An analysis of the stopped-flow kinetics of gaseous ligand uptake and release by adult mouse erythrocytes

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Ligand uptake and release by the haemoglobin contained within adult mouse erythrocytes was studied by using dual-wavelength stopped-flow techniques. The rate of O_2 uptake is very much lower than that expected for an equivalent concentration of haemoglobin in free solution. The O_2 -concentration-dependence found in uptake experiments is greater than first-order. CO uptake shows the same pattern of reactivity as does O_2 , but the associated rates of uptake are lower and the concentration-dependence of the CO rates is first-order. O_2 release from the adult erythrocytes was measured by stopped-flow mixing with $Na_2S_2O_4$. Under these circumstances the deoxygenation of intracellular haemoglobin shows accelerating time courses. The apparent rate-constant-dependence on dithionite concentration shows a rate limit at high reductant concentrations. Computer simulations of both ligand uptake and release processes were carried out by using a three-dimensional model. The simulations clearly indicate that in rapid-mixing experiments the rather slow experimentally observed O_2 uptake rate is due to rate-limiting diffusion through an extracellular stagnant solvent layer. In the case of O_2 release, however, the major rate-controlling process is the rate of O_2 dissociation from the haemoglobin molecules, which accelerates during the deoxygenation process.

INTRODUCTION

During their early rapid-mixing studies on O_2 reactions with haemoglobin Hartridge & Roughton (1927) discovered that the rate of oxygenation of haemoglobin within the sheep erythrocytes was 40 times lower than that expected for the reaction of an equivalent concentration of haemoglobin free in solution. Hartridge & Roughton (1927) also observed that the reverse reaction, namely the deoxygenation of oxygenated erythrocytes by $Na_2S_2O_4$ occurred at a rate only slightly lower than that expected for free haemoglobin. Since these initial reports a large number of independent studies have verified these original data (Gibson *et al.*, 1955; Sirs & Roughton, 1963; Lawson *et al.*, 1965; Harrington *et al.*, 1977; Coin & Olson, 1979; Vandergriff & Olson, 1984a,b,c).

The rather slow uptake of O_2 by erythrocytes was originally identified by Hartridge & Roughton (1927) as possibly arising from a number of causes, but Roughton and colleagues in later studies presented theoretical arguments in favour of a slowing of O_2 uptake caused by a highly diffusion-resistant erythrocyte membrane (Roughton, 1932; Nicholson & Roughton, 1951). A number of workers have independently criticized this early analysis, and substantial evidence is now available showing that the erythrocyte membrane does not present a significant diffusion barrier (Gibson *et al.*, 1955; Kreuzer & Yahr, 1960; Kutchai, 1975). It has been well documented by Olson and his colleagues (Coin & Olson, 1979; Vandergriff & Olson, 1984a,b,c) that the low rate of O_2 uptake by erythrocytes, observed experimentally in rapid-mixing studies, is due largely to the existence of a stagnant layer of solution surrounding the erythrocyte. This layer is rapidly depleted of O_2 on mixing erythrocytes with oxygenated buffer, and the thickness of this layer grows with time as the hydrodynamic characteristics of

the erythrocyte suspension change with time. Olson and his co-workers have also developed mathematical models to simulate the time courses of gaseous ligand uptake and release, which provide a fully quantitative description of the process in three dimensions and can thus be directly compared with experimental results (Vandergriff & Olson, 1984a). This model presents significant advances on the earlier models (Holland & Forster, 1966; Holland *et al.*, 1985; Yamaguchi *et al.*, 1985), which used initial-rate or apparent-rate studies and are at best semi-quantitative. Owing mainly to the mathematical complexity of this three-dimensional approach it has previously only been applied by Olson and his co-workers. In the present study we describe an analysis that employs the methodology of Vandergriff & Olson (1984a,b,c) applied to the kinetics of both O_2 and CO uptake by adult mouse erythrocytes together with the kinetics of deoxygenation of those same cells on mixing with dithionite. Simulations were made by using Vandergriff's (1984) three-dimensional disc model and comparison is made with the experimental data. This model has allowed us to study the dynamics of the stagnant layer surrounding the adult mouse erythrocyte and to describe both the process of gaseous uptake and release in a quantitative manner for this type of erythrocyte.

MATERIALS AND METHODS

The blood of adult C57BL/6J mice was collected into heparinized 0.9% NaCl at 4 °C. The erythrocytes were washed three times with the experimental iso-osmotic buffer (77.5 mM-NaCl/10 μ M- $CaCl_2$ /58 mM-sodium phosphate buffer, pH 7.4) and centrifuged at 4 °C be-

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tween washings. Cell size was determined by calibrated microscopic examination, and cell volume and internal haemoglobin concentration were determined by standard methods. For kinetic experiments with oxygenated cells the cells were used within 30 min of the mice being killed. In experiments requiring deoxygenated cells the cell suspension was deoxygenated by blowing O_2 -free N_2 over the surface of a shaking suspension of cells for 30 min, and the cells were employed within 1 h of the mice being killed.

Solutions containing various concentrations of dissolved gases were prepared by dilution of a stock solution with degassed iso-osmotic buffer by using standard anaerobic syringe techniques. Dithionite solutions were prepared according to the method of Coin & Olson

followed that previously described by Vandergriff & Olson (1984a,b,c). In this model the erythrocyte is approximated by a disc of radius and volume equal to that of the erythrocyte. In this way the three-dimensional problem of ligand exchange is reduced to a two-dimensional problem, owing to the radial symmetry of the disc, while still retaining a reasonable approximation of the complex erythrocyte geometry.

The simulation requires the solution of a set of equations describing both the diffusional changes in ligand concentration, expressed in the form of Fick's Laws, and the changes due to chemical reaction. These equations for the cytosolic concentrations of O_2 and oxygenated haemoglobin during the uptake of ligand thus have the form:

$$\frac{\partial [O_2]}{\partial t} = D(O_2) \left(\frac{\partial^2 [O_2]}{\partial r^2} + \frac{1}{r} \cdot \frac{\partial [O_2]}{\partial r} + \frac{\partial^2 [O_2]}{\partial z^2} \right) - k' [O_2] [Hb] + k [HbO_2] \quad (1)$$

$$\frac{\partial [HbO_2]}{\partial t} = D(HbO_2) \left(\frac{\partial^2 [HbO_2]}{\partial r^2} + \frac{1}{r} \cdot \frac{\partial [HbO_2]}{\partial r} + \frac{\partial^2 [HbO_2]}{\partial z^2} \right) + k' [O_2] [Hb] - k [HbO_2] \quad (2)$$

(1979), and both stock and diluted samples were assayed for dithionite concentration by anaerobic titration against $K_3Fe(CN)_6$ by using the published molar absorption coefficient ϵ_{420} $1026 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Creutz & Sutin, 1973).

Stopped-flow measurements were made with an Aminco DW2a spectrophotometer in the dual-wavelength mode in order to remove light-scattering artifacts. Rapid mixing was obtained by using an Aminco Morrow stopped-flow attachment. For O_2 -uptake and O_2 -release experiments a reference wavelength of 577 nm was employed and a reaction wavelength of 560 nm. For CO-uptake experiments the corresponding wavelength pair 535 nm and 560 nm was employed.

In order to minimize cell lysis in the apparatus the drive system was operated at 0.2 MPa (30 lbf/in²). This necessarily low flow rate led to an increase in the dead-time of the apparatus to 15 min. This time was determined by using the method outlined by Hiromi (1979). Optical absorption changes associated with ligand binding or release were captured on a BBC micro-computer, and the final data used in comparison with simulations were the averages for four independent time courses. The molecular rate constants for O_2 binding to and O_2 dissociation from haemoglobin were taken from the literature (Vandergriff & Olson, 1984a,b,c; Brittain *et al.*, 1986). The CO association rate constant was determined by stopped-flow measurements of CO binding to the haemoglobin from lysed erythrocytes by using the procedure of Coin & Olson (1979). The rate of monomerization of dithionite to the reductively active sulphur dioxide radical $SO_2^{\cdot -}$ was determined by measurement of the lag period observed when oxymyoglobin, in oxygenated solution, was rapidly mixed with dithionite (Coin & Olson, 1979).

O_2 -equilibrium-binding curves were obtained by thin-film methods as previously described (Lapennas *et al.*, 1981; Wells, 1982).

COMPUTATIONAL ANALYSIS

The approach taken in our computer simulation studies of gaseous ligand uptake and release by erythrocytes

where $D(O_2)$ and $D(HbO_2)$ are the diffusion coefficients for O_2 and oxyhaemoglobin and have values of $8.5 \times 10^{-6} \text{ cm}^2 \cdot \text{s}^{-1}$ and $8 \times 10^{-8} \text{ cm}^2 \cdot \text{s}^{-1}$ respectively, r is radial distance and z axial distance and k' and k are the rate constants for O_2 binding to and O_2 dissociation from haemoglobin and have the values of $3 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ and 29 s^{-1} respectively. In these equations the terms appearing in the large parentheses represent the diffusional components and the following two terms represent the chemical reaction components of the overall time-dependence of the concentrations of O_2 (eqn. 1) and oxyhaemoglobin (eqn. 2).

Additional computational complexity arises from the fact that in a complete simulation of erythrocyte kinetics separate expressions must be used to describe the concentration changes of the various components in the different compartments of the system, namely the bulk solvent, stagnant layer and cell cytoplasm, together with the appropriate boundary conditions applicable to the cell membrane, edge of the stagnant layer and cell centre. All of these complexities and their resolution have previously been thoroughly discussed by Olson and his colleagues (Vandergriff & Olson, 1984a,b,c). As no analytical expressions exist for such equations, the simulation first expresses the partial-differential equations in finite different form, dividing the various compartments in the system into finite space elements. The time course of the concentration changes in the various space elements are then calculated by using a modified form of an Euler predictor-corrector numerical integration method (Moll, 1969; Crank, 1975; Smith, 1978; Vandergriff & Olson, 1984a,b). In the case of ligand uptake the reaction of haemoglobin with CO or O_2 was found to be adequately represented by using a simple one-step process, as ligand uptake is dominated by diffusional constraints imposed by the growing stagnant layer surrounding the erythrocytes. In the case of O_2 -release experiments the simulation must not only include such equations as eqns. (1) and (2), to express the change in the intracellular concentrations of O_2 and oxyhaemoglobin, but also in the external diffusion sublayer must take account of dithionite reactivity:

$$\frac{\partial[\text{O}_2]}{\partial t} = D(\text{O}_2) \left(\frac{\partial^2[\text{O}_2]}{\partial r^2} + \frac{1}{r} \cdot \frac{\partial[\text{O}_2]}{\partial r} + \frac{\partial^2[\text{O}_2]}{\partial z^2} \right) - k_s[\text{S}_2\text{O}_4^{2-}] \quad (3)$$

$$\frac{\partial[\text{S}_2\text{O}_4^{2-}]}{\partial t} = D(\text{S}_2\text{O}_4^{2-}) \left(\frac{\partial^2[\text{S}_2\text{O}_4^{2-}]}{\partial r^2} + \frac{1}{r} \cdot \frac{\partial[\text{S}_2\text{O}_4^{2-}]}{\partial r} + \frac{\partial^2[\text{S}_2\text{O}_4^{2-}]}{\partial z^2} \right) - k_s[\text{S}_2\text{O}_4^{2-}] \quad (4)$$

where $[\text{S}_2\text{O}_4^{2-}]$ refers to dithionite concentration and k_s to the first-order rate constant for the reaction of dithionite with O_2 . Under the conditions employed in the O_2 -release studies the reaction was found to be dominated by the rate of release of O_2 from haemoglobin. As this process is known to be sensitive to both pH and the degree of saturation of the haemoglobin from which dissociation is occurring (Dalziel & O'Brien, 1961; Antonini & Brunori, 1971), it is necessary to include in the simulation the Bohr effect and an expression for the co-operative nature of the O_2 -binding process, in order to obtain an adequate simulation of the time courses of O_2 -release from the erythrocytes. The data necessary to define the Bohr effect and the saturation-dependence of the O_2 dissociation rate were obtained directly from O_2 equilibrium titration measurements and input into the simulation program according to the methods of Vandergriff & Olson (1984b).

The time-dependence of the thickness of the stagnant layer surrounding the erythrocyte was modelled by using the expression (Levich, 1962; Rice, 1980; Vandergriff & Olson, 1984a):

$$d(t) = d(0) + Kt^A \quad (5)$$

where $d(t)$ is the thickness of the stagnant layer at time t , $d(0)$ is the initial stagnant layer thickness, K is a proportionality constant and A is the rate parameter for the growth of the stagnant layer. Although the dependence of the value of K on particle size and kinematic viscosity has been established (Landau & Liftshitz, 1959; Rice, 1980; Vandergriff, 1984), its absolute value is dependent on the nature of the flow particular to a given apparatus and the mode in which the apparatus is operated. In the present study the value of K was determined empirically. Beginning with an initial value, obtained from the literature (Vandergriff & Olson, 1984a), for the value of K determined in a similar study ($K = 0.0041 \text{ cm} \cdot \text{s}^{-\frac{1}{2}}$), simulated time courses were calculated as outlined above. The simulated curves were then compared with the experimental data and the sum of the squares of the differences was computed. The value of K was then altered and the process repeated. This procedure was continued iteratively until a value of K was obtained ($0.002 \text{ cm} \cdot \text{s}^{-\frac{1}{2}}$) that gave a minimum sum of the squares of the differences between the simulated and experimental data. The value of the rate parameter A has been shown to have a limiting value of 0.41 at short times after mixing, during turbulent flow, and a limiting value of 0.58 at longer times, during laminar flow (Landau & Liftshitz, 1959). As our apparatus has a dead time of 15 ms, as operated in this study, we have employed a compromise value of 0.5 (Vandergriff, 1984). Hydrodynamic principles (Levich, 1962; Rice, 1980) together with empirical investigations indicate a value for $d(0)$ of 1–2 μM in a conventional stopped-flow apparatus such as that employed in these studies (Vandergriff & Olson, 1984a).

For comparison of simulated and experimental data all reaction curves were normalized to the apparatus

Fig. 1. Model dependence of rate of O_2 uptake by erythrocytes

The experimental time course for O_2 uptake by adult mouse erythrocytes is shown (●) together with the time courses predicted by a spherical-cell model (---), a three-dimensional disc model, with a value of K (see eqn. 5) of $0.002 \text{ cm} \cdot \text{s}^{-\frac{1}{2}}$ (□) and that expected for the reaction of free haemoglobin in solution (—). The O_2 concentration was $146 \mu\text{M}$. The sphere was calculated to have the same cell volume as the disc model, and the free haemoglobin concentration was identical with that used experimentally ($20 \mu\text{M}$).

Fig. 2. Ligand-concentration-dependence of the rate of ligand uptake by erythrocytes

The apparent rate of ligand uptake (obtained as $\ln 2/t_{1/2}$) is shown for uptake into adult erythrocytes. Experimental data are shown for both CO (●) and O_2 (○) with a haemoglobin concentration of $20 \mu\text{M}$.

Table 1. Parameters used in simulations

Parameter	Value	Origin
Diffusion coefficient for O ₂ [$D(O_2)$]	$8.5 \times 10^{-6} \text{ cm}^2 \cdot \text{s}^{-1}$	Kreuzer (1970)
Diffusion coefficient for CO [$D(\text{CO})$]	$6.1 \times 10^{-6} \text{ cm}^2 \cdot \text{s}^{-1}$	Roughton (1959)
Diffusion coefficient for Na ₂ S ₂ O ₄ [$D(\text{Na}_2\text{S}_2\text{O}_4)$]	$1.0 \times 10^{-5} \text{ cm}^2 \cdot \text{s}^{-1}$	Coin & Olson (1979)
Diffusion coefficient for oxyhaemoglobin [$D(\text{HbO}_2)$]	$8.0 \times 10^{-8} \text{ cm}^2 \cdot \text{s}^{-1}$	Kreuzer (1970)
O ₂ -dissociation rate (k)	29 s^{-1}	Brittain <i>et al.</i> (1986)
O ₂ -binding rate (k')	$3 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$	Coin & Olson (1979)
Hill coefficient (h)	2.3	Present study
Cell radius (r)	$2.7 \text{ }\mu\text{M}$	Present study
Mean cell thickness (z)	$1.24 \text{ }\mu\text{M}$	Present study
Expansion const (K)	$0.002 \text{ cm} \cdot \text{s}^{-\frac{1}{2}}$	Present study
Initial stagnant-layer thickness [$d(0)$]	$1 \text{ }\mu\text{M}$	Vandergriff & Olson (1984a)
Haemoglobin concentration ([Hb])	$20 \text{ }\mu\text{M}$	Present study

Fig. 3. Time courses for O₂ uptake into erythrocytes

Experimental time courses (●) are shown for O₂ uptake into adult erythrocytes together with simulated time courses obtained by using the three-dimensional disc model (—). The haemoglobin concentration was 20 μM . The curves show time courses for 114 μM -, 148 μM -, 280 μM - and 447 μM -O₂ from top to bottom.

dead-time. Experimental data are presented as fractional deoxygenation and are compared with simulation values presented as the ratio of the volume-weighted average of the grid-point oxyhaemoglobin concentration to the total internal haemoglobin concentration.

A fuller description of the mathematical model is given by Vandergriff (1984).

The computer programs employed in these simulations are modified forms of programs very kindly made available by Professor J. S. Olson (William Rice University, Houston, TX, U.S.A.). The programs are written in FORTRAN 77 and were run on an IBM 4341 computer. Typical run times for simulation of ligand uptake are 40 min and for O₂ release, with a co-operative dissociation process and Bohr effect, approx. 2 h.

RESULTS

O₂ uptake

The reaction of adult mouse erythrocytes with O₂ was found to follow an accelerating time course that was

about 30 times slower than that expected for an equal concentration of haemoglobin in free solution (Fig. 1). Furthermore the O₂-concentration-dependence of the apparent rate constant for this reaction of erythrocyte with O₂ showed a higher-than-first-order dependence on ligand concentration (Fig. 2). The O₂-binding time courses at various ligand concentrations were simulated by using the model outlined above. The reaction curve at 148 μM -O₂ was arbitrarily chosen to fit the best value for K of eqn. (5) iteratively, in order to allow the calculation of the rate of growth of the stagnant solvent layer surrounding the cells, under the flow conditions employed in this study. A best-fit value of 0.002 was obtained. This value was fixed in all subsequent calculations, and the other time courses were simulated with variation of only the O₂ concentration employed. The various parameters necessary as input into the simulations are shown in Table 1 and a comparison of the simulated and experimental O₂-uptake time courses are shown in Fig. 3.

CO uptake

The apparent rate of CO uptake, at low ligand concentrations, was lower than that of O₂ uptake by about 2-fold. In the case of CO uptake the ligand-concentration-dependence of the apparent rate of reaction was found to be closely first-order over the range 100–400 μM , in contrast with the dependence observed for O₂. Simulations of the time course made with the use of the three-dimensional disc model were performed as above, by employing a diffusion coefficient for CO of 6.1×10^{-6} (calculated from the formula of Roughton, 1959). A comparison of the calculated and experimental curves is shown in Fig. 4.

O₂ release

When rapidly mixed with a large excess of dithionite the deoxygenation of intracellular haemoglobin in the adult erythrocytes followed an accelerating time course. The apparent rate constant for the deoxygenation process approached a limiting rate at high reductant concentration but showed a marked deviation from linearity at low concentrations (Fig. 5).

Simulations of O₂-release time courses made with the use of the disc model that included a simple equilibrium formulation of O₂ binding to and dissociation from haemoglobin did not give satisfactory correspondence to the experimental time courses. The model was thus extended to include the experimentally determined Bohr

Fig. 4. Time courses for CO uptake into erythrocytes

Experimental time courses (●) are shown for CO uptake into erythrocytes together with stimulated time courses obtained by using the three-dimensional disc model (—). The haemoglobin concentration was $25 \mu\text{M}$. The time courses shown are for $95 \mu\text{M}$ -, $135 \mu\text{M}$ -, $180 \mu\text{M}$ -, $265 \mu\text{M}$ - and $345 \mu\text{M}$ -CO from top to bottom.

Fig. 5. Dithionite-concentration-dependence of the rate of O_2 release from erythrocytes

The apparent rate of O_2 release from erythrocytes, obtained as $\ln 2/t_{1/2}$, is shown for the reaction of initially oxygenated erythrocytes with excess dithionite. The initial O_2 concentration was $140 \mu\text{M}$.

effect and co-operativity. Under these conditions a good correspondence between prediction and experimental results was obtained (Fig. 6).

DISCUSSION

Over many years the processes of O_2 uptake and release by erythrocytes have been analysed with the use of a variety of physical models. In the early years analysis was often based on the rather improbable infinite-sheet model (Moll, 1969; Coin and Olson, 1979). It is only in the past few years, with the ready availability of substantial computing power, that more realistic models have been able to be analysed. These more recent developments have led to the modelling of the processes of ligand uptake and release in three dimensions (Vandergriff, 1984). However, some workers have employed the numerical methods developed by Olson and his co-workers (Vandergriff & Olson, 1984a,b,c) using improb-

Fig. 6. Time courses for O_2 release from erythrocytes

Experimental time courses for the release of O_2 from erythrocytes are shown (●) together with simulated time courses obtained by using the three-dimensional disc model incorporating the alkaline Bohr effect and co-operative ligand binding. The initial O_2 concentration was $140 \mu\text{M}$. The time courses shown are for 1.7 mM -, 6.7 mM - and 26.8 mM -dithionite from top to bottom.

able geometries. Hook *et al.* (1988) used a hollow-sphere model for the biconcave-disc-shaped adult erythrocyte and found it necessary not only to vary the surface area of the cell but also the diffusion coefficients for the various solutes involved in order to fit the simulated time courses with those obtained experimentally from stopped-flow studies.

On the other hand Olson and his co-workers (Vandergriff & Olson, 1984a,b,c) have employed a three-dimensional disc model for the erythrocyte from a number of species without recourse to these iterative fitting procedures for what are usually experimentally determined parameters. Unfortunately the erythrocytes used by Vandergriff & Olson (1984a,b,c) were aged for up to 48 h before use and so exhibited very high O_2 affinities ($p_{50} = 5 \text{ mmHg}$), well removed from the normal physiological region. As such this model has not previously been tested on cells showing reasonably physiological O_2 -binding properties. In the present study we have used cells no more than 1 h old and have thus studied cells with O_2 affinities closely similar to those observed under physiological conditions. However, our findings on adult mouse erythrocytes in general concur quite closely with those obtained for other species by Vandergriff & Olson (1984a,b,c) when account is taken of differences in O_2 affinity, erythrocyte size etc.

Adult mouse erythrocytes showed a ligand-concentration-dependence of rate of O_2 uptake that was greater than first-order (Fig. 2). This phenomenon has previously been shown to be consistent with diffusion of extracellular O_2 through a growing stagnant solvent layer surrounding the cells after rapid mixing has stopped (Vandergriff & Olson, 1984a), and clearly rules out any slowing of the reaction due to simple diffusion barriers, which could be expected themselves to show a first-order dependence on ligand concentration (Coin & Olson, 1979). This diffusion process through the stagnant layer is of major importance to the case of O_2 uptake where the ligand-binding reaction is rapid. It may be noted that the quantitative role of the

stagnant layer depends on the ligand that is binding to the intracellular haemoglobin. The rate of reaction of CO with haemoglobin is much lower than for O₂, and in consequence the effect of diffusion through the stagnant layer is not limiting and so we observe little deviation from a first-order dependence of rate on ligand concentration for the reaction of CO (Fig. 2).

Very good agreement between experimental data and the predictions of the three-dimensional disc model for ligand uptake by erythrocytes was obtained without resort to any variable parameters, other than ligand concentration, by using an initial stagnant-layer thickness of 1 μ m that grows according to the formula $0.002 \times (\text{time})^{0.5}$. It is clear that the uptake of ligand into adult mouse erythrocytes is closely represented by the three-dimensional disc model for both O₂ and CO over a range of ligand concentration (Figs. 3 and 4).

In O₂-release studies the rate of O₂ release is independent of dithionite concentration at high concentrations of the reductant (Fig. 5). Under these conditions sufficient reductant is present in the solvent element immediately surrounding the cell to reduce fully all the intracellular O₂. In this situation diffusion of ligand through the stagnant layer is not limiting, and the rate of haemoglobin deoxygenation is dependent on the rate of dissociation from the haemoglobin molecules. Under these conditions a good correspondence between experiment and simulation is only obtained if the O₂-dissociation process is rigorously modelled. A simple binding-equilibrium approximation is insufficient. In order to obtain a good correlation between experiment and simulation we found it necessary to treat the O₂ binding to haemoglobin as a co-operative process, and furthermore it was necessary to include the alkaline Bohr effect and the known dissociation rate-dependence on the extent of haemoglobin ligation (Antonini & Brunori, 1971; Vandergriff & Olson, 1984b). On incorporating these added factors we obtained good agreement between calculation and observation, although these added refinements increase the computational time required by approximately a magnitude (Fig. 6).

In summary, we conclude that in the case of ligand uptake, if reaction with haemoglobin is rapid, as for O₂, then the existence of a diffusional barrier provided by a growing stagnant layer surrounding the erythrocytes is sufficient to account quantitatively for the slowing of the reaction observed in stopped-flow studies as compared with reaction with haemoglobin free in solution (within the context of a three-dimensional disc model). O₂ release, however, at high reductant concentration, is little affected by a stagnant layer, and is essentially entirely controlled by the process of O₂ dissociation from the haemoglobin molecules.

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