Stopped-flow kinetics of oxygen uptake and release by embryonic red blood cells

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The processes of O₂ uptake and release by the three embryonic haemoglobins contained within early mouse embryonic red blood cells have been studied using dual-wavelength stopped-flow kinetic spectroscopy. The rate of O₂ uptake into the pseudo-spherical, nucleated, embryonic red blood cells exhibits a greater than first-order dependence on O₂ concentration. The time courses for the release from the red blood cells into dithionite-containing solutions tends towards a limiting rate at high dithionite concentrations. The rates of both the uptake and release processes observed in the embryonic cells are compared with those previously seen for adult mouse red blood cells. A new mathematical model is described which accurately simulates both uptake and release experimental data for the nucleated embryonic red blood cells.

INTRODUCTION

Over the past sixty years, since the pioneering studies of Hartridge & Roughton (1927), many studies have been made of O₂ uptake and release of whole red blood cells with the aim of establishing a working model of red blood cell function, culminating in the three-dimensional disc model of Vandergriff (1984). This model has been employed to accurately describe O₂ uptake and release from a range of adult red blood cell types (Vandergriff, 1984; Vandergriff & Olson, 1984a,b,c; Brittain & Simpson, 1989). However, this model has not previously been developed to describe ligand-exchange processes in any other than biconcave disc-shaped erythrocytes. As part of an ongoing series of studies on the functional characteristics of embryonic haemoglobins (Brittain & Wells, 1982; Purdie *et al.*, 1983; Brittain *et al.*, 1986; Brittain, 1987) we have employed the experimental approaches of Vandergriff (1984) in order to study the O₂ uptake and release processes in the embryonic red blood cells of mice at the polychromatophilic pre-erythrocyte stage of development. We report here these experimental findings, together with a modification to the original three-dimensional disc model of Vandergriff (1984) which allows us to accurately simulate these ligand exchange processes in the large, nucleated, pseudo-spherical embryonic red blood cells.

MATERIALS AND METHODS

Mice of the strain C57 BL/6J were time-mated according to the usual methods (Whitten, 1958; Purdie *et al.*, 1983), and pregnant females were killed at 12 days' gestation. Embryos were dissected into heparinized cold saline (0.9% NaCl). The embryonic red blood cells were washed by repeated suspension in cold saline followed by low-speed centrifugation. Care was taken at each suspension step not to shear the cells, and the final cell pellet was resuspended in iso-osmotic buffer solution (Coin & Olson, 1979). In all the procedures outlined above, the cell suspensions were kept in plasticware, as we have observed significant crenellation of the cells if glassware

is used (Bessis, 1973). As each embryo yielded approx. 1 µl of blood, each experiment required the blood from approx. 50 embryos.

Red blood cell suspensions were microscopically examined to obtain geometric parameters. The pseudo-spherical cells were found to have an average diameter of 11 µm and the cell nucleus a radius of 3.5 µm. At 12 days' gestation, the cells appeared to be an essentially homogeneous population (> 97%; Craig & Russell, 1964). Haemocrit and total haemoglobin concentration were determined using standard procedures (Baker & Silverston, 1976).

For O₂ uptake experiments, the embryonic red blood cells were deoxygenated by gently shaking the cells under a stream of O₂-free N₂. This process required approx. 1 h for the embryonic cells. In O₂ release experiments, air-equilibrated cell suspensions were employed.

Dithionite solutions were prepared according to the procedures of Coin & Olson (1979) and the final solutions were calibrated for activity by titration against standard ferricyanide solutions (Creutz & Sutin, 1973; Brittain & Simpson, 1989).

Dual-wavelength stopped-flow was performed as described previously (Brittain & Simpson, 1989). In stopped-flow studies of embryonic cells, the degree of cell lysis produced by shear forces in the stopped-flow apparatus was approx. 20%.

Data capture and analysis were performed as previously outlined (Brittain & Simpson, 1989), with the exception that in a few experiments where the sample was very limited, such that a final haemoglobin concentration of only 1.5 µM was used, Fourier filtering of the reaction traces was employed to remove the noise generated mainly by the optical chopper of the dual-wavelength stopped-flow apparatus (Brittain, 1989).

O₂ equilibrium measurements were made using the thin film techniques previously described (Lapennas *et al.*, 1981; Wells, 1982).

COMPUTATIONAL ANALYSIS

The mathematical approach used in this study to

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Fig. 1. The hollow-sphere model

The model of the nucleated embryonic red blood cell uses a central nucleus of radius (r_n) $3.5 \mu\text{m}$ placed in a spherical cell with a hollow cytoplasmic volume of thickness (C_r) $2 \mu\text{m}$. The cell is surrounded by a stagnant layer of solvent of thickness d_i which grows with time (t) after flow stops.

describe embryonic red blood cell kinetics was that developed by Vandergriff & Olson (1984a,b,c,) for biconcave red blood cells as described recently by Brittain & Simpson (1989). The rate equations employed in the analysis must take account of both diffusional and chemical reaction terms (Vandergriff, 1984), as in whole cell studies a homogeneous system of reaction is not appropriate. Using the geometric model which is illustrated in Fig. 1 (which can be considered to be a 'hollow-sphere' model) we arrive at equations of the form:

$$\frac{\partial[\text{O}_2]}{\partial t} = D_{\text{O}_2} \left(\frac{\partial^2[\text{O}_2]}{\partial r^2} + \frac{2}{r} \cdot \frac{\partial[\text{O}_2]}{\partial r} \right) - k^1[\text{O}_2][\text{Hb}] + k[\text{HbO}_2] \quad (1)$$

$$\frac{\partial[\text{HbO}_2]}{\partial t} = D_{\text{HbO}_2} \left[\frac{\partial^2[\text{HbO}_2]}{\partial r^2} + \frac{2}{r} \cdot \frac{\partial[\text{HbO}_2]}{\partial r} \right] + k^1[\text{O}_2][\text{Hb}] - k[\text{HbO}_2] \quad (2)$$

where D_{O_2} ($1.2 \times 10^{-5} \text{ cm}^2 \cdot \text{s}^{-1}$) and D_{HbO_2} ($1.9 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$) are the diffusion coefficients for O_2 and oxyhaemoglobin and r is the radial distance from the centre of the cell (account being taken of the volume excluded by the cell nucleus). As three distinct compartments exist within this model, namely the bulk solvent, stagnant layer and cell cytoplasm, similar equations must be derived for each compartment (Vandergriff, 1984; Brittain & Simpson, 1989). Account must also be taken of the fact that the hydrodynamics of the stopped-flow apparatus are such that the thickness of the stagnant layer surrounding the red blood cell grows with time after flow stops. The time-dependence of the thickness of the stagnant solvent layer surrounding the red blood cells was modelled using the expression:

$$d_i = d_0 + Kt^A \quad (3)$$

(Levich, 1962; Rice, 1980; Vandergriff & Olson, 1984a; Brittain & Simpson, 1989), where d_i is the stagnant layer thickness at time t , d_0 is the initial stagnant layer thickness, which we set at $1 \mu\text{m}$ (Brittain & Simpson, 1989), K is a proportionality constant and A is a rate parameter to which we assign a value of 0.5 (Vandergriff & Olson, 1984a; Brittain & Simpson, 1989).

The proportionality constant K has previously been determined to be $0.002 \text{ cm} \cdot \text{s}^{-\frac{1}{2}}$ for adult red blood cells in the particular apparatus used in this study (Brittain & Simpson, 1989). As it has previously been shown that $K \propto r^{\frac{1}{2}}$, where r is the radius of the particle in flow (Landau & Lifshitz, 1959; Rice, 1980; Vandergriff & Olson, 1984c) in this study we have employed a value of $0.0026 \text{ cm} \cdot \text{s}^{-\frac{1}{2}}$ derived from the above relationship using the measured embryonic red blood cell radius (Table 1). Hence we have removed the necessity to fit the experimental data set to a best-fit value for this parameter (Brittain & Simpson, 1989). Furthermore, when considering the nucleated red blood cell, it is necessary to establish new boundary conditions to describe the nuclear-cytoplasmic interface. We employed for this situation the conditions:

$$\frac{\partial[\text{O}_2]}{\partial r} = 0 \text{ and } \frac{\partial[\text{HbO}_2]}{\partial r} = 0$$

Table 1. Parameters used in simulations

Parameter	Value	Origin
Diffusion coefficient for O_2 [D_{O_2}]	$1.2 \times 10^{-5} \text{ cm}^2 \cdot \text{s}^{-1}$	Kreuzer (1970)
Diffusion coefficient for $\text{Na}_2\text{S}_2\text{O}_4$	$1.0 \times 10^{-5} \text{ cm}^2 \cdot \text{s}^{-1}$	Coin & Olson (1979)
Diffusion coefficient for HbO_2 [D_{HbO_2}]	$1.9 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$	Kreuzer (1970)
O_2 dissociation rate	50 s^{-1}	*
O_2 association rate	$3 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$	Coin & Olson (1979)
Cell radius	$5.5 \mu\text{m}$	This study
Nucleus radius	$3.5 \mu\text{m}$	This study
Expansion coefficient	$0.0026 \text{ cm} \cdot \text{s}^{-\frac{1}{2}}$	This study
Initial stagnant layer	$1 \mu\text{m}$	Vandergriff & Olson (1984a)
[Hb]	12 mM	This study

* Calculated from p_{50} and association rate as described by Vandergriff & Olson (1984c).

Fig. 2. O_2 -binding curves for 12-day-old embryonic red blood cells

O_2 -binding curves were obtained for 12-day embryonic red blood cells suspended in iso-osmotic buffers at pH 7.8 (---) and pH 7.4 (—) using standard thin layer techniques. The fractional saturation is shown as a function of O_2 concentration.

Having derived the appropriate set of partial differential equations, it is necessary to solve them in terms of time and space. However, no analytical solutions exist. We therefore took the approach of finite difference approximation in which, by dividing the system into a series of imaginary space elements consisting of concentric spherical shells, the spacial partial differentials are approximated by a function of the concentrations in adjacent elements (Crank, 1975; Smith, 1978). The resulting set of equations, now expressed in terms of very small but finite intervals of time and space, need to be integrated to obtain time courses for the changes in concentration of O_2 and oxyhaemoglobin.

The integration procedure employed was a modified Euler predictor-corrector method previously described in detail by Moll (1969) and Vandergriff & Olson (1984a,b).

In O_2 release simulations, it was found that the hollow-sphere model would not accurately predict the observed time courses, using equations of the form shown in (1) and (2). It was found to be necessary to modify the chemical reaction terms in eqns. (1) and (2) to take account of the shape of the complex binding curves obtained for the embryonic cells (Purdie *et al.*, 1983; Brittain & Wells, 1982; Brittain, 1987) together with the measured Bohr effect (Fig. 2).

The co-operative and Bohr processes were modelled by fixing the O_2 association rate at $3 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ and obtaining a value for the intracellular pO_2 and oxygen dissociation rate using the procedures outlined by Vandergriff (1984) and Moll (1969). The time-dependent O_2 dissociation rate (k) was then included in the chemical reaction term of equations analogous to eqns. (1) and (2). The pO_2 was obtained as a weighted average of the pO_2 values from the two equilibrium curves obtained at pH 7.4 and pH 7.8, measured at each 5% interval of fractional saturation (Vandergriff, 1984). For comparison of simulated and experimental data, all reaction curves were normalized to the apparatus dead time. Experimental data are presented as fractional deoxygenation and are compared with simulated values which are obtained as the ratio of the volume weighted average of the grid point oxyhaemoglobin concentration of the

hollow-sphere volume to the total internal haemoglobin concentration.

Computer programs were written in FORTRAN 77 and run on an IBM 4341 computer. Typical run times for the simulation of a single uptake time course was 20 s and for a single release time course approx. 15 min.

RESULTS

At 12 days' gestation, the population of embryonic red blood cells was found to contain > 97% nucleated polychromatophilic pre-erythrocytes with a mean cell diameter of $11 \mu\text{m}$ and containing haemoglobin at a concentration of 12 mM. When these cells, in the deoxygenated form, were rapidly mixed with oxygenated iso-osmotic buffer in the stopped-flow apparatus, accelerating time courses were observed for the conversion of the intracellular deoxyhaemoglobin to oxyhaemoglobin. The apparent rate of reaction of these cells, obtained from the half-time of the reaction traces, showed a greater than first-order dependence on the solution O_2 concentration (Fig. 3). Simulation of the experimental time courses was performed using the 'hollow sphere' model and is shown in Fig. 4 together with the fit to experimental data. The fit between experimental and simulation data was found to be satisfactory even if the O_2 -binding reaction was treated as a simple process, as in these experiments the dominant factor controlling the character of the time courses arises from rate-limiting diffusion through the extracellular stagnant solvent layer.

Fig. 3. O_2 concentration dependence of the apparent rate of uptake by embryonic red blood cells

The apparent rate of O_2 uptake (k_{app}), obtained as $\ln 2/t_{1/2}$, is shown for uptake into nucleated embryonic red blood cells. Both the experimental (○) and theoretically predicted (—) dependences are shown.

Fig. 4. Time courses for O_2 uptake into embryonic red blood cells

Experimental time courses (●) are shown compared with simulated time courses (—) employing the hollow sphere model. Haemoglobin concentration was $7 \mu M$ and the O_2 concentrations were $70 \mu M$, $140 \mu M$, $280 \mu M$ and $700 \mu M$ for the curves shown from top to bottom.

Fig. 5. Time courses for the release of O_2 from embryonic red blood cells

Experimental (●) and simulated (—) time courses are shown for the reaction of air-equilibrated embryonic red blood cells with 3 mM-, 6 mM- and 30 mM-dithionite, shown from top to bottom.

When oxygenated nucleated embryonic red blood cells were rapidly mixed with iso-osmotic solutions containing dithionite, the absorbance changes arising from the conversion of oxyhaemoglobin to deoxyhaemoglobin followed slower, but slightly accelerating, time courses. In simulations of these experiments, the use of a simple equilibrium description of O_2 release from the intracellular oxyhaemoglobin complex produced quite large systematic deviations from the observed time courses. The inclusion of the measured Bohr effect (see Fig. 2), together with the saturation-dependent rate of O_2 dissociation, yielded a good correspondence between simulated and experimental time courses (Fig. 5) and predicted well the observed dithionite concentration-dependence of the apparent rate of O_2 release from the red blood cells (Fig. 6).

Fig. 6. Dithionite concentration-dependence of the apparent rate of O_2 release from embryonic red blood cells

The apparent experimental rate (○) of O_2 release (k_{app}), obtained as $\ln 2/t_{1/2}$, is compared with simulated data (—) for the reaction of air-equilibrated embryonic red blood cells with dithionite.

DISCUSSION

In order to describe the physiological processes of O_2 uptake and release by circulating red blood cells, a detailed knowledge of the reactivity of haemoglobin is insufficient. It is necessary to study the reactions of whole red blood cells. At present, and indeed for the past fifty years, the only reasonable experimental method available for such studies has been dual-wavelength stopped-flow. Over this period, a number of different quantitative models have been proposed to account for the observed reactivity of red blood cells (Hartridge & Roughton, 1927; Coin & Olson, 1979; Vandergriff & Olson, 1984a,b,c). These models have culminated in a three-dimensional disc model for the adult anucleate red blood cell (Vandergriff, 1984) which appears to accurately simulate the observed O_2 uptake and release experiments (Vandergriff & Olson, 1984a,b; Brittain & Simpson, 1989).

Although a number of studies have reported experimental observations relating to comparative ligand reactivity of various red blood cell types (Holland & Forster, 1966; Holland *et al.*, 1977, 1985), only one major study has appeared to be concerned with the application of a fully quantitative model to cells of different morphology (Vandergriff & Olson, 1984c). In that report, all cell types were investigated using the three-dimensional disc model of Vandergriff, and particularly in the case of the nucleated *Amphiuma* red blood cells, produced systematic deviations between simulations and experiments (Vandergriff & Olson, 1984c). In the present study we have investigated the nature of the processes of O_2 uptake and release by relatively large, nucleated, spheroidal embryonic red blood cells and have simulated the experimental time course using an adapted form of the original model of Vandergriff (1984). We have developed a model which is geometrically more realistic for nucleated cells whilst still retaining the basic

hydrodynamic features described by Vandergriff & Olson (1984a,b). It should be pointed out that a somewhat similar geometric model has been employed by Hook *et al.* (1988) as a model for the adult biconcave red blood cell, mainly to reduce the computational complexity of the disc model. When applied to adult cells this rather inappropriate geometry required that both diffusion coefficients and red cell volume be iteratively altered to obtain a reasonable fit to the experimental data even when a fixed stagnant layer thickness was employed (Hook *et al.*, 1988). In the present study, as the hollow sphere geometry is appropriate to the nucleated embryonic red blood cells, no such iterative fitting is necessary and independently determined physical parameters were employed in all calculations, with only the experimental O₂ or dithionite concentrations being variables for any given data set (Table 1).

The shape of the embryonic red blood cell reaction time courses and their concentration dependence for both uptake and release show a remarkable similarity to those seen for adult mouse red blood cells (Brittain & Simpson, 1989). However, the absolute rate of O₂ uptake by the adult cells is much faster than that seen for the embryonic cells, as the rate of uptake is governed largely by diffusional processes through the extracellular stagnant layer. It is thus expected that the larger embryonic cell should show a slower rate of uptake (Middleman, 1972; Huxley & Kutchai, 1983). In quantitative terms, the embryonic cells show a rate of uptake very close to that predicted on the basis of their surface area/volume ratio as described by Vandergriff & Olson (1984c).

The rate of O₂ release from embryonic cells is very similar to that reported for the adult cells (Brittain & Simpson, 1989). As this process is largely governed by the rate of O₂ dissociation from oxyhaemoglobin, it is not surprising that the differences in geometry between the adult and embryonic cells is of secondary significance in this process. The heterogeneous nature of the intracellular haemoglobin population in the embryonic cells seems to have little effect on the release experiments. At 12 days' gestation, the embryonic red blood cells contain approx. 30 % EI, 45 % EII and 25 % EIII (Purdie *et al.*, 1983). However, the Bohr effect and co-operativity of EII and EIII are similar to that seen for adult mouse haemoglobin (Purdie *et al.*, 1983). The high O₂ affinity of EI coupled with its much lower O₂ dissociation rate (Brittain *et al.*, 1986) means that its effects are only expected to become significant in the latest stages of O₂ release. The good correspondence between simulations and experiments for release experiments suggests that the effects of EI are only minimal.

In terms of their physiological functioning, the results alluded to above seem consistent with the roles of the adult and embryonic cells. In the adult, the passage of the red blood cells through the lungs can be considered as a transient process and hence the cell must be optimized for rapid uptake of O₂. As uptake is diffusion-regulated, it is apparent that the biconcave disc would serve as an optimum compromise between a sheet-like structure, for rapid uptake, and the need for a relatively compact, robust cell structure capable of passage through small capillaries. In the embryo, a relatively constant supply and demand for O₂ exists and as such, before placental function is established, the embryonic red blood cell has something of an O₂ buffer role. Under these conditions it

would appear that it is not the kinetic but rather the equilibrium O₂-binding properties of the red blood cells which are of paramount importance, and therefore the embryonic cells need not be structurally optimized for rapid O₂ uptake; hence their spheroidal shape.

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