similar behavior is expected for discoidal cells, although the exact power dependence has not been predicted theoretically.

Equation 1 is an oversimplification of the real situation in stopped-flow, rapid mixing experiments. Even after convective mixing has ceased to occur, the oxygen concentration gradient in the external aqueous layer is not a linear function of the distance from the cell surface (see Fig. 2 of Vandegriff and Olson, 1984a). In addition, the thickness of the unstirred layer varies with the time. In the initial turbulent flow within the mixing chamber, the cells are well mixed and only a thin layer of solution surrounding the surface remains unstirred (Rice, 1980). As flow continues into the observation chamber and the turbulence begins to dissipate, the cells become entrained in microscopic eddies that grow even larger after

² "Materials and Methods" are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 3650 Rockville Pike, Bethesda, MD 20814. Request Document No. 84 M-387, cite the authors, and include a check or money order for $1.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
O₂ Exchange by Red Cells

TABLE 1
Shape parameters and rates of O₂ uptake by red blood cells

<table>
<thead>
<tr>
<th>Red blood cell</th>
<th>MCV µm³</th>
<th>Diameter µm</th>
<th>MCT</th>
<th>S/V</th>
<th>Experimental k₁</th>
<th>Theoretical k₁</th>
<th>K</th>
<th>d(t₁/₂) µm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artificial</td>
<td>0.0042</td>
<td>0.20</td>
<td></td>
<td>30</td>
<td>900</td>
<td>1400</td>
<td>0.0001</td>
<td>0.02</td>
</tr>
<tr>
<td>Goat</td>
<td>0.020</td>
<td>3.43</td>
<td>1.38</td>
<td>2.4</td>
<td>188</td>
<td>162</td>
<td>0.0033</td>
<td>8.0</td>
</tr>
<tr>
<td>Sheep</td>
<td>0.035</td>
<td>5.29</td>
<td>1.55</td>
<td>2.05</td>
<td>137</td>
<td>107</td>
<td>0.0055</td>
<td>9.5</td>
</tr>
<tr>
<td>Horse</td>
<td>0.045</td>
<td>5.92</td>
<td>1.62</td>
<td>1.9</td>
<td>45</td>
<td>92</td>
<td>0.0057</td>
<td>10.5</td>
</tr>
<tr>
<td>Dog</td>
<td>0.071</td>
<td>7.52</td>
<td>1.61</td>
<td>1.79</td>
<td>69</td>
<td>77</td>
<td>0.0039</td>
<td>12.0</td>
</tr>
<tr>
<td>Rabbit</td>
<td>0.074</td>
<td>7.52</td>
<td>1.67</td>
<td>1.73</td>
<td>76</td>
<td>75</td>
<td>0.0039</td>
<td>12.0</td>
</tr>
<tr>
<td>Man</td>
<td>0.088</td>
<td>8.36</td>
<td>1.60</td>
<td>1.72</td>
<td>69</td>
<td>71</td>
<td>0.0041</td>
<td>13.0</td>
</tr>
<tr>
<td>Frog</td>
<td>0.068</td>
<td>16.0</td>
<td>3.38</td>
<td>0.61</td>
<td>19</td>
<td>22</td>
<td>0.0055</td>
<td>30.0</td>
</tr>
<tr>
<td>Amphiuma</td>
<td>0.108</td>
<td>53.0</td>
<td>5.50</td>
<td>0.44</td>
<td>8.2</td>
<td>9.4</td>
<td>0.0074</td>
<td>76.0</td>
</tr>
</tbody>
</table>

where t(t) is the thickness of the unstirred layer at time t; d(0), the thickness of the layer during turbulent flow in the mixer (i.e. t = 0); and K, a proportionality constant. The absolute value of K must be determined empirically, but the dependence on particle size and solvent viscosity is given by (Landau and Lifschitz, 1959; Rice, 1980; and Vandegriff and Olson, 1984a):

\[ K \approx R^{1/3}D^{1/2}S^{7/12} \]  

where R is the radius of the circular surface of the red cell; D, the oxygen diffusion constant, and ν, the kinematic viscosity. In our analyses, the thickness of the unstirred layer is evaluated as the reaction proceeds; the oxygen concentrations at this outer diffusion boundary is fixed equal to that in the bulk solvent; and then the oxygen concentrations between the outer boundary and the cell surface are allowed to vary according to Fick’s second law (Vandegriff and Olson, 1984a). The red cells are modeled as discs, and the unstirred layer is assumed to project the same distance, d(t), in the r and z directions. For the oxygen uptake simulations presented in Figs. 1A and 2 and Table I, the oxygen-hemoglobin reaction is assumed to be a simple, one-step process. Coin and Olson (1979) and others have shown that this is a reasonable approximation as long as the total O₂ concentration is great enough to achieve a final saturation value ≥ 90% (see the next section). The association rate constant, k⁺, was taken to be 3 × 10⁶ M⁻¹ s⁻¹, and the dissociation rate constant was computed from kₐ⁻(O₂)γ=0.6, where (O₂)γ=0.5 is the concentration of free oxygen that is required to saturate 50% of the hemoglobin molecules at equilibrium.

The solid line in Fig. 2 represents the predicted dependence of the apparent bimolecular rate of uptake on the surface area to volume ratio of discoidal red cells and was calculated using our three-dimensional model and Equation 2. The value of K for human cells was obtained empirically by fitting sets of oxygen uptake time courses at various O₂ concentrations (Vandegriff and Olson, 1984a); the values for the other cells were computed from Equation 3. The thickness of the unstirred layer at the half-time of the various reactions with air-equilibrated buffer, d(t₁/₂), is listed in the last column in Table I. As shown, this thickness is roughly proportional to the radius of the red cell and is in agreement with the simpler analyses of Middleman (1972) and Huxley and Kutchai (1963). In addition to simulating the dependence on S/V ratio, the more rigorous three-dimensional analysis predicts the correct absolute rates of oxygen uptake for cells varying in diameter from 0.2 to 50 µm (Table I).

Cooperativity and Oxygen Uptake—In the previous analyses, the reaction of intracellular hemoglobin was assumed to be a simple, one-step equilibrium. Although adequate for describing oxygen uptake at high gas concentrations, this assumption is unsatisfactory for analyzing deoxygenation time courses. In order to take into account cooperativity, Moll’s (1969) procedure was adopted, and the oxygen dissociation rate constant was varied with hemoglobin saturation according to the observed equilibrium curve (Vandegriff and Olson, 1984b). When this algorithm was used to reanalyze uptake time courses using the stirring parameters obtained empirically from the simple equilibrium analysis, small but significant deviations between the experimental and calculated results were observed. As shown in Fig. 3, a much better fit is obtained when K is decreased from 0.0041 to 0.0031 cm

...
was assumed where red cells at pH and artificial cells, respectively.

eters are listed in Table data observed for Amphiuma cells.

theoretical time courses. Cellular parameters used in the calculations to vary with fractional degree of saturation according to the equilibrium curves.

heme concentrations were 18, 21, and 6 mM for Amphiuma, human, and artificial cells, respectively. Closed triangles represent experimental data observed for Amphiuma cells. Open circles represent the observed data for human cells. Closed squares represent the data observed for artificial cells (AC). The solid lines in A and B represent theoretical time courses. Cellular parameters used in the calculations are given in Table I, and theoretical diffusion constants are given in Table II. A, observed and calculated time courses of O₂ uptake. Experimental time courses were obtained by mixing deoxygenated red cells in isotonic buffer at pH 7.4 and 25 °C with oxygenated buffer at 1 atm O₂ to give final concentrations of 0.025 mM O₂ and 0.020 mM total heme after mixing. For the simulations, a simple equilibrium was assumed where k' = 3 × 10⁶ M⁻¹ s⁻¹ and k = 12, 29, and 69 s⁻¹ for the artificial, human, and Amphiuma cells, respectively. B, observed and calculated time courses of O₂ release. The reaction conditions are given in Table III. For simulations, a cooperative equilibrium was assumed in which k' = 3 × 10⁶ M⁻¹ s⁻¹ and k was allowed to vary with fractional degree of saturation according to the equilibrium curves (C, see Vandegriff and Olson, 1984b). The Bohr effect was included for simulations of O₂ release by the artificial and human cells (Vandegriff and Olson, 1984b). C, oxygen equilibrium curves for red cells at pH 7.4 and 25 °C. The solid lines were drawn through the data points and do not represent theoretical fits. Equilibrium parameters are listed in Table III.

Experimental and theoretical time courses of O₂ uptake and release by Amphiuma, human, and artificial erythrocytes and their oxygen equilibrium curves. Intracellular heme concentrations were 18, 21, and 6 mM for Amphiuma, human, and artificial cells, respectively. Closed triangles represent experimental data observed for Amphiuma cells. Open circles represent the observed data for human cells. Closed squares represent the data observed for artificial cells (AC). The solid lines in A and B represent theoretical time courses. Cellular parameters used in the calculations are given in Table I, and theoretical diffusion constants are given in Table II. A, observed and calculated time courses of O₂ uptake. Experimental time courses were obtained by mixing deoxygenated red cells in isotonic buffer at pH 7.4 and 25 °C with oxygenated buffer at 1 atm O₂ to give final concentrations of 0.025 mM O₂ and 0.020 mM total heme after mixing. For the simulations, a simple equilibrium was assumed where k' = 3 × 10⁶ M⁻¹ s⁻¹ and k = 12, 29, and 69 s⁻¹ for the artificial, human, and Amphiuma cells, respectively. B, observed and calculated time courses of O₂ release. The reaction conditions are given in Table III. For simulations, a cooperative equilibrium was assumed in which k' = 3 × 10⁶ M⁻¹ s⁻¹ and k was allowed to vary with fractional degree of saturation according to the equilibrium curves (C, see Vandegriff and Olson, 1984b). The Bohr effect was included for simulations of O₂ release by the artificial and human cells (Vandegriff and Olson, 1984b). C, oxygen equilibrium curves for red cells at pH 7.4 and 25 °C. The solid lines were drawn through the data points and do not represent theoretical fits. Equilibrium parameters are listed in Table III.

Experimental and theoretical dependence of the apparent second order O₂ uptake rate, k', on the surface area to volume ratio (micrometer⁻¹) of red cells from different species. Closed circles represent the data of Holland and Forster (1966) plus the data for Amphiuma and human cells measured in this study (see Fig. 1A). The solid line represents the calculated dependence using cylindrical models for the red cells. Table I presents a list of the species and the corresponding size parameters and rates.

Experimental and theoretical time courses of O₂ uptake by human red cells. Open circles represent observed data at pH 7.4 and 25 °C for human erythrocytes (K. D. V., 21 mm internal heme). Cellular and diffusion parameters are given in Tables I and II, respectively. Reaction conditions and equilibrium parameters are given in Table III. The long-dashed dotted line (—-) represents the calculated time course using a simple equilibrium with k' = 3 × 10⁶ M⁻¹ s⁻¹ and k = 29 s⁻¹ and the stirring parameter, K (Equation 3) = 0.0041 cm s⁻¹/². The long-dashed short-dashed line (-----) represents the calculated time course using a cooperative equilibrium with k' = 3 × 10⁶ M⁻¹ s⁻¹ and k varying with the middle equilibrium curve in Fig. 1C (C), and K (Equation 3) = 0.0041 cm s⁻¹/². The solid line (——) represents the same cooperative model and K (Equation 3) = 0.0031 cm s⁻¹/². The dashed line (----) represents the theoretical time course using the cooperative model, K = 0.0031 cm s⁻¹/², and the Bohr effect, ΔpH = 0.4 (Vandegriff and Olson, 1984b).

s⁻¹/² (Equation 2). This adjustment suggests that the simple equilibrium analysis underestimates the extent of convective mixing by about 25%. However, since Moll’s (1969) technique for taking into account cooperativity is itself an approximation, it is difficult to judge which stirring parameter is more valid. Viewed in a different perspective, these results provide the upper and lower limits of K and a ±25% estimate for the error in the calculated values of the unstirred layer. The simple equilibrium analysis (K = 0.0041 cm s⁻¹/²) was used to describe the time courses in Fig. 1A and the results in Table I because oxygen equilibrium curves were not available for the data of Holland and Forster (1966). If the lower value of K were used in these analyses, the observed rates would be increased ≤10%, d(tᵣₑ) would be decreased by ~25%, but the dependence on S/V would be the same. For oxygen release
time courses, there is no detectable difference between curves calculated with either \( K = 0.0041 \) or \( 0.0031 \text{ cm s}^{-1/2} \), however, the cooperativity algorithm must be used to obtain a satisfactory fit to the experimental data. Consequently, in all the succeeding analyses cooperativity was taken into account and \( K \) was set equal to \( 0.0031 \text{ cm s}^{-1/2} \) (see Table II).

**Differences between Uptake and Release Experiments**

Time courses for oxygen release from Amphiuma, human, and artificial cells are shown in Fig. 1B. In these experiments, the half-time observed for the Amphiuma cells was only 2 and 10 times greater than the corresponding half-times for human and artificial cells, respectively. This much smaller variation with cell size in comparison to that observed for oxygen uptake (Figs. 1A and 2) is due to the boundary conditions of the oxygen release reaction and to differences between the oxygen affinities of the three preparations of red cells. First, in deoxygenation experiments, high concentrations (26 mM) of dithionite are present adjacent to the cell surface so that the limiting effects of the unstirred layer are reduced considerably. As a result, the additional dependence on red cell size due to external diffusion sublayers is much smaller, and the apparent rate should depend only on the first power of the surface area to volume ratio.

Second, the rates of oxygen flux at the cell surface are much more dependent on the affinity of the intracellular hemoglobin in deoxygenation experiments than in uptake measurements. This is a reflection of the external boundary conditions and can be understood qualitatively in terms of Equation 1. In most uptake experiments, deoxygenated cells are mixed with an air-equilibrated buffer so that \( C_s \approx 0.125 \text{ mM} \). Assuming a uniform intracellular oxyhemoglobin distribution, the value of \( C_s \) at the half-time of the reaction is roughly equal to the concentration of free oxygen that is required to half-saturate the intracellular hemoglobin in equilibrium experiments, \((O_2)_{Y=0.5}\). Oxygen equilibrium curves for the artificial, human, and Amphiuma cells are shown in Fig. 1C and the values of \((O_2)_{Y=0.5}\) are 0.004, 0.011, and 0.023 mM, respectively (see Table III). All of these concentrations are much smaller than \( C_s \) so that the external oxygen fluxes in uptake experiments at \( t_{1/2} \) are given by \( \sim D \cdot C_s/d \) and roughly independent of the hemoglobin binding parameters. In contrast, \( C_s \approx 0 \) in deoxygenation experiments and the external flux at \( t_{1/2} \) is given by \( \sim -D \cdot (O_2)_{Y=0.5}/d \). Thus, for release experiments there is a direct dependence of the observed rate on the apparent equilibrium dissociation constant for oxygen binding to intracellular hemoglobin. As a result, the size difference between Amphiuma and human cells is almost compensated by the 2-fold greater \((O_2)_{Y=0.5}\) value of the salamander erythrocytes, and the observed rates of release for the two suspensions (Fig. 1B) are much closer in magnitude than the rates of oxygen uptake (Fig. 1A). Similar considerations apply to comparisons with the much smaller artificial cells.

**Solid lines** in Fig. 1B represent theoretical time courses computed with the more rigorous, three-dimensional disc model. Cooperative oxygen binding to hemoglobin, extracellular diffusion through unsterred layers, and finite rates of the \( O_2 \)-dithionite reaction are all taken into account (Vandegriff and Olson, 1984b). These calculations confirm quantitatively the ideas and conclusions presented in the preceding paragraph: uptake is limited primarily by external diffusion processes and is markedly affected by cell size and shape; \( O_2 \) release in the presence of high concentrations of dithionite does depend on cell size but is affected to a greater extent by the oxygen binding parameters of intracellular hemoglobin.

**Table II**

**Intracellular diffusion constants as a function of heme concentration**

These values were taken from Kreuzer (1970) for the diffusion of \( O_2 \) and hemoglobin at 25°C. The diffusion constant for \( O_2 \) in solvent is \( 2.1 \times 10^{-5} \text{ cm²/s} \). The heme concentrations correspond to the data points in Fig. 5.

<table>
<thead>
<tr>
<th>Internal heme concentration</th>
<th>Oxygen diffusion constant</th>
<th>Hemoglobin diffusion constant</th>
</tr>
</thead>
<tbody>
<tr>
<td>mM</td>
<td>( \times 10^6 \text{ cm}^2/\text{s} )</td>
<td>( \times 10^6 \text{ cm}^2/\text{s} )</td>
</tr>
<tr>
<td>2</td>
<td>1.9</td>
<td>0.0600</td>
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<tr>
<td>4</td>
<td>1.7</td>
<td>0.0480</td>
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<tr>
<td>10</td>
<td>1.5</td>
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<td>16</td>
<td>1.4</td>
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<tr>
<td>20</td>
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<td>25</td>
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<td>0.94</td>
<td>0.0090</td>
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<td>50</td>
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<td>60</td>
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<tr>
<td>70</td>
<td>0.76</td>
<td>0.0067</td>
</tr>
<tr>
<td>80</td>
<td>0.72</td>
<td>0.0042</td>
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<tr>
<td>90</td>
<td>0.70</td>
<td>0.0040</td>
</tr>
<tr>
<td>100</td>
<td>0.65</td>
<td>0.0032</td>
</tr>
</tbody>
</table>

**Table III**

**Effects of chemical reaction parameters on the rates of \( O_2 \) uptake and release by red blood cells at 25°C**

\((O_2)_{Y=0.5}\) is the free concentration of oxygen required to give 50% saturation in equilibrium titration experiments with the same cell suspension used in the kinetic experiments; \( n \) is the observed Hill coefficient at \( Y = 0.5 \); \( k \) is the apparent bimolecular uptake rate for cells and was calculated from the half-time of the time course measured at 0.125 mM \( O_2 \) after mixing (i.e. \( k = (\ln 2)/(t_{1/2} - (O_2)) \)). \( k \) is the apparent deoxygenation rate measured by mixing cells in air equilibrated buffer with 52 mM sodium dithionite before mixing (again, \( k = (\ln 2)/(t_{1/2}) \)). Buffer, reconstitution, and exact reaction conditions and techniques are given under "Materials and Methods" (see also Vandegriff and Olson, 1984a, 1984b; Vandegriff, 1984). All data represent experimental observations.

<table>
<thead>
<tr>
<th>Cell preparation and conditions</th>
<th>((O_2)_{Y=0.5})</th>
<th>( n )</th>
<th>( k' )</th>
<th>( k )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artificial cells (liposomes)</td>
<td>4.0</td>
<td>2.3</td>
<td>( \sim 14000 )</td>
<td>14.0</td>
</tr>
<tr>
<td>Amphiuma</td>
<td>22.8</td>
<td>2.2</td>
<td>5.5</td>
<td>2.1</td>
</tr>
<tr>
<td>Human, pH studies</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. pH 6.6</td>
<td>27.5</td>
<td>2.6</td>
<td>54.4</td>
<td>9.1</td>
</tr>
<tr>
<td>b. pH 7.0</td>
<td>18.0</td>
<td>2.5</td>
<td>64.0</td>
<td>6.8</td>
</tr>
<tr>
<td>c. pH 7.4</td>
<td>11.0</td>
<td>2.3</td>
<td>68.8</td>
<td>4.4</td>
</tr>
<tr>
<td>d. pH 7.8</td>
<td>6.1</td>
<td>2.2</td>
<td>78.4</td>
<td>2.7</td>
</tr>
<tr>
<td>e. pH 8.2</td>
<td>3.4</td>
<td>2.1</td>
<td>80.8</td>
<td>2.9</td>
</tr>
<tr>
<td>Human, pyruvate-insoln treated, pH 7.4 (20 mM DPG)</td>
<td>21.9</td>
<td>2.7</td>
<td>60.0</td>
<td>7.1</td>
</tr>
<tr>
<td>Reconstituted human cells, pH 7.4, 10 mM heme</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Without inositol hexaphosphate</td>
<td>((7.6)^{a} (3.0)^{a})</td>
<td>144.0</td>
<td>7.0</td>
<td></td>
</tr>
<tr>
<td>b. With 2.5 mM inositol hexaphosphate</td>
<td>((63.7)^{a} (2.7)^{a})</td>
<td>112.0</td>
<td>15.0</td>
<td></td>
</tr>
<tr>
<td>Cynate treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Control, without cyanate</td>
<td>((11.0)^{a} (2.9)^{a})</td>
<td>69.6</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>b. With 50 mM cyanate</td>
<td>((6.0)^{a} (3.0)^{a})</td>
<td>68.0</td>
<td>2.2</td>
<td></td>
</tr>
</tbody>
</table>

*Equilibrium parameters were taken from Imai and Yonetani (1975) for stripped hemoglobin ± 2 mM inositol hexaphosphate 25°C, pH 7.2.

1 Equilibrium parameters were taken from Kilmarin et al. (1973) for normal and carboxymethylated hemoglobin at pH 7.2. The original values were measured at 37°C; those reported in the table were corrected to 25°C using a Q10 equal to 1.8 (Imai and Yonetani, 1975).
Internal Heme Concentration—Experimental time courses for O₂ uptake and release by red cells containing 21 and 10 mM internal heme concentrations are shown in Fig. 4. The higher heme concentration represents native cells (K. D. V.); the lower concentration was obtained by the reconstitution procedure of Coin and Olson (1979, see Miniprint). For both types of kinetic experiments, the observed rates nearly doubled when the internal hemoglobin concentration was decreased 2-fold. The solid lines in Fig. 4 represent simulations using identical cell and O₂ binding parameters; the only differences considered were internal hemoglobin concentrations and greater internal diffusion constants at the lower protein concentration (see Table II). As shown, both the half-times and the shapes of the time courses can be fitted quantitatively for both uptake and release experiments.

A summary of available data for the dependence of oxygen exchange rates on internal heme concentration is shown in Fig. 5. The symbols represent experimental half-times and the solid lines connect theoretical half-times calculated using the cylindrical disc model. As pointed out by Coin and Olson (1979), the proportionality between the half-time of O₂ uptake and internal heme concentration demonstrates that this process is limited exclusively by diffusion and not chemical reaction. The internal heme concentration determines the amount of mass transfer that must occur in order to saturate the cells. Since the rate of this transfer is roughly independent of hemoglobin concentration (see Equation 1), the time for completion of the uptake reaction is proportional to the number of available heme sites. In addition, the internal oxygen diffusion constant decreases 3-fold as the intracellular protein concentration increases from 0 to ~25 mM heme (Table II).

A somewhat similar situation is observed for deoxygenation kinetics; there is a roughly linear dependence of t½ on internal heme concentration. In this case, however, chemical reaction begins to limit the rate of oxygen release at lower heme concentrations (≤5 mM). This is observed as a finite y intercept in Fig. 5B, and the resultant value, 20 s⁻¹, is approximately equal to the apparent rate of deoxygenation of isolated hemoglobin. Again, the release rate is more dependent on the exact oxygen-binding characteristics of intracellular hemoglobin.

One potential problem with our theoretical analyses is that there could be a wide distribution of heme concentrations in the cell populations which were examined experimentally. Adopting the procedures of Corash et al. (1974), a discontinuous density gradient of Stractan I₁ was used to separate fresh red cells (K. D. V.) into four fractions. The percentage of the total and the intracellular heme concentration of these fractions were: 49% at 19 ± 1 mM, 34% at 23 ± 1 mM, 7% at 24 ± 1 mM, and 10% at 28 ± 1 mM. The intracellular heme concentration of the unfractionated cells was measured to be 22 ± 1 mM. Thus, there is a rather narrow distribution of internal heme concentration centered about the mean value for freshly drawn cell preparations (1–2 days old). If the proportion of more dense cells (28 mM heme) had been significantly greater, the time courses for the unfractionated preparation would have exhibited biphasic character, and the assumption of a single population of cells containing the mean intracellular heme concentration would not be valid. Time courses for O₂ uptake by the four fractions and the original cell suspensions were simulated, and experimental and theoretical half-times are presented in Fig. 5A. The agreement is quite good, and although not shown, the rates of O₂ uptake for all five preparations could be fitted over a 10-fold range of O₂ concentrations (Vandegriff, 1984).

The Effects of pH, Organic Phosphates, and Cyanate—The
effects of pH on the rates of \( O_2 \) uptake and release are shown in Fig. 6. Oxygen equilibrium curves for the red blood cell preparations were presented previously (Fig. 4 in Vandegriff and Olson, 1984b) and the binding parameters are summarized in Table III. There is little dependence of the uptake reaction on pH, whereas the rate of release decreases markedly with increasing pH. Again, these results can be understood qualitatively in terms of Equation 1. In uptake experiments the oxygen flux at the cell surface is determined primarily by the external oxygen concentration, \( C_e \), since the internal concentration of free oxygen, \( C_i \), is kept 5-40-fold lower due to chemical combination with hemoglobin. The small increase (≈25%) in uptake rate with increasing pH is readily explained in terms of the \((O_2)_{y=0.6}\) values in Table III. At the half-time of the reaction, the oxygen flux at the cell surface is roughly equal to \( D(0.125 \text{ mm} - (O_2)_{y=0.5})/d \) (Equation 1). As the pH is raised from 6.6 to 8.2, \((O_2)_{y=0.6}\) decreases and the apparent flux becomes ≈25% greater. In deoxygenation experiments, the flux at the cell surface and at the half-time of the reaction is approximately equal to \(-D(0.125 \text{ mm} - (O_2)_{y=0.6})/d \) since \( C_i = 0 \). As shown in Fig. 6B, there is a 5-fold decrease in the rate of deoxygenation in going from pH 6.6 to 8.2 which correlates directly with the 8-fold decrease in \((O_2)_{y=0.6}\) over the same pH with the 8-fold decrease in \((O_2)_{y=0.6}\) over the same pH range.

The solid lines in Fig. 6 represent a quantitative analysis of these pH effects. A detailed description of the simulation of oxygen release experiments was presented in the previous paper (Vandegriff and Olson, 1984b), and kinetic Bohr effect corrections were made at pH 7.0, 7.4, and 7.8. As shown in Fig. 6A, the uptake rates are expected to exhibit little dependence on pH, and identical theoretical rates and time courses were obtained with and without the Bohr effect corrections (Fig. 3, see also Vandegriff, 1984). The agreement between the observed and calculated rates in Fig. 6B confirms quantitatively the inverse relationship between oxygen affinity and the rate of release by red cell preparations.

The effects of elevated concentrations of 2,3-diphosphoglycerate on time courses for oxygen uptake and release are shown in Fig. 7. Under normal conditions (control in Fig. 7) the concentration of DPG is 5 mM which is approximately one organic phosphate molecule/hemoglobin tetramer. This level can be raised 4-fold (+2,3-DPG, Fig. 7) by incubating freshly drawn and washed red blood cells with inorganic phosphate, pyruvate, and inosine (Salhany et al., 1971; Deticke et al., 1971; see “Materials and Methods”). Oxygen equilibrium parameters for this condition are listed in Table III. As predicted by Equation 1, there was little change in the rate of oxygen uptake even though the elevated concentration of DPG caused \((O_2)_{y=0.6}\) to increase 2-fold. In agreement with the earlier experimental work of Salhany et al. (1971), the rate of release increased approximately 2-fold.

As pointed out by Salhany et al. (1971), the increase in \((O_2)_{y=0.6}\) with increasing DPG levels is due to a 0.2 unit decrease in internal pH. At an extracellular pH of 7.4 and (DPG) ~ 20 mM, the internal pH is 7.6 (Duhm, 1971; Salhany et al., 1971);...
et al., 1971). A number of other physiological factors are also changed at the higher level of DPG: 1) the intracellular Bohr effect is decreased from 0.7 to 0.5 H+/heme during deoxygenation (Benesch et al. 1969; Duhm, 1976a); 2) the buffering capacity of the cell is reduced from 2.5 to 2.3 mM H+/mM heme-pH unit (Duhm, 1976b); and 3) the cellular volume is decreased (from 88 to 74 μm³) and the hemoglobin concentration is increased (from 21 to 25 mM) due to water movement out of the cell. The latter changes and the internal pH changes are the result of a shift in the Donnan equilibrium ratio (Duhm, 1976a). All of these alterations were taken into account when simulating the time courses observed for the pyruvate/inosine-treated cells (+2,3-DPG in Fig. 7).

The agreement between the observed and calculated time courses in Fig. 7 is reasonable. As in the case of heme concentration, it is likely that there is a distribution of DPG concentrations within the populations of both native and pyruvate/inosine-treated red blood cells. The magnitude of this complication can be judged from the value of the Hill coefficient measured in equilibrium titrations (Table III). At pH 7.4, the n value is 2.3 for the native cell suspension. When these cells are lysed and the hemoglobin is isolated (O₂)y=0.5 decreases to about 0.005 mM but the n value increases to 2.7. Thus, the lower n value observed in cell suspensions is thought to be an expression of heterogenous cell populations with respect to internal organic phosphate concentrations (Samaja and Winslow, 1979). Sirs (1969) reported that the deoxygenation rate of freshly drawn human erythrocytes decreases considerably in a period of 1–4 h, and we have observed similar phenomena. Salbany et al. (1971) interpreted this change in terms of a decrease in intracellular DPG concentration as the red cells become depleated of nutrients. This variability led us to use cells which had been aged for at least 12 h at 4°C. These suspensions exhibited reproducible kinetic data, and the internal DPG concentration was found to stabilize at ~5 mM.

Two other experiments involving modification of the activity of intracellular hemoglobin are presented in Table III. First, inositol hexaphosphate was incorporated into red cells by resealing erythrocytes in the presence of 10 mM heme and 2.5 mM organic phosphate. Like DPG, inositol hexaphosphate binds to tetramers and lowers dramatically the affinity of hemoglobin for oxygen (Imai and Yonetani, 1975). The absolute rates for uptake and release by the control reconstituted cells were ~2-fold greater than those for normal cells due to the lower heme concentration (see Fig. 4). Since (O₂)y=0.5 for inositol hexaphosphate-treated cells increases markedly, the release rate was both expected and observed to increase markedly, whereas the uptake rate decreased only slightly (Table III). Second, human red cells were treated with potassium cyanate which carbamylates the N-terminal valine residues in the α and β chains of hemoglobin. This reaction causes an approximately 2-fold increase in oxygen affinity by preventing the modified protein molecules from binding DPG (Kilmartin et al., 1973). Again, the expected effects on the cellular oxygen-exchange kinetics were observed. The rate of deoxygenation decreased by ~50%, whereas there was little effect on the rate of uptake (Table III).

**DISCUSSION**

The analysis of oxygen-exchange kinetics requires an accurate knowledge of red cell size and shape (Fig. 2) internal heme concentration (Fig. 5), and the oxygen affinity of the hemoglobin molecules (Figs. 6 and 7 and Table III). This paper represents our attempt to quantitate the dependence of O₂ uptake and release rates on these morphological and physiological parameters. The results can be used to interpret data collected under other, sometimes pathological conditions. Twenty-five years ago, Carlsen and Comroe (1958) measured the rates of CO and NO uptake by human erythrocytes under various osmotic conditions. In slightly hypotonic media (0.6% saline, ~200 mosm), the red cells swelled into spherocytes; however, the observed rates of uptake were the same as those measured in isotonic media (510 mosm). Carlsen and Comroe (1958) suggested that the lack of any change in rate in going from the normal discoidal to the spherical shape indicated that membrane resistance was the major rate-limiting process during O₂ uptake by red cells. They assumed that the decrease in the S/V ratio in going from discs to spheres would decrease the rate of uptake if internal diffusion processes were limiting. However, they failed to consider the concomitant dilution of the internal hemoglobin which causes the rate to increase due to a higher value of D₀, in the cell (Table II). The net result is little change in the speed of the overall reaction. We have observed a similar phenomenon in O₂ release experiments: the rate of deoxygenation of human red cells is unaffected by swelling in slightly hypotonic media (Vandegriff, 1984). In contrast, crenation of human red cells in hypertonic media (620 mosm) caused a 45% decrease in the rate of oxygen uptake and a 23% decrease in the rate of oxygen release. Under these conditions, red cells lose a large amount of water and become spherical. In this case, the S/V ratio decreases and the cytoplasmic viscosity increases. Both effects contribute to a decrease in the overall rate of O₂ uptake. The smaller decrease observed in the deoxygenation experiments was due to the fact that (O₂)y=0.5 increased from 0.011 to 0.018 mM when the cells were placed in hypertonic media (Vandegriff, 1984).

Previous data obtained with blood from individuals suffering from sickle cell anemia are more difficult to interpret, primarily because not all of the relevant physiological parameters were measured. Rotman et al. (1974) observed a 2-fold decrease in the rate of oxygen uptake in going from normal cell samples to those from homozygous sickle cell patient. In contrast, a 2-fold increase in the rate of deoxygenation with sodium dithionite was observed. Unfortunately, these workers did not measure internal heme concentrations or oxygen equilibrium curves for their blood samples. A more thorough investigation was carried out by Harrington et al. (1977). They observed a 60% decrease in the uptake rate in going from isolated normal (AA) cells to sickle (SS) cells; however, no increase in the deoxyxgenation rate was observed for the isolated cell preparations. Harrington et al. (1977) suggested that the decreased uptake rate was due to increased intracellular viscosity of the sickle cells; however, this effect should also have been expressed as a decrease in the deoxygenation rate. The differential effect on uptake and release and the differences between the data of Rotman et al. (1974) and Harrington et al. (1977) could probably be explained if the oxygen equilibrium parameters and internal heme concentrations of the various samples were known. In addition, the kinetics of gelation and its influence on the chemical reaction parameters need to be taken into account. Charache et al. (1970) have shown that freshly isolated sickle cells contain more DPG than normal cells and exhibit a lower affinity for oxygen. This would explain the greater rates of deoxygenation observed by Rotman et al. (1971). In experiments by Harrington et al. (1977) the cells were isolated and allowed to stand for up to 24 h; this could have caused a loss of DPG and could have eliminated the differences between the O₂ equilibrium binding properties of the normal and sickle cells. In any case, it is clear that these experiments should be repeated with
more careful measurements of the exact morphological and O₂ binding characteristics of the sickle cell suspensions.

Finally, the boundary considerations (Equation 1) and ideas developed here for interpreting in vitro rapid mixing experiments are directly applicable to the in vivo capillary situation.

Oxygen uptake in alveolar capillaries is limited extensively by extracellular diffusion. The oxygen flux is determined primarily by the partial pressure of oxygen in the gas spaces which specify the concentration of O₂ dissolved in the lung tissue (C₀ in Equation 1); the exact chemical properties of the erythrocyte hemoglobin are less important. Only when the oxygen affinity of the protein is extremely low do the binding characteristics influence the rate and extent of uptake. In the case of oxygen release in contracting muscle tissue, the rate and extent of oxygen release in nonpulmonary tissue are extremely sensitive to the chemical reaction parameters of the hemoglobin molecules.

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The discontinuous pressure was made by lowering the four solutions, from the damen to the higher peaks, into culloidin distillation tubes filled with glass beads. Each solution was in a weight of 600 mg and placed in a beaker of distilled water at 37°C.

Oxygen uptake was measured on the gaseous phase in an oxygen gasel. The gasel was made of a 250 ml. volume of 0.1 M KHCO₃, 0.01 M HCl, and 0.01 M KCl, and placed in a beaker of distilled water at 37°C.

The oxygen flux was determined using the equation of transport in a finite element picture. The oxygen flux was determined using the equation of transport in a finite element picture.