Measurement of the distribution of anion exchange function in normal human red cells

Julia E. Raftos*†‡, Robert M. Bookchin§ and V. L. Lew*

*The Physiological Laboratory, University of Cambridge, Cambridge CB2 3EG, UK, †Department of Physiology, Monash University, Clayton, 3168 Victoria, Australia and §Department of Medicine, Albert Einstein College of Medicine, Bronx, NY 10461, USA

1. The aim of the present work was to investigate cell-to-cell variation in anion exchange turnover in normal human red cells. Red cells permeabilized to protons and K^+ dehydrate extremely rapidly by processes that are rate-limited by the induced K^+ permeability or by anion exchange turnover. Conditions were designed to render dehydration rate-limited by anion exchange turnover. Cell-to-cell variation in anion exchange function could then be measured from the distribution of delay times required for dehydrating cells to attain resistance to haemolysis in a selected hypotonic medium.

2. Red cells were suspended at 10% haematocrit in a low-K^+ solution and, after a brief pre-incubation with 20 μM SITS at 4 °C, were warmed to 24 °C, and the protonophore CCCP was added (20 μM) followed 2 min later by valinomycin (60 μM). Delay times for cells to become resistant to lysis were measured from the instant of valinomycin addition by sampling suspension aliquots into thirty volumes of 35 mM NaCl. After centrifugation the per cent lysis was estimated by measuring the haemoglobin concentration in the supernatant. Typical median delay times with this standardized method were 4–5 min.

3. The statistical parameters of the delay time distributions report the population spread in the transport function that was limiting to dehydration. In the absence of SITS and CCCP, dehydration was limited by the diffusional Cl^- permeability (P_{Cl}). Delay time distributions for P_{Cl}- and anion exchange-limited dehydration were measured in red cells from three normal donors. For both distributions, the coefficients of variation ranged between 13·0 and 15·2%, indicating a high degree of uniformity in P_{Cl} and anion exchange function among individual red cells.

The present work is part of a more general study of cell-to-cell variation in the activity of red cell membrane transporters, for which only mean population values are available. The importance of such studies has come to light in recent years from the realization that minor functional differences among red cells, of no apparent physiological significance, may influence the response of individual cells to pathological stress and ultimately determine the abnormality of volume, shape or lytic susceptibility of each cell. The relevance of variable transporter expression in reticulocytes to the generation of mature red cells with diverse abnormal densities was recently demonstrated in sickle cell anaemia (Bookchin, Ortiz & Lew, 1991; Lew & Bookchin, 1992). In normal red cells, gradual increases in uniform Ca^{2+} permeabilization were shown to generate extremely heterogeneous Ca^{2+} distributions (García-Sancho & Lew, 1988). Most haematological disorders affecting red cells are associated with characteristic heterogeneities in their morphology, function or life spans. To distinguish whether the origin of these heterogeneities is in stochastic or deterministic factors, we must obtain information about the variability among cells. Only then can hypotheses on possible mechanisms for diverse cell responses to similar perturbations be formulated.

We describe here the development and application of a new method to measure the distribution of anion exchange activity among red cells. The anion exchanger, with about 10^6 copies per cell, is the most abundant transport protein in the red cell membrane (Cabantchik & Rothstein, 1974). In the body, it mediates an electroneutral 1 : 1 exchange of Cl^- for HCO_3^-, essential for the transport of CO_2 from tissues to lungs (Gunn, Dalmark, Tosteson & Wieth, 1973). Anion traffic through the exchanger is about 10^6 times faster than the cation traffic through all the combined cation transporters, and about 10^4 times faster than the diffusional

‡ To whom correspondence should be addressed at the Department of Physiology, Monash University, Clayton, 3168 Victoria, Australia.

anion traffic, thought to represent conductive Cl^- transport through the exchanger (Vestergaard-Bogind & Lassen, 1974; Knauf, Fuhrmann, Rothstein & Rothstein, 1977; Gunn, 1978; Knauf, Law & Marchant, 1983; Fröhlich, 1984). The distribution of diffusional Cl^- permeabilities (P_{Cl}) in normal human red cells was recently investigated by Raftos, Bookchin & Lew (1996) in populations of K^+-permeabilized, dehydrating red cells, by following time-dependent changes in the profile of haemolysis curves, as they migrated towards lower tonicities. The methodological limit in their estimates of profile invariance was 7·5%, and the results fell within this limit, suggesting a high degree of uniformity in P_{Cl}. However, the 'profile migration' method used in those studies cannot be readily applied to the investigation of the distribution of anion exchange function, because the time course of cell dehydration limited by exchanger activity is too fast for lysis curve sampling. The method developed and applied here measures the distribution of 'delay times', the time needed for a cell to reach a critical dehydration stage, which is the same for all the cells, under conditions in which dehydration is rate-limited by the activity of the anion exchangers in each cell. As with P_{Cl}, the results showed a high degree of uniformity in anion exchange function among normal human red cells. The method also helped to establish the validity of a predicted statistical correlation between critical haemolytic volume and osmolyte content in populations of normal human red cells (Lew, Raftos, Sorette, Bookchin & Mohandas, 1995).

METHODS

Principle and design of delay time method

Preliminary simulations with the Lew–Bookchin red cell model (Lew & Bookchin, 1986) indicated that red cells suspended in low-K^+ solutions and permeabilized to both K^+ and H^+ would dehydrate extremely fast, with cell volumes decreasing by 20% within 2–3 min (Fig. 1). Analysis of model data, partly confirmed by earlier observations, showed that under such conditions K^+ efflux is accompanied by rapid H^+ influx driven by the highly hyperpolarized membrane potential (Macey, Adorante & Orme, 1978). Cell HCO_3^- ions, lost by buffering the incoming H^+, are replaced by extracellular HCO_3^- that enters the cell through the anion exchange protein in exchange for cell Cl^- (Wieth, 1980). The result is a rapid efflux of KCl and water limited by the induced K^+ or H^+ permeability or by anion exchange turnover.

When experimental conditions are set to make anion exchange the predominant rate-limiting process, any significant difference in anion exchange turnover among the cells should affect their velocities of dehydration. Such differences may be detected by the spread of delay times for cells to reach a selected dehydrated state from an initial common condition. The selected dehydrated state may be exposed by the extent of haemolysis occuring in a fixed hypotonic medium, as done here, or by density separation through a fixed density layer.

Human red cells can be permeabilized to K^+ and H^+ by exposure to valinomycin and to the proton ionophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP) (Macey *et al.* 1978; Wieth, 1980). In Fig. 1, the red cell model (Lew & Bookchin, 1986; model available in source code or compiled version on request) was used to illustrate the predicted cell volume changes that occur during dehydration in the presence of CCCP and valinomycin, and during subsequent hypotonic shock. Initially, H^+ ions are at electrochemical equilibrium across the cell membrane, so that CCCP has no effect on their distribution. Addition of valinomycin triggers rapid dehydration, shown as the decrease in relative cell volume (V/V°, where V° is the cell volume before the commencement of the ionophore-induced dehydration). At 1 min intervals after the addition of valinomycin, cell samples are mixed into the selected hypotonic saline, causing a sudden rise in relative cell volume. The cells sampled at 1 and 2 min rapidly reach the critical cell volume of V/V° of ~1·7 (Ponder, 1948) and lyse. At about 3 min after valinomycin addition, the cells have dehydrated to V/V° of ~0·77, and they no longer lyse in the hypotonic medium, since they have

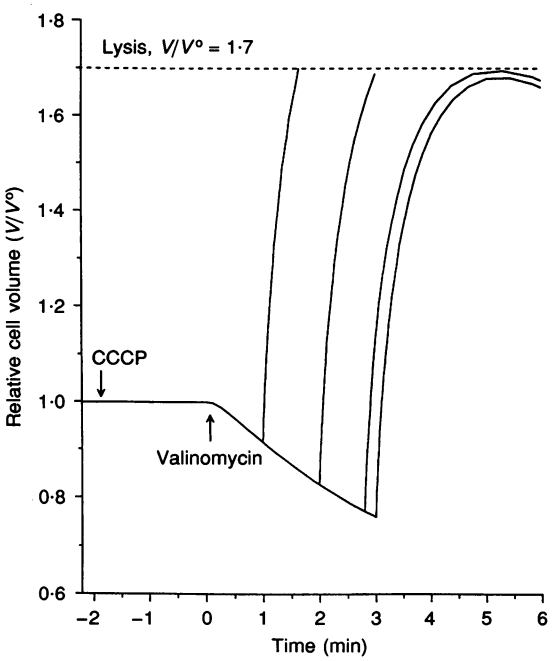

Figure 1. Predicted red cell volume after treatment of red cells with CCCP and valinomycin, and during measurement of delay times to haemolysis protection

Simulations were based on the Lew–Bookchin red cell model (Lew & Bookchin, 1986). A 10% suspension of red cells in low-K^+ medium is incubated at 24 °C. Addition of 20 μM CCCP and 60 μM valinomycin was modelled by increasing the membrane conductances for H^+ and K^+ to 10^{11} and 80 h^{-1}, respectively. To approximate the change in conditions as 50 μl of the suspension is mixed with 1·5 ml of hypotonic saline (at 1, 2, 2·8 and 3 min), the ionic composition of the extracellular solution in the model was reset to 35 mM NaCl and the haematocrit was reduced to 0·3%. Lysis occurred when cells reached the critical haemolytic volume (set at $V/V^\circ = 1·7$). In addition to osmotic re-equilibration of water, the volume changes predicted by the model for the cells exposed to the hypotonic saline result from: (i) minor reductions in cell osmolytes during prelytic swelling, due to loss of HCl through the Jacobs–Stewart mechanism (Lew & Bookchin, 1986) driven by the outward Cl^- gradient in the hypotonic saline, (ii) continued net KCl loss, and (iii) reduction in the osmotic coefficient of haemoglobin by its dilution. The proposed pathway for HCl cotransport through the band 3 protein (Bisognano, Dix, Pratap, Novak & Freedman, 1993) has not been incuded in the model.

lost enough osmolyte (mostly KCl) to equilibrate in the hypotonic medium without swelling beyond their critical haemolytic volume. Furthermore, once these cells escape lysis initially, they continue to dehydrate in the hypotonic medium (note the downward curving in the relative volume of the unlysed cells in Fig. 1). This ensures no subsequent lysis of the cells that did not lyse instantly, a convenient feature which allows delayed centrifugation of samples. Therefore, for the separation of dehydrating cell subpopulations, lysis methods have a crucial advantage over density separation methods which require immediate centrifugation of samples, a procedure incompatible with a high frequency of sampling.

The model simulation in Fig. 1 represents the predicted volume response of a single cell (or a population of identical cells under uniform initial conditions); i.e. an abrupt change from 100 to 0% haemolysis with a delay time of about 2·8 min. The volume response of the model cell is determined by three parameters: protonophore- and valinomycin-induced H^+ and K^+ permeabilities, and anion exchange turnover rate (Wieth, 1980). In a population of real red cells, differences in initial osmolyte contents, volumes and membrane areas will also affect the delay times of the individual cells. Therefore, interpretations of observed delay time distributions must distinguish between effects of initial cell differences and those resulting from variations in their anion exchange turnovers (see Discussion for analysis).

It was important to choose a hypotonic (lytic) NaCl solution of osmolality C_L that would expose intercellular differences in the rate-limiting parameter. On the one hand, differences in dehydration states among cells, reflecting the heterogeneity of dehydrating velocities in the cell population, need time to develop. However, on the other hand, as the cells approach maximal dehydration, differences in their *rates* of dehydration are no longer detectable. Analysis of model predictions and preliminary experimental results indicated that the crucial compromise in the choice of C_L for a standardized delay time assay was between: (i) good spread of delay times, which increases with time, (ii) reduced mean delay times, to minimize the influence of factors other than the performance of the rate-limiting transporter on cell dehydration rates, and (iii) convenience for high frequency sampling, to ensure accuracy in the measurement of the integrated curves, the derivatives of which provide the delay time distribution curves. A C_L of about 70 ideal mosmol kg^{-1} (35 mM NaCl) fulfilled these criteria adequately, and was chosen for the standardized assay.

Preparation of red cell suspensions

Heparinized venous blood was obtained from healthy, unpaid volunteers, after they had given their written consent, and the red cells were washed 4 times and suspended to a haematocrit of 10% in a pH 7·5 solution containing (mM): NaCl, 150; KCl, 2; Hepes-Na, 2; EGTA, 0·1; $NaHCO_3$, 1; and 120 units ml^{-1} carbonic anhydrase.

Measurement of anion exchange-limited and P_{Cl}-limited delay times

As described in the Results, initial experiments showed that anion exchange-limited delay times could be achieved by pre-incubation for 20 min at 4 °C with 20 μM SITS (4-acetamido-4′-isothiocyanatostilbene-2-2′-disulphonic acid) (Cabantchik & Rothstein, 1972). The suspensions were then warmed to 24 °C with continuous stirring that was maintained throughout. The ionophores CCCP and valinomycin were dissolved in DMSO. Two minutes after adding 20 μM CCCP, 60 μM valinomycin (suspension concentrations) was added to initiate cell dehydration. For P_{Cl}-limited delay times, CCCP and SITS were omitted.

After addition of valinomycin (time zero), 50 μl samples of the suspension, taken at the intervals indicated in the figures, were mixed immediately into 1·5 ml of 35 mM NaCl. After centrifugation, the supernatant haemoglobin concentration was measured spectrophotometrically as cyanomethaemoglobin, and the percentage haemolysis, relative to the 100% haemolysis values obtained immediately after valinomycin addition, was plotted as a function of the sampling times to generate the delay time curves (see Fig. 2).

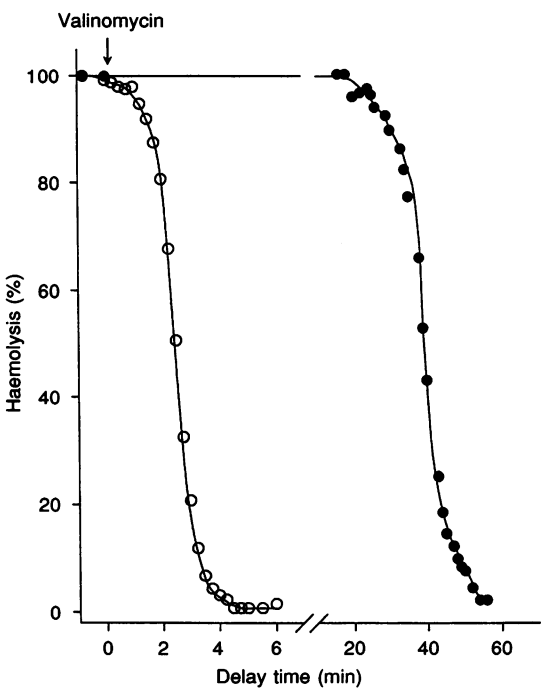

Figure 2. Delay time curves for valinomycin-treated red cells in the presence and absence of CCCP

A 10% suspension of fresh red cells in the standard low-K^+ solution (see Methods) was divided in two; 20 μM CCCP was added to one suspension, and 60 μM valinomycin was added to both, at time zero. Timed 50 μl samples were then mixed with 1·5 ml of 35 mM NaCl and, after centrifugation, the percentage haemolysis measured. The curves were drawn by hand through the experimental points. The median delay time at 50% haemolysis was 2·8 min in the presence of CCCP (○), and 39 min in its absence (●).

RESULTS

Conditions required for dehydration to be rate-limited by anion exchange

An initial series of experiments defined the conditions under which cell dehydration induced by the combined action of CCCP and valinomycin was rate-limited by anion exchange. In the presence of CCCP, dehydration was extremely rapid (Fig. 2, ○), so that after the addition of valinomycin the median delay time, when haemolysis was reduced to 50%, was 2·2 ± 0·4 min (mean ± S.D. with $n = 4$ different donors). When cells were exposed to 60 μM valinomycin without CCCP, cell dehydration was rate-limited by P_{Cl} (Hunter, 1977; Freeman, Bookchin, Ortiz & Lew, 1987; Raftos *et al.* 1996), and was much slower (Fig. 2, ●). Haemolysis protection began about 20 min after valinomycin addition (Fig. 2), with an average median delay time of 38·7 ± 1·2 min ($n = 4$). If bicarbonate and carbonic anhydrase were simultaneously omitted from the incubation, median delay times for cells treated with both CCCP and valinomycin increased markedly to 5·6 ± 1·6 min ($n = 2$; not shown), as expected from the contribution of anion exchange to the dehydration. Corresponding changes in the rate of pH equilibration in the absence of bicarbonate and carbonic anhydrase were observed previously by Wieth (1980).

The next series of experiments measured the effects of varying concentrations of valinomycin, between 8 and 120 μM, on the delay times of the cells in the presence of 20 μM CCCP. With increasing concentrations of valinomycin, there was a gradual decrease in the delay times for all the cells, as seen by the left shift in their delay time curves (Fig. 3), until a saturating concentration of valinomycin was reached at 60 μM.

Taken together, these results indicate that both valinomycin-induced K^+ permeability and anion exchange contributed to the dehydration rate. To make anion exchange rate-limiting, its activity had to be reduced without introducing spurious heterogeneities among the cells. Pre-incubation of red cells for 20 min at 4 °C with increasing concentrations of SITS progressively increased the delay times (Fig. 4).

Model simulations were carried out to determine the concentrations of SITS required for anion exchange to be the main rate-limiting factor for cell dehydration, with minimal contributions from K^+ and Cl^- permeabilities. Model parameters for electrodiffusional Cl^- and K^+ permeabilities (P_{Cl} and P_K) were set at measured P_{Cl} (Fig. 6; Raftos *et al.* 1996) and maximal valinomycin-induced P_K values (Fig. 3). We then asked what the average delay time should be for cells to attain haemolysis protection in a medium of 35 mM NaCl so that the combined contribution of K^+ and Cl^- permeabilities to the limitation of cell dehydration rate was kept below 5%. Model simulations showed that a mean delay time within 4–7 min satisfied these constraints, and the concentration of 20 μM SITS was chosen accordingly.

To determine whether SITS binds and inhibits anion exchange uniformly in the cells, a suspension was divided into three aliquots which received either 66 μM, 33 μM or no SITS. After 20 min incubation at 4 °C, equal volumes of the SITS-free suspension and the suspension with 66 μM SITS were warmed to 24 °C and mixed for 4 min before the addition of CCCP and valinomycin. The delay time curve for this mixture was indistinguishable from that of a suspension pre-incubated with 33 μM SITS (Fig. 5). Both curves were smooth, with no suggestion of the bimodality expected if the distribution or effect of SITS was heterogeneous. Since SITS is water-soluble and, under the incubation conditions used here, inhibits the anion exchange by non-covalent binding (Cabantchik & Rothstein, 1972), its rapid redistribution was not surprising. These results show that SITS can make anion exchange rate-limiting for red cell dehydration under these conditions, without itself changing

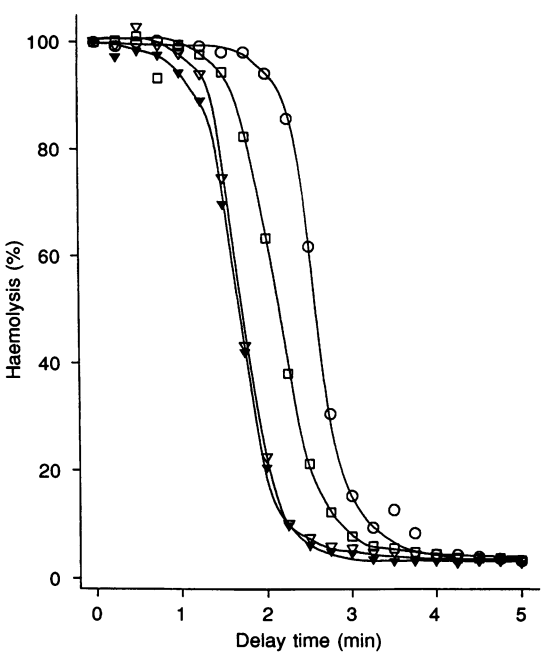

Figure 3. The effect of valinomycin concentration on the delay times of red cells treated with CCCP

A 10% red cell suspension in the standard low-K^+solution, containing 20 μM CCCP, was divided into aliquots. At zero time valinomycin was added to give final concentrations of 8 (○), 30 (□), 60 (▽) and 120 μM (▼), and delay time curves were obtained.

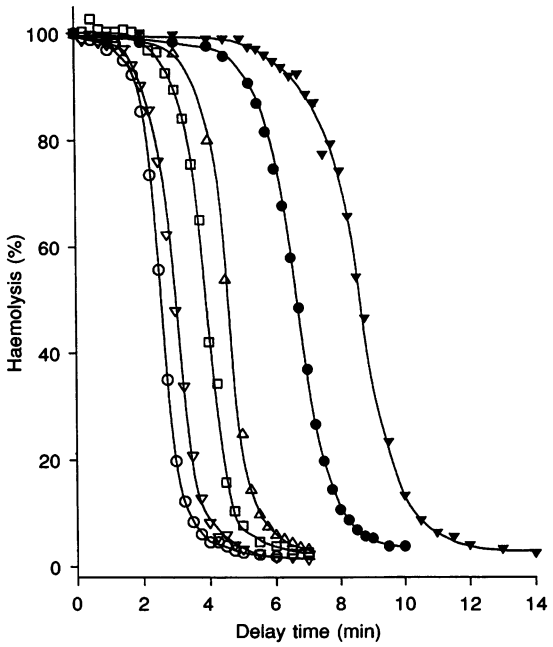

Figure 4. The effect of SITS concentration on the delay times of red cells treated with CCCP and valinomycin
Cells suspended at 10% haematocrit in the standard low-K^+ solution were pre-incubated for 20 min at 4 °C in aliquots containing the following SITS concentrations: 0 (○), 2 (▽), 8 (□), 16 (△), 24 (●), and 32 μM (▼). The suspensions were then warmed to 24 °C, 20 μM CCCP and 30 μM valinomycin were added, and delay time curves were obtained.

the distribution of delay times. Therefore, the delay times obtained from suspensions with 20 μM SITS, 20 μM CCCP and a saturating concentration of valinomycin (60 μM) reflect the distribution of anion exchange function among the cells. These standardized conditions were used in the studies below.

Measurement of P_{Cl} and anion exchange distributions among red cells from individual donors

P_{Cl}- and anion exchange-limited delay time curves, measured in red cells from three donors, are shown in the upper panels of Figs 6 and 7, respectively. The histograms (lower panels of Figs 6 and 7) show the percentage of cells reaching haemolysis protection within each delay time interval of 2 min (Fig. 6) or 15 s (Fig. 7). Gaussian curves fitted to the histogram data provided the mean delay time and standard deviation of each distribution, and the coefficients of variation (c.v. = s.d./mean). The c.v. values of the distribution of P_{Cl}-dependent delay times ranged from 13·0 to 14·0% and those for the anion exchange-limited process from 13·2 to 14·8% (Table 1).

It is interesting to note the variability in the individual means of anion exchange relative to P_{Cl}-limited delay times (Table 1). The values obtained for these three donors were consistent and reproducible and therefore attributable more to genuine donor differences than to minor variations in SITS concentrations. A larger sample of donors will be required to confirm and quantify this variability before further speculation on its meaning is justified.

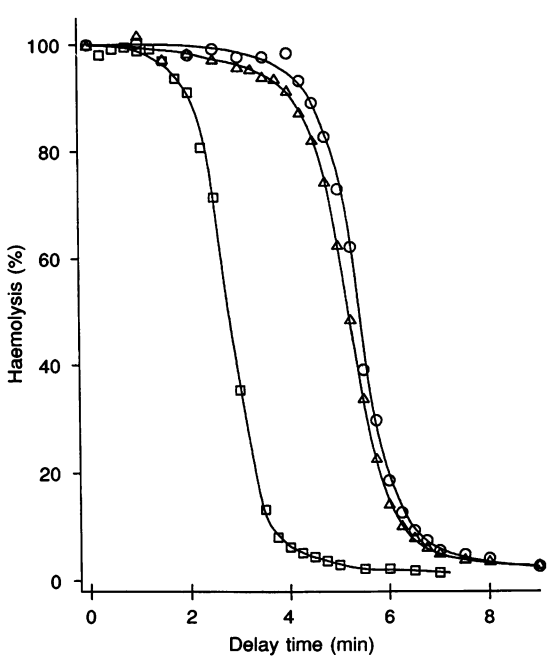

Figure 5. Redistribution of SITS among red cells
Three aliquots of a 10% cell suspension in the standard low-K^+ solution received either no SITS (□), 33 μM SITS (○), or 66 μM SITS followed by 20 min pre-incubation at 4 °C. After warming to 24 °C, an additional suspension was prepared by mixing equal volumes of the suspensions pre-incubated in 0 and 66 μM SITS (△). After 4 min mixing at 24 °C, delay time curves were obtained in the presence of 20 μM CCCP and 60 μM valinomycin. (The 66 μM SITS suspension was only used for subsequent mixing, not for its own delay time curve.)

Table 1. Statistical parameters of the distribution of delay times

P_{Cl}-limited delay times (min)	Anion exchange-limited delay times (min)
40·0 ± 5·6 (14·0)	5·80 ± 0·86 (14·8)
43·7 ± 5·8 (13·2)	4·80 ± 0·73 (15·2)
40·1 ± 5·2 (13·0)	3·85 ± 0·51 (13·2)

The distributions of P_{Cl}- and anion exchange-limited delay times were obtained by fitting Gaussian curves to the histograms in Figs 6 and 7, respectively. Means ± S.D. and c.v. values (in parentheses) for each Gaussian curve are shown. For further details see legends to Figs 6 and 7.

DISCUSSION

We report here the development and application of a new delay time method to assess the heterogeneity of red cell anion exchange function and diffusional Cl^- permeability. The results show that the delay time distributions of P_{Cl} and anion exchange-limited dehydration can be fitted by bell-shaped Gaussian curves with c.v. values of 13–15%.

To interpret these results and assess their significance it is necessary to understand first which cell properties are expressed in the delay time distribution. In the analysis below we demonstrate that delay time distributions reflect the change in time of the osmolyte content and lytic volume distributions in the cell population as it dehydrates, and that the similarity in c.v. values between delay time and original cell volume distributions indicates uniformity of P_{Cl} and anion exchange function among the cells.

The delay time distributions report the lag with which individual cells, dehydrating at a rate limited by their P_{Cl} or by the turnover of the anion exchanger, attain an osmolyte loss which protects them from haemolysis when diluted into a specific hypotonic medium. Therefore, delay time distributions should relate to and reflect the change with time of the osmolyte content and volume distributions of the dehydrating cells. This can be described more precisely

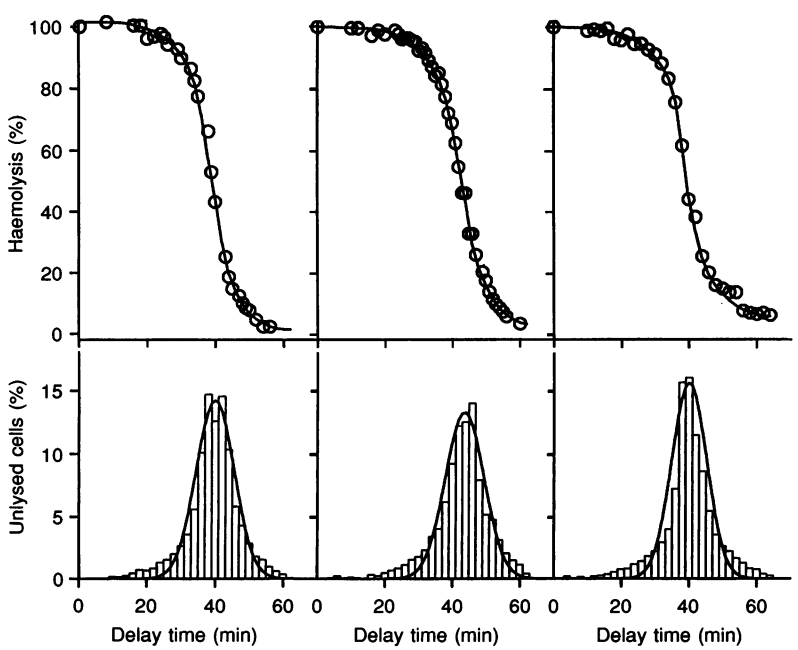

Figure 6. Distribution of P_{Cl}-limited delay times in red cells from three donors

Below each of the three delay time curves in the upper panels, obtained after the addition of 60 μM valinomycin but no CCCP and no SITS, corresponding histograms show the fraction of cells reaching their delay times (unlysed) within each 2 min delay time interval. Thus defined, the Gaussian curves fitted to the histograms correspond to the derivatives of the delay time curves. These Gaussian curves report the mean and S.D. of the P_{Cl}-limited delay time distribution for each cell population (see Table 1).

as follows. For any dehydrating cell in osmotic equilibrium at lysis, $C_L = \Sigma Q^L / V_W^L$, where C_L is the selected osmolality for the haemolytic medium (constant), ΣQ^L is the residual osmolyte content of the cell at the instant of lysis and V_W^L is the critical haemolytic volume of cell water. If the residual osmolyte content of a cell at the time of sampling, ΣQ, is higher than ΣQ^L, the cell will lyse on contact with the C_L medium because it will swell beyond its critical haemolytic volume; if ΣQ is lower than ΣQ^L, the cell will attain osmotic equilibrium with $V_W < V_W^L$ and will not lyse (we neglect effects of minor prelytic osmolyte losses, comparable for all cells – see legend to Fig. 1). Under the selected conditions, as the cells dehydrate, their ΣQ values all eventually fall below ΣQ^L, and that is why the lysis decreases from 100 to 0% with advancing time along the delay time curve. These curves therefore contain information on both the original distributions of ΣQ (ΣQ^o) and V_W^L among the cells, and the distribution of velocities at which the cells approach ΣQ^L as they dehydrate.

We next need to know the ΣQ^o and V_W^L distributions of normal human red cells. In a previous analysis of the volume, haemoglobin content, haemoglobin concentration and haemolytic distributions of normal human red cells, it was shown that the c.v. values of the distributions of ΣQ^o and V_W^L corresponded to those of the original cell volumes (Lew *et al.* 1995). In that study, flow cytometric measurements indicated that the distribution of original cell volumes was Gaussian, with a c.v. between 12·8 and 14·8% (mean c.v. ± S.E.M. = 13·65 ± 0·03; $n = 22$), in agreement with earlier estimates from microscopic measurements (Canham & Burton, 1968). Therefore, $c.v.^o \approx c.v._{\Sigma Q} \approx c.v._{V_W^L}$ with values within the 13–15% range. Thus defined, c.v.^{o} represents the c.v. value common to all these distributions, derived from direct measurement of the original red cell volume distribution.

After dehydration is triggered by the addition of valinomycin, how are the osmolyte and volume distributions expected to change with time? To address this point we will first assume that all the cells dehydrate at the same velocity, i.e. that the rate of osmolyte loss is the same for all the cells. For K^+-permeabilized cells, dehydrating by net loss of KCl and water, limited by anion movement, and undergoing the treatments described for the delay time method, the mean velocity of osmolyte loss is given by:

$$\overline{\Delta\Sigma Q}/\overline{\Delta t} = \overline{v}, \quad (1)$$

where $\Delta\Sigma Q$ represents the osmolyte lost in the interval Δt, v is the velocity of osmolyte loss, provisionally assumed equal for all cells, and the bars over symbols indicate mean values. The value of the mean v may be estimated from:

$$\overline{v} = (\overline{\Sigma Q^o} - \overline{\Sigma Q^L})/\overline{\Delta t}, \quad (2)$$

where mean ΣQ^o and ΣQ^L are the osmolyte contents of cells at time zero, when they are in osmotic equilibrium with an

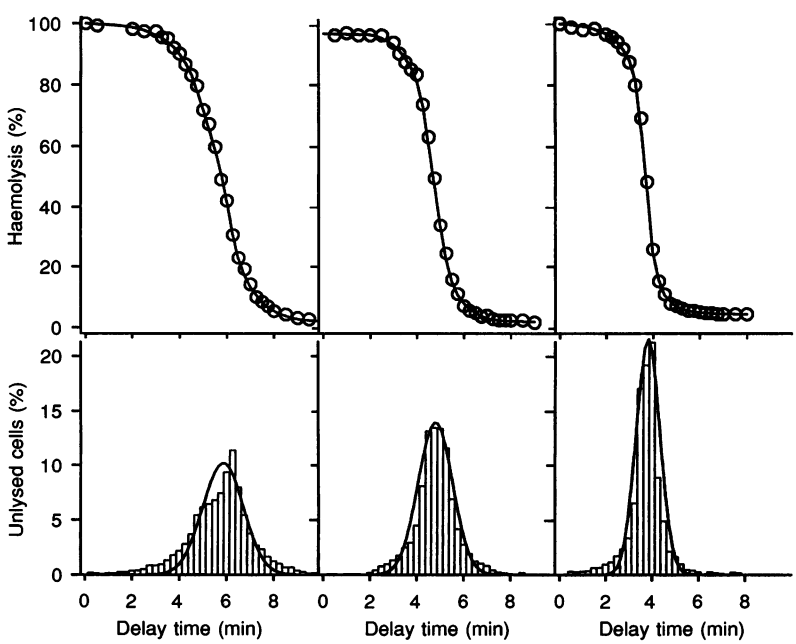

Figure 7. Distribution of anion exchange-limited delay times in red cells from three donors

The delay time curves limited by P_{Cl} (Fig. 6) and by anion exchange (this figure) were measured on the same cell suspensions from each donor, and are shown in the corresponding upper panels. After 20 min preincubation at 4 °C with 20 μM SITS the red cells were warmed to 24 °C and received 20 μM CCCP and 60 μM valinomycin. The histograms and Gaussian curve fits in the lower panels (see legend to Fig. 6) provide the statistical parameters of the anion exchange-limited delay time distributions (see Table 1).

isotonic plasma-like buffer of osmolality C_B, and at mean time Δt, when they reach osmotic equilibrium with the haemolytic buffer of osmolality C_L, respectively. The mean ΣQ^o and ΣQ^L are given by:

$$\overline{\Sigma Q^o} = \overline{V_W^o} C_B \quad (3)$$

and

$$\overline{\Sigma Q^L} = \overline{V_W^L} C_L, \quad (4)$$

where the corresponding mean V_W values represent the volumes of cell water at time zero (V_W^o, original condition of the cells) and at the time when the dehydrating cells reach osmotic equilibrium in C_L without further haemolysis (V_W^L).

We now ask how the statistical parameters of the delay time curve (c.v.$_{dt}$) relate to c.v.^{o}. We first designate ΣQ^o and V_W^L at their mean values $\pm$ 1 S.D., using the subscripts 'low' and 'high' to indicate -1 S.D. and $+1$ S.D. from their mean values, respectively.

$$\Sigma Q^o_{low} = \overline{\Sigma Q^o}(1 - \text{c.v.}^o), \quad (5)$$

$$\Sigma Q^o_{high} = \overline{\Sigma Q^o}(1 + \text{c.v.}^o), \quad (6)$$

$$V^L_{low} = \overline{V_W^L}(1 - \text{c.v.}^o), \quad (7)$$

$$V^L_{high} = \overline{V_W^L}(1 + \text{c.v.}^o). \quad (8)$$

The assumption that all the cells dehydrate at the same rate implies that the c.v. of the ΣQ distribution is not disturbed during dehydration. Therefore, c.v.$_{\Sigma Q^o}$ = c.v.$_{\Sigma Q}$. This, together with the common value of c.v.^{o} for ΣQ^o and V_W^L, allows pairing lows and highs from eqns (5) and (7), and (6) and (8) in calculating the loss of cell osmolyte needed to reach osmotic equilibrium with C_L, $\Delta\Sigma Q$. Thus:

$$\Delta\Sigma Q_{low} = (\overline{\Sigma Q^o} - C_L\overline{V_W^L})(1 - \text{c.v.}^o) \quad (9)$$

and

$$\Delta\Sigma Q_{high} = (\overline{\Sigma Q^o} - C_L\overline{V_W^L})(1 + \text{c.v.}^o). \quad (10)$$

Substituting the value of $(C_L\overline{V_W^L})$ from eqn (4) into eqns (9) and (10), we obtain:

$$\Delta\Sigma Q_{low} = (\overline{\Sigma Q^o} - \overline{\Sigma Q^L})(1 - \text{c.v.}^o) \quad (11)$$

and

$$\Delta\Sigma Q_{high} = (\overline{\Sigma Q^o} - \overline{\Sigma Q^L})(1 + \text{c.v.}^o). \quad (12)$$

We can now estimate the time for low and high cells to reach haemolysis protection, Δt_{low} and Δt_{high}, as follows:

$$\Delta t_{low} = \overline{\Delta t}(\Delta\Sigma Q_{low}/\overline{\Delta\Sigma Q}), \quad (13)$$

$$\Delta t_{high} = \overline{\Delta t}(\Delta\Sigma Q_{high}/\overline{\Delta\Sigma Q}). \quad (14)$$

Substituting eqns (1), (2), (11) and (12) into (13) and (14), we obtain:

$$\Delta t_{low} = \overline{\Delta t}(1 - \text{c.v.}^o), \quad (15)$$

$$\Delta t_{high} = \overline{\Delta t}(1 + \text{c.v.}^o). \quad (16)$$

Based on the properties of Gaussian distributions, we may define c.v.$_{dt}$ by:

$$\text{c.v.}_{dt} = \tfrac{1}{2}(\Delta t_{high} - \Delta t_{low})/\overline{\Delta t}. \quad (17)$$

Substituting eqns (15) and (16) into (17) we obtain:

$$\text{c.v.}_{dt} = \text{c.v.}^o, \quad (18)$$

which predicts that c.v.$_{dt}$ and c.v.^{o} should be similar *if the velocity of dehydration among the cells is uniform*, as was assumed. Conversely, if c.v.$_{dt} \approx$ c.v.^{o}, then cell-to-cell variation in rate-limiting permeability must be minimal, since any significant variation would have expanded c.v.$_{dt}$ relative to c.v.^{o} to a detectable extent. Thus the Gaussian distributions of delay times, with the c.v. values of 13–15% observed here, indicate a high degree of uniformity of dehydration rates limited by either P_{Cl} or anion exchange turnover.

The uniformity of P_{Cl} and anion exchange distributions is consistent with the notion that anion conductance is primarily through the anion exchange protein (Vestergaard-Bogind & Lassen, 1974; Knauf *et al.* 1977; Gunn, 1978; Knauf *et al.* 1983; Fröhlich, 1984), and also indicates a uniform amount of anion exchange protein in each cell. The present results, by documenting that c.v.$_{dt} \approx$ c.v.^{o}, add further indirect support to the proposed statistical correlation between red cell osmolyte content and V_W^L (Lew *et al.* 1995). The delay time method described here provides a new approach to studies of the distribution of transport functions in various abnormal red cells.

Bisognano, J. D., Dix, J. A., Pratap, P. R., Novak, T. S. & Freedman, J. C. (1993). Proton (or hydroxide) fluxes and the biphasic osmotic response of human red blood cells. *Journal of General Physiology* **102**, 99–123.

Bookchin, R. M., Ortiz, O. E. & Lew, V. L. (1991). Evidence for a direct reticulocyte origin of dense red cells in sickle cell anemia. *Journal of Clinical Investigation* **87**, 113–124.

Cabantchik, Z. I. & Rothstein, A. (1972). The nature of the membrane sites controlling anion permeability of human red blood cells as determined by studies with disulfonic stilbene derivatives. *Journal of Membrane Biology* **10**, 311–330.

Cabantchik, Z. I. & Rothstein, A. (1974). Membrane proteins related to anion permeability of human red blood cells. *Journal of Membrane Biology* **15**, 207–226.

Canham, B. P. & Burton, A. C. (1968). Distribution of size and shape in populations of normal human red cells. *Circulation Research* **22**, 405–422.

FREEMAN, C. J., BOOKCHIN, R. M., ORTIZ, O. E. & LEW, V. L. (1987). K-permeabilized human red cells lose an alkaline, hypertonic fluid containing excess K over diffusible anions. *Journal of Membrane Biology* **96**, 235–241.

FRÖHLICH, O. (1984). Relative contributions of the slippage and tunneling mechanisms to anion net efflux from human erythrocytes. *Journal of General Physiology* **84**, 877–893.

GARCÍA-SANCHO, J. & LEW, V. L. (1988). Detection and separation of human red cells with different calcium contents following uniform calcium permeabilization. *Journal of Physiology* **407**, 505–522.

GUNN, R. B. (1978). Considerations of the titratable carrier model for sulfate transport in human red blood cells. In *Membrane Transport Processes*, ed. HOFFMAN, J. F., pp. 61–77. Raven Press, New York.

GUNN, R. B., DALMARK, M., TOSTESON, D. C. & WIETH, J. O. (1973). Characteristics of chloride transport in human red blood cells. *Journal of General Physiology* **61**, 185–206.

HUNTER, M. J. (1977). Human erythrocyte anion permeabilities measured under conditions of net charge transfer. *Journal of Physiology* **268**, 35–49.

KNAUF, P. A., FUHRMANN, G. F., ROTHSTEIN, S. & ROTHSTEIN, A. (1977). The relationship between anion exchange and net anion flow across the human red blood cell membrane. *Journal of General Physiology* **69**, 363–386.

KNAUF, P. A., LAW, F. Y. & MARCHANT, P. J. (1983). Relationship of net chloride flow across the human erythrocyte membrane to the anion exchange mechanism. *Journal of General Physiology* **81**, 95–126.

LEW, V. L. & BOOKCHIN, R. M. (1986). Volume, pH and ion content regulation in human red cells: analysis of transient behavior with an integrated model. *Journal of Membrane Biology* **92**, 57–74.

LEW, V. L. & BOOKCHIN, R. M. (1992). Role of reticulocyte transport heterogeneity in the generation of mature sickle cells with different volumes. *Biochemical Society Transactions* **20**, 797–800.

LEW, V. L., RAFTOS, J. E., SORETTE, M., BOOKCHIN, R. M. & MOHANDAS, N. (1995). Generation of normal human red cell volume, hemoglobin content and membrane area distributions, by "birth" or regulation? *Blood* **86**, 334–341.

MACEY, R. I., ADORANTE, J. S. & ORME, F. W. (1978). Erythrocyte membrane potentials determined by hydrogen ion distribution. *Biochimica et Biophysica Acta* **512**, 284–295.

PONDER, E. (1948). *Hemolysis and Related Phenomena*. Grune and Stratton, New York.

RAFTOS, J. E., BOOKCHIN, R. M. & LEW, V. L. (1996). Distribution of chloride permeabilities in normal human red cells. *Journal of Physiology* **491**, 773–777.

VESTERGAARD-BOGIND, B. & LASSEN, U. V. (1974). Membrane potential of *Amphiuma* red cells: hyperpolarizing effect of phloretin. In *Comparative Biochemistry and Physiology of Transport*, ed. BOLIS, L., BLOCH, K., LURIA, S. E. & LYNEN, F., pp. 346–353. Elsevier/North-Holland Biomedical Press, Amsterdam.

WIETH, J. O. (1980). Interaction between two types of pH-equilibrating proton and hydroxyl ion carriers. In *Membrane Transport in Erythrocytes. Relations between Function and Molecular Structure*, ed. LASSEN, U. V., USSING, H. H. & WIETH, J. O., *Alfred Benzon Symposium 14*, pp. 512–519. Munksgaard, Copenhagen.

Acknowledgements

We thank the British Heart Foundation, The Wellcome Trust, the National Health and Medical Research Council (Australia) and NIH (USA; grants HL28018 and HL28905) for funds, and Mrs J. Gray for excellent technical assistance.

Author's email address

J. E. Raftos: j.raftos@med.monash.edu.au

Received 16 July 1996; accepted 29 October 1996.