Anion Transport in Dog, Cat, and Human Red Cells

Effects of Varying Cell Volume and Donnan Ratio

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ABSTRACT Membrane potential and the rate constants for anion self-exchange in dog, cat, and human red blood cells have been shown to vary with cell volume. For dog and cat red cells, the outward rate constants for SO₄ and Cl increase while the inward rate constant for SO₄ decreases as cells swell or shrink. These changes coincide with the membrane potential becoming more negative as a result of changes in cell volume. Human red cells exhibit a similar change in the rate constants for SO₄ and Cl efflux in response to cell swelling, but shrunken cells exhibit a decreased rate constant for SO₄ efflux and a more positive membrane potential. Hyperpolarization of shrunken dog and cat red cells is due to a volume-dependent increase in $P_{\text{Na}}$. If this increase in $P_{\text{Na}}$ is prevented by ATP depletion or if the outward Na gradient is removed, the response to shrinking is identical to human red cells. These results suggest that the volume dependence of anion permeability may be secondary to changes in the anion equilibrium ratio which in red cells is reflected by the membrane potential. When the membrane potential and cell volume of human red cells were varied independently by a method involving pretreatment with nystatin, it was found that the rate of anion transport (for SO₄ and Cl) does not vary with cell volume but rather with membrane potential (anion equilibrium ratio); that is, the rate constant for anion efflux is decreased and that for influx is increased as the membrane potential becomes more positive (internal anion concentration increases) while the opposite is true with membrane hyperpolarization (a fall in internal anion concentration).

INTRODUCTION

Unlike human and most other mammalian red blood cells, cat and dog red cells are low in K and high in Na (cf. Bernstein, 1954). Cat and dog red cells
also exhibit unique transport properties. The cells do not display any ouabain-sensitive active transport of Na or K (Miles and Lee, 1972) nor do their membranes possess any Na, K-ATPase activity (Chan et al., 1964). Davson (1942) and Parker and Hoffman (1976) have reported that the cation permeability of dog red cells is volume dependent, that is, that the Na permeability, $P_{Na}$, decreases and the K permeability, $P_{K}$, increases as cells swell while the opposite occurs in shrunken cells. Sha'afi and Hajjar (1971) found a similar volume dependence for cation permeability in cat red cells.

Recently the properties of anion transport in cat and dog red cells have also been investigated. Anion self-exchange is approximately twofold higher in dog red cells compared to cat or human (Deuticke and Gruber, 1970; Wieth et al., 1974). Although the rates differ, the anion exchange systems of these red cell types exhibit many common properties. The dependence of anion influx on the intracellular anion concentration, the pH dependence, and competitive inhibition between anions are almost identical in dog, cat, and human red blood cells (Castranova et al., 1976, 1979; Gunn et al., 1973; Passow and Wood, 1974; Dalmark, 1975). Parker et al. (1977) have measured net Cl permeability in dog red cells and have found it to be similar to values reported for human red cells (Sachs et al., 1975). These data indicate that the $P_{Cl}$ is normally so large that the membrane potential is reflected by the Cl equilibrium ratio.

Sha'afi and Pascoe (1972) have reported volume-dependent variations in anion permeability in cat red blood cells; i.e., the rate constant for SO$_4$ influx decreases as cells shrink and increases as cells swell. These results differ from those of Funder and Wieth (1976) which show no volume dependence for anion transport in human red cell ghosts. Gunn et al. (1975) suggested that this discrepancy might be explained by competitive inhibition between Cl and SO$_4$ since, in the former study, cell volume was altered by variation in external NaCl concentration.

The purpose of the present study was to determine whether anion transport in cat and dog red cells is indeed volume dependent and to compare the characteristics of these types of cells to human red cells which lack the striking volume dependence of cation transport. We conclude that the observed changes in anion transport are due to changes in the anion distribution ratio rather than cell volume, per se. We also discuss the apparent dependence of the anion self-exchange rate on the Donnan ratio for anions in relation to recent results by Dalmark (1975) and Knauf (1979). A brief account of this work has been reported previously (Castranova et al., 1976).

**METHODS**

**Red Cells**

Venous blood was drawn into a heparinized container to prevent clotting (1 mg heparin/10 ml blood). The blood was centrifuged at 12,000 g at 2°C and theuffy coat was removed. The red cells were then washed three times by alternate resuspension and centrifugation with 10 vol of medium appropriate to the particular experiment being performed (see below).
SO₄ Self-Exchange

Measures of SO₄ transport were obtained by determining the rate constants for ³⁵SO₄ efflux and influx under equilibrium exchange conditions at 37°C. The method for measuring efflux is a modification of that described in detail by Gardos et al. (1969) and used previously by us (Castranova and Hoffman, 1979, Castranova et al., 1979).

**Efflux**  Cells washed in SO₄ flux media (millimolar), 80 NaCl, 5 KCl, 10 Na₂SO₄, 5 NaHEPES (N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid), 5 glucose, 0-200 sucrose (pH 7.4), were incubated at 10% hematocrit for 3 h at 37°C to ensure equilibration of cell volumes in media of different osmolarities (varied with sucrose) and to load cells with ³⁵SO₄ (NEX-041, New England Nuclear, Boston, Mass.) which was added to cell suspensions to give approximately 10 μCi/ml. It was independently shown that cell volume and anion contents reach a steady state within 3 h of incubation and are essentially constant during the time periods required for flux measurements. After incubation, the suspensions were centrifuged, the radioactive media was removed, and the cells were washed again three times in the cold (2°C) with nonradioactive SO₄ flux media of appropriate osmolarities. In order to commence the efflux these washed, ³⁵SO₄-loaded cells were then added to flasks containing the appropriate media to give a hematocrit of 0.5% and incubated at 37°C with gentle shaking.

At given times after the addition of cells to the various media, suspension samples were taken and the cells were packed by centrifugation (12,300 g at 2°C). Supernate samples were taken, acidified with trichloroacetic acid (TCA), and centrifuged. Aliquots of this supernate were added to aqueous counting scintillant (ACS) fluor (Amersham/Searle Corp., Arlington Heights, Ill.).

The amount of ³⁵SO₄ appearing in the medium at time t (Pₜ) was determined by liquid scintillation counting of these supernate samples. The radioactivity in the medium at time infinity (P∞) was determined from the cell suspension. Samples of the cell suspensions were acidified with TCA and centrifuged to remove precipitated protein. Aliquots of the supernate were added to ACS fluor and ³⁵SO₄ determined by liquid scintillation counting.

**Influx**  Cells were equilibrated in nonradioactive SO₄ flux media of varying composition for 3 h at 37°C at a 10% hematocrit as described above for efflux. Influx was initiated by adding a tracer amount of ³⁵SO₄ to each cell suspension. Suspension samples were taken at 0, 5, 10, and 15 min and 3 h after this addition. Cells were separated from the radioactive medium by centrifugation (12,300 g, at 2°C) and washed three times by centrifugation in iced (2°C) nonradioactive SO₄ flux media of appropriate osmolarity containing 10 μM 4-acetamide-4'-isothiocyanostilbene-2,2'-disulfonate (SITS). The SITS was added as a precautionary measure to minimize any loss of ³⁵SO₄ from the cells during washing since SITS is known to inhibit SO₄ efflux (Knauf and Rothstein, 1971; Castranova et al., 1976, 1979). The washed cells were then hemolyzed and an aliquot of hemolysate was acidified with TCA and centrifuged to remove precipitated protein. The radioactivity of each time sample was determined by liquid scintillation counting. A second aliquot of each hemolysate sample was taken to determine the hemoglobin absorption, A₅₄₀. This value was divided by the A₅₄₀ of a known volume of packed cells to determine ³⁵SO₄/liter cells at time t. ³⁵SO₄/liter cells at equilibrium (T = ∞) was obtained in the same manner from the 3-h sample.

Cl Self-Exchange

Cells were washed in Cl flux media of different osmolarities (millimolar), 93 NaCl, 5 KCl, 5 NaHEPES, 5 glucose, 0-200 sucrose (pH 7.4). For volume equilibration, red
cells were incubated at 10% hematocrit in the respective media containing 1 μCi/ml 32Cl (ICN Pharmaceuticals Inc., Irvine Calif., no. 63005) for 3 h at 37°C. In preparation for efflux measurements, cell suspensions were incubated 30 min at 0°C. For measurement of efflux rate, a sample of cell suspension was centrifuged (using a Sorvall HB-4 rotor [DuPont Instruments-Sorval, DuPont Co., Newtown, Conn.] at 2°C and 27,000 g for 5 min), the radioactive supernate was removed, and the efflux was initiated by adding the unwashed 32Cl-containing cells to isotope-free medium of proper osmolarity at 0°C. The hematocrit was 1%. A rapid filtering technique was used to obtain supernate samples at intervals of a few seconds (Wieth et al., 1973). The amount of 32Cl in samples was determined by liquid scintillation counting as described previously for 35SO4 efflux.

**Calculation of Rate Constants**

Anion transport in cat, dog, and human red cells appears to follow a two-compartment model (Gardos et al., 1969) which is described by the equation:

\[ \ln(1 - P_t/P_w) = -kt, \]

where \( k \) is the rate constant, \( t \) is time, and \( P_t, P_w \) are the amounts of isotope in the compartment being filled at times \( t \) and \( \infty \). Thus, we plot \( \ln(1 - P_t/P_w) \) vs. time and take the slope of the line, fit to the data by the method of least squares, as the rate constant. The correlation coefficients for the data reported here are 0.98 or above.

Efflux, for constant surface area, is dependent on cell volume as follows: \( ^oM = \frac{\kappa}{V} \) \( ^oC_i \), where \( ^oM \) is efflux in millimoles per hour, \( \kappa \) is the rate constant for efflux in hour\(^{-1} \); \( V \) is the intracellular volume in liter cell water; and \( ^oC_i \) is the intracellular concentration of Cl or SO4 in millimoles/liter cell water. Thus, it is necessary to correct the results for differences in cell volume to be able to assess changes in membrane permeability. For this reason all rate constants for efflux were corrected for differences in relative cell volume as follows: corrected \( ^o\kappa \) = (measured \( ^o\kappa \) ) (fraction of control volume). Therefore the corrected \( ^o\kappa \) will reflect actual volume dependence of the membrane permeability to anions. In this study, relative efflux (millimoles per liter cell H2O × hour) can be obtained from the product of the corrected \( ^o\kappa \) and \( ^oC_i \).

In addition to correction for cell volume differences, a further calculation is required to obtain the influx rate constant in the proper units. These units have been shown by Sachs (1971) to be (millimoles per liter cell H2O × hour) (millimoles per liter medium)\(^{-1} \). Therefore, SO4 influx rate constants \( (k_{SO4}) \), corrected for volume differences and in the proper units, were calculated from the equation:

\[ \frac{k_{SO4}}{k_{SO4}} = (\kappa) \text{ (fraction of control volume) } \text{ (SO4 cell/SO4 medium)}, \]

where \( \kappa \) = slope of the line where \( \ln(1 - P_t/P_w) \) is plotted against time and has units of hour\(^{-1} \). Thus, in this study, relative influx (millimoles per liter cell H2O × hour) can be obtained from the product of the corrected \( \kappa \) and \( C_o \), i.e., the extracellular concentration of substrate in millimoles per liter medium.

**Determination of Relative Cell Volume**

Values of cell volumes relative to control were obtained either from the equation:

\[ \text{Fraction of control volume} = 1 + 0.72 \left( \frac{T_c}{T_t} - 1 \right) \]

(Ponder, 1948); where \( T_c \) and \( T_t \) are the osmolarities measured by freezing point depression (Advanced osmometer, Advanced Instruments, Inc., Needham Heights, Mass.) of media containing control and test cells, respectively, or from the equation:
Fraction of control volume \( = \frac{(1 - CW_c)}{(1 - CW_t)} \)

(Parker and Hoffman, 1976); where \( CW_c \) and \( CW_t \) are fractional cell water contents (% vol/vol determined by wet weight-dry weight differences, using 1.097 as the specific gravity of cells) of cells in control and test media, respectively. The relative cell volumes determined from these two equations were comparable.

**Membrane Potentials**

Membrane potentials were either calculated from equilibrium anion distribution ratios or determined by the fluorescence technique described by Hoffman and Laris (1974).

For measurement of anion distributions, cells were equilibrated with either \( {}^{36}\text{Cl} \) or \( {}^{36}\text{SO}_4 \) in the appropriate media of various osmolarities as described above for measurement of fluxes. Cell suspensions were then centrifuged at 27,000 g for 10 min and the distribution of \( {}^{36}\text{Cl} \) or \( {}^{36}\text{SO}_4 \) between cells and medium determined from an aliquot of supernate or of packed cells obtained after supernate had been removed. Details of the technique have been described previously (Hoffman and Laris, 1974). Cell water content (% vol/vol) was determined, using a sample of the remaining packed cells, by wet weight-dry weight measurements.

Chloride and \( \text{SO}_4 \) ratio \( (r_{\text{Cl}}, r_{\text{SO}_4}) \) and corresponding membrane potentials \( (E_m) \) were calculated as follows:

\[
\frac{r_{\text{Cl}}}{r_{\text{SO}_4}} = \left( \frac{\text{cellular radioactivity}}{\text{supernatant radioactivity}} \right) \div \text{cell water content}
\]

\[
E_m = 54 \log_{10}(r_{\text{Cl}}) \text{ for } r_{\text{Cl}} \text{ determined at } 0^\circ\text{C}.
\]

\[
E_m = 61 \log_{10}(r_{\text{SO}_4}) \text{ for } r_{\text{SO}_4} \text{ determined at } 37^\circ\text{C}.
\]

To measure potential with the dye, diS-C5(5), fluorescence was measured using an Aminco Bowman spectrofluorophotometer (American Instrument Co., Silver Spring, Md.) with a narrow band pass filter (640, Baird-Atomic, Inc., Bedford, Mass.) and a cut-off filter (RG-665, Schott Inc., New York) to eliminate interference in signal due to the light scattering of the red cells. Excitation and emission wavelengths were set at 640 and 665 nm, respectively.

Red cells were equilibrated for 3 h in media of various osmolarities. Changes in fluorescence were initiated by addition of the K ionophore, valinomycin \((10^{-8}\text{M} \text{ final})\), and were measured in cell suspensions \((0.33\% \text{ hematocrit})\) equilibrated with 66 \( \mu \text{g/ml} \) diS-C5 (5). Stock solutions of fluorescent dye \((0.2 \text{ mg/ml})\) and valinomycin \((0.3 \text{ mM})\) were made in ethanol. Membrane potential was determined from measurement of the null point for K, i.e., the extracellular concentration of K at which there is no change in fluorescence upon the addition of valinomycin, as described by Hoffman and Laris (1974) and by Parker et al. (1977). At the K null point, the membrane potential (determined at 22°C) can be identified as the Nernst potential for K:

\[
E_m = -58 \log_{10} \left( \frac{\text{millimoles K/liter cell water}}{\text{millimoles K/liter medium, at null}} \right)
\]

Therefore, intracellular and null concentrations of K were determined for red cells with different cellular volumes and used to calculate the membrane potential.
Nystatin Treatment

Using a modification of the nystatin treatment of Cass and Dalmark (1973) which markedly increases the membrane permeability to Na and K, we were able to alter the ionic content of human red cells and obtain cells of either different volume and constant membrane potential or constant volume and different potential, potentials being calculated from anion distribution ratios.

Red cells were washed three times (12,100 g at 2°C) in a pretreatment medium (millimolar), 150 KCl, 10 NaHEPES, 5 glucose, 30 sucrose (pH 7.2), and then resuspended at 20% hematocrit in this medium. Nystatin (5 mg/ml in methanol) was added to give 50 μg/ml and cell suspensions were incubated at 0°C for 30 min to allow the nystatin to interact with the membrane. After this, cells were centrifuged, resuspended in equilibration media of desired composition (see below) to a 20% hematocrit, and incubated at 0°C for 20 minutes with 35 μg/ml nystatin.

To obtain cells of different volume but constant membrane potential the compositions of the equilibration media were (millimolar): 110, 140, 170, or 200 KCl; 10 NaCl; 5 NaHEPES; 5 dextrose; and 30 sucrose (pH 7.2). After equilibration with media of the desired KCl concentration, nystatin was effectively removed from cells, with concomitant restoration of the membrane’s original permeability to Na and K, by washing at 25°C eight times (12,000 g at 25°C, 10% hematocrit per wash) in the respective equilibration media and then once in the flux medium (millimolar): 80 NaCl, 5 KCl, 10 Na2SO4, 5 NaHEPES, 5 glucose, 100 sucrose (pH 7.4). Nystatin-free cells were then incubated at 37°C for 3 h in the flux medium containing 36SO4 in preparation for measuring \( k_{\text{SO4}} \) and cell water content as described above. During incubation in the flux medium, cells treated in a hypotonic equilibration medium shrink, their internal ion content rising toward the control value. On the other hand, cells treated in a hypertonic equilibration medium swell and the internal ion content falls toward the control level. Thus, cells are obtained with different volume but constant membrane potential. Cells of constant volume but different anion ratios were obtained by nystatin treatment in the following equilibration media (millimolar): 140, 200, or 260 KCl; 10 NaCl; 5 NaHEPES; 5 dextrose; and 30 sucrose (pH 7.2). As before, cells were washed at 25°C eight times in the respective equilibration media and once in a flux medium of the same osmolarity as the equilibration medium. The compositions of the flux media (pH = 7.4) were (millimolar): 150 NaCl; 5 KCl; 5 NaHEPES; 5 glucose; and 0, 100, or 200 sucrose. The cells were incubated at a 10% hematocrit for 2 h at 37°C and 1 h at 0°C to load with 36Cl. Then the \( k_{\text{Cl}}, \tau_{\text{Cl}}, \) and cell water content were measured as described above.

RESULTS AND DISCUSSION

The effects of varying cell volume on the efflux rate constant for sulfate self-exchange (\( k_{\text{SO4}} \)) in cat, dog, and human red blood cells are shown in Fig. 1. In contrast to previous work (Sha’afi and Pascoe, 1972) concerning the volume dependence of SO4 transport in cat red cells, cell volume was altered by variations in external sucrose concentration rather than changes in external NaCl. Indeed, Cl has been shown to inhibit SO4 transport in cat and dog red cells by Castaño, et al. (1976, 1979) and in human red cells by Passow and Wood (1974). Thus by maintaining the external ionic strength constant, it is possible to avoid variation in Cl inhibition of SO4 efflux during studies of SO4 transport vs. cell volume. Fig. 1 shows that the volume dependence of SO4 self-exchange is very similar in cat and dog red blood cells. In these low K-
high Na cells, the rate constant for SO₄ efflux increases in both swollen and shrunken cells. In human red cells, %k₉₀₄ also increases as the cells swell. However, in contrast to the situation in cat and dog cells, %k₉₀₄ decreases as human red cells shrink.

![SO₄ transport in cat, dog, and human red cells at different volumes.](image)

**Figure 1.** SO₄ transport in cat, dog, and human red cells at different volumes. Cells (10% hematocrit) were incubated at 37°C for 3 h in media containing (millimolar) 80 NaCl, 5 KCl, 10 Na₂SO₄, 5 HEPES, 0-200 sucrose, 5 glucose (pH 7.4), and 100 μCi³⁵SO₄/ml packed cells. Rates for ³⁵SO₄ efflux (%k₉₀₄) were determined as described in Methods and cell volumes were calculated from the Ponder equation: relative volume = 1 + 0.72(Tc/T - 1). These data represent means of two determinations. Individual determinations vary by 2%. (A) %k₉₀₄ as a function of relative cell volume for cat (□), dog (■), and human (×) red blood cells. (B) %k₉₀₄ for cat (□), dog (■), and human (×) cells, expressed as fraction of control rates vs. relative cell volume.

It was known from the work of Van Slyke et al. (1923), Jacobs and Parpart (1931), as well as from Cook (1967), Gary-Bobo and Solomon (1968, 1971), and Freedman and Hoffman (1979) that changes in cell volume alter the intracellular pH and the net charge on hemoglobin. Such a change in
hemoglobin charge would in turn alter the Donnan equilibrium of red cells. They predict that at pH = 7.4 a decrease in cell volume would result in membrane depolarization while cells would hyperpolarize as their volume increased. The effect of cell volume on membrane potential, $E_m$, is given in Table I. Membrane potentials were calculated either from the equilibrium distribution of $^{38}\text{SO}_4$ and $^{36}\text{Cl}$, or using the fluorescent dye technique of Hoffman and Laris (1974). In human red blood cells, the Donnan ratio and $E_m$ vary with cell volume as expected, i.e., $E_m$ becomes more negative as the cells swell and more positive as they shrink. Dog red cells exhibit a different response to cell volume changes. In dog red cells, $E_m$ is hyperpolarized compared to control for both swollen and shrunken cells. The difference in $E_m$

| TABLE I |
| EFFECT OF CELL VOLUME ON DONNAN DISTRIBUTION |
| RATIO OF $\text{SO}_4$ ($r_{\text{SO}_4}$) AND MEMBRANE POTENTIAL ($E_m$) |

| Cells   | Fraction of control volume* | $r_{\text{SO}_4}$ | $E_m$ | $E_m$ |
|---------|-----------------------------|-------------------|-------|-------|
|         |                             | mV                | mV    |       |
| Dog     | 1.42                        | 0.76              | -7.3  | -1.8  |
|         | 1.00                        | 0.97              | -0.8  | +2.5  |
|         | 0.71                        | 0.81              | -5.6  | -3.1  |
| Human   | 1.29                        | 0.84              | -4.6  |       |
|         | 1.00                        | 1.05              | +1.3  |       |
|         | 0.89                        | 1.24              | +5.7  |       |

Data represent means of two determinations. Individual determinations vary by 1%.

* Calculated from cell water contents (% V/V) as described in Methods. Control % H$_2$O: 69.8 (dog), 69.0 (human).

± $r_{\text{SO}_4} = [\text{SO}_4]_{\text{cell}}/[\text{SO}_4]_{\text{medium}}$; 37°C.

§ $E_m = 61\log r_{\text{SO}_4}$. (In other experiments, membrane potentials calculated from $r_{\text{Cl}} = (\text{Cl})_{\text{cell}}/(\text{Cl})_{\text{medium}}$ showed the same relative dependence on cell volume as $E_m$ calculated from $r_{\text{SO}_4}$).

¶ Obtained using the fluorescent dye technique of Hoffman and Laris (1974); 22°C.

† Fresh dog cells.

between shrunken human and dog red cells may have some bearing on the difference in the volume dependence of SO$_4$ movement in these cells, i.e., $^\circ k_{\text{SO}_4}$ seems to increase as $E_m$ becomes more negative and decrease with more positive potentials.

Results presented in Table I indicate that in cat and dog red cells internal Cl decreases as cells swell and shrink. It is possible that the increases in $^\circ k_{\text{SO}_4}$ that occur with changes in cat and dog cell volume as shown in Fig. 1 result from a decreased Cl inhibition of SO$_4$ efflux. To test this, the volume dependence of the rate constant for Cl efflux was determined (Table II). As with $^\circ k_{\text{SO}_4}$, $^\circ k_{\text{Cl}}$ increases as cat and dog red cells swell and shrink. Therefore, the volume dependence of $^\circ k_{\text{SO}_4}$ is not due to changes in the intracellular competition of SO$_4$ and Cl for transport by mediated exchange.
Values for the rate constant of SO₄ influx, \( k_{SO_4} \), at different cell volumes in cat and dog red cells are listed in Table III. Note that \( k_{SO_4} \) decreases with any change in cell volume. These results differ from those reported by Sha'afi and Pascoe (1972) for cat red cells where \( k_{SO_4} \) was found to decrease in shrunken cells and increase in swollen cells. It seems likely that these discrepancies are due to the inhibition of SO₄ transport by Cl in the Sha'afi and Pascoe (1972) study.

Data shown in Fig. 1 and Table III indicate that there is a reciprocal relationship between the volume dependence of \( k_{SO_4} \) and \( k_{SO_4} \) in cat and dog red blood cells. This relationship is of interest in light of changes in \( E_m \) which occur with alterations of cell volume (Table I). It seems that \( k_{SO_4} \) increases as \( E_m \) becomes more negative while \( k_{SO_4} \) decreases.

Clearly both cell volume and \( E_m \) varied simultaneously in the above studies. We suspected that \( E_m \) might be the more important variable. The studies described below were conducted in an effort to assess the separate effects of cell volume and \( E_m \).

In dog and cat red blood cells, Na permeability (\( P_{Na} \)) is very dependent on cell volume, i.e., \( P_{Na} \) increases in shrunken cells and decreases in swollen cells (Davson, 1942; Parker and Hoffman, 1965, 1976; Sha'afi and Hajjar, 1971). Results on the half time of Na movement in shrunken dog red cells (Parker and Hoffman, 1976) in combination with measurements of \( P_{Cl} \) in these cells (Parker et al., 1977) indicate that \( P_{Na}/P_{Cl} \) can approximate 0.6 as dog red cells shrink. Under the conditions of our experiment, there is a large outwardly directed concentration gradient for Na. Therefore, in shrunken cat and dog red cells where \( P_{Na} \) is large and approaches \( P_{Cl} \), the contribution of the Na gradient to the membrane potential becomes significant and would tend to hyperpolarize the membrane. This could explain the negative \( E_m \) that is seen in shrunken cat and dog red cells.

### Table II

| Cells | Osmolarity* (mosM) | \( k_{Cl} \) at 0°C | \( k_{Cl} \) |
|-------|-------------------|---------------------|-------------|
|       | mosM              | min⁻¹               |             |
| Cat   | 225               | 6.50                | 1.31        |
|       | 300               | 4.96                | 1.00        |
|       | 375               | 6.67                | 1.34        |
| Dog   | 225               | 11.39               | 1.31        |
|       | 300               | 8.69                | 1.00        |
|       | 375               | 11.81               | 1.36        |

* Estimates of relative volume are 1.24 and 0.85 for 225 and 375 mosM, respectively, where cell volume at 300 mosM is taken as unity. See Methods for comments regarding cell volume measurements.

† Values for \( k_{Cl} \) are means of two determinations. Determinations of \( k_{Cl} \) vary by less than 5%.
Since the volume dependence of Na permeability in cat and dog red cells can be abolished by depletion of the cellular energy stores (Hoffman, 1966; Sha'afi and Pascoe, 1973), it should be possible to prevent the observed hyperpolarization in shrunken cat and dog red cells if energy depleted cells are used. This is in fact the case. Results of energy depletion on the volume dependence of \( E_m \) and \( \kappa_{SO_4} \) in cat and dog red cells are given in Table IV. Note that \( E_m \) in energy-depleted dog red cells becomes more negative as the cells swell but more positive as they shrink. Note also that, after energy depletion, \( \kappa_{SO_4} \) increases in swollen cat and dog red cells but decreases in shrunken cells. Thus, the volume dependence of \( E_m \) and \( \kappa_{SO_4} \) in energy-depleted cat and dog red cells is very similar to that found in fresh human red cells.

The results presented in Table IV suggest a connection between \( E_m \) and \( \kappa_{SO_4} \), which is further supported by the data given in Table V. Here the

| TABLE III |
| --- |
| THE RATE OF SO₄ INFLUX (\( \kappa_{SO_4} \)) AS A FUNCTION OF CELL VOLUME FOR CAT AND DOG RED BLOOD CELLS |

| Cells | Fraction of control volume* | \( \kappa_{SO_4} \) at 37°C (mmol/liter cells x 1 mM Na) | Fraction of control \( \kappa_{SO_4} \) |
| --- | --- | --- | --- |
| Cat | 1.34 | 1.25 | 0.90 |
| 0.69 | 1.39 | 1.00 |
| Dog | 1.35 | 1.86 | 0.74 |
| 0.76 | 2.52 | 1.00 |

Data represent means of two determinations. Individual determinations vary by 2%.

* Calculated from cell water contents as described in Methods. Control % H₂O: 69.2 (cat), 69.3 (dog).

change in membrane potential resulting from the increased \( P_Na \) of shrunken cat and dog red cells was varied by varying the outwardly directed gradient for Na. To do this, the same-external NaCl concentrations were used for shrunken and control cells of a given pair, but the concentration of NaCl was varied to obtain cells with different Na gradients for different shrunken/control pairs. As in the previous experiments, cell volume was changed by varying medium osmolarity with sucrose. As the outward gradient for Na decreased (increasing \( Na_o \)), the potential in shrunken cells became less negative compared to control. Coincident with this less negative \( E_m \) is a decrease in the \( \kappa_{SO_4} \) of shrunken cat and dog red cells compared to control. Note that \( \kappa_{SO_4} \) from control cells decreases as external NaCl is raised. This is probably due to an increase in Cl inhibition of sulfate efflux. It is for this reason that the data are analyzed by comparing each shrunken/control pair.

The results presented in Tables IV and V show that it is possible to produce a volume dependence of anion transport rates in cat and dog red cells that is
qualitatively similar to that found for human red cells. This can be done by
preventing hyperpolarization in shrunken cat and dog cells either by elimi-
nating the increase in $P_{Na}$ which accompanies shrinking or by removing the
outward gradient for Na.

The importance of $E_m$ in determining the rate of anion transport is now

### TABLE IV

VOLUME DEPENDENCE OF SO$_4$ EFFLUX ($\kappa_{SO_4}$) AND ANION
DISTRIBUTION RATIO ($r_{SO_4}$) IN FRESH AND ENERGY-
DEPLETED CAT AND DOG RED BLOOD CELLS

| Cells | Osmolarity* | Fresh | Depleted† |
|-------|-------------|-------|-----------|
|       | mosM        |       |           |
| Cat   | 225         | 1.18  | 1.13      |
|       | 300         | 1.00  | 1.00      |
|       | 350         | 1.19  | 0.88      |
| Dog   | 225         | 1.19  | 1.22      |
|       | 300         | 1.00  | 1.0       |
|       | 350         | 1.55  | 0.83      |

| Cells | Osmolarity | Fresh | Depleted† |
|-------|------------|-------|-----------|
|       | mosM       |       |           |
| Dog   | 225        | 0.80  | 0.79      |
|       | 300        | 0.89  | 0.88      |
|       | 350        | 0.80  | 1.17      |

| Cells | Osmolarity | Fresh | Depleted† |
|-------|------------|-------|-----------|
|       | mosM       |       |           |
| Dog   | 225        | -5.9  | -6.2      |
|       | 300        | -3.1  | -3.4      |
|       | 350        | -5.9  | +4.2      |

Data are means of two determinations. Individual determinations vary by 2%.

* Estimates of relative volume are 1.24 and 0.90 for 225 and 350 mosM, respectively.

† Control rate constants (h$^{-1}$): fresh cat cells = 1.0; depleted cat cells = 0.73; fresh
dog cells = 2.4; depleted dog cells = 1.69.

‡ Red cells were incubated for 24 h at 25°C in sulfate flux medium (see Methods)
without glucose to deplete the cells of energy. After incubation, these red cells were
washed three times and $\kappa_{SO_4}$ and $r_{SO_4}$ were determined as usual (see Methods) in
sulfate flux medium without glucose. Energy depletion was verified by measurements
of intracellular ATP (data not shown).

‖ $r_{SO_4} = [(SO_4)_{out}/(SO_4)_{medium}]^{1/2}$, 37°C.

§ $E_m = 61 - \log_{10} r_{SO_4}$.

shown by experiments with human red cells. Using the nystatin procedure of
Cass and Dalmark (1973) and media of appropriate osmolarity and ionic
strength, it is possible to obtain either cells of different volume but constant
$E_m$, or cells of constant volume but different $E_m$. Table VI shows that the rate
constant for SO$_4$ efflux is relatively unchanged when volume is varied 40% under
conditions of constant $E_m$. This is in contrast to the more complicated
situation shown in Fig. 1 B, where $E_m$ varies and a 40% change in volume produces a 45% change in $\delta K_{SO_4}$.

Table VII shows the results of varying $E_m$ at constant cell volume. Note that $\delta K_{Cl}$ decreases about 50% as $E_m$ is changed by +12 mV. Fig. 1 B and Table I show that a 35% decrease in $\delta K_{SO_4}$ is found in normal (non-nystatin treated) human red cells when $E_m$ varies by +10.5 mV.

**TABLE V**

**EFFECT OF Na\textsubscript{o} ON THE VOLUME DEPENDENCE OF SO\textsubscript{4} EFFLUX ($\delta K_{SO_4}$) AND SO\textsubscript{4} DISTRIBUTION RATIO ($r_{SO_4}$) IN CAT AND DOG RED BLOOD CELLS**

| Sulfate efflux | $\delta K_{SO_4}$ | Fraction of control $\delta K_{SO_4}$ |
|---------------|------------------|-------------------------------------|
| Cells         | Na\textsubscript{o} | Control | Shrunken |
|               | mM               | h\textsuperscript{-1} |         |
| Cat           | 100              | 1.32    | 2.20     | 1.66    |
|               | 125              | 1.13    | 1.66     | 1.47    |
|               | 150              | 1.09    | 1.05     | 0.97    |
| Dog           | 100              | 2.56    | 3.69     | 1.40    |
|               | 125              | 2.22    | 2.85     | 1.28    |
|               | 150              | 2.07    | 1.91     | 0.92    |

Equilibrium anion ratio

| $r_{SO_4}$ | $\Delta E_m$ |
|------------|--------------|
| Cells      | Na\textsubscript{o} | Control | Shrunken | $\Delta E_m$ |
|            | mM            |         |         | mM           |
| Cat        | 100           | 0.95    | 0.76    | -5.9         |
|            | 125           | 0.99    | 0.81    | -4.2         |
|            | 150           | 0.76    | 0.84    | +2.7         |

Data represents means of two determinations. Individual determinations of $\delta K_{SO_4}$ vary by 5% while variation of $r_{SO_4}$ is 2%.

* Composition of control media (millimolar): 100, 125, or 150 NaCl; 5 KCl; 10 Na\textsubscript{2}SO\textsubscript{4}; 5 NaHEPES; 5 glucose; 100, 50, or 0 sucrose (pH = 7.4). Composition of hypertonic media (millimolar): 100, 125, or 150 NaCl; 5 KCl; 10 Na\textsubscript{2}SO\textsubscript{4}; 5 NaHEPES; 5 glucose; 175, 125, or 75 sucrose (pH = 7.4).

† Cells in 375 mosM medium compared to control cells in 300 mosM medium.

§ $r_{SO_4} = [(SO_4)_{cell}/(SO_4)_{medium}]^{1/2}$; 37°C.

|| $E_m$ (shrunken) - $E_m$ (control); $E_m = 61 \cdot \log_{10} r_{SO_4}$.

From our results with normal cat, dog, and human red cells and with nystatin-treated human red cells, we conclude that changes in cell volume per se are not responsible for the observed variations in anion exchange rates. Rather these variations are related to changes in $E_m$ which ordinarily accompany cell volume alterations (see Jacobs and Parpart (1931) and Freedman and Hoffman (1979) for further references and discussion). The conclusion that volume, per se, does not affect anion transport agrees with results obtained
with human red cell ghosts, where volume can be changed without affecting membrane potential since little or no hemoglobin is present (Funder and Wieth, 1976).

Our results indicate a correlation between membrane potential and the rate constant for anion transport. It should be noted that with the steady-state experiments presented here no distinction can be made between changes in membrane potential and changes in anion distribution ratios. Therefore our results could be interpreted to indicate that the rate constant for anion transport is directly dependent on the anion distribution ratio rather than the electrical potential.

This suggestion agrees with measurements of chloride self-exchange reported by Dalmark (1975). He has shown that $k_{Cl}$ increases as the $E_m$ of human red cells is made more negative by alkalinization. He has also shown that the $k_{1/2}$, the half-saturation constant, for internal anions declines as the membrane is hyperpolarized while the $k_{1/2}$ for external anions rises. As a result of these observations Dalmark (1975) proposed a model for the anion transport system in red blood cells in which the distribution of anion carriers across the membrane is asymmetric in response to the anion distribution, i.e.,

$$\frac{\text{number of carriers outer surface}}{\text{number of carriers inner surface}} = \left[ \frac{\text{Cl}_i}{\text{Cl}_o} \right].$$

This model is certainly consistent with our results. Knauf (1979) has reviewed this model in some detail. He has concluded that anion transport can be

| Fraction of control volume | $\%SO_4$ | $E_m$ $mV$ | $k_{SO_4}^\%$ |
|---------------------------|----------|-----------|--------------|
| 1.21                      | 1.22     | +5.3      | 0.98         |
| 1.12                      | 1.24     | +5.7      | 1.06         |
| 1.00                      | 1.24     | +5.7      | 1.00         |
| 0.83                      | 1.31     | +7.2      | 1.04         |

Human red cells with relative volumes of 0.83, 1.00, 1.12, and 1.21, respectively, were obtained by nystatin pretreatment in media of the following composition (millimolar): 110, 140, 170, or 200 KCl; 10 NaCl; 5 NaHEPES; 5 glucose; and 30 sucrose (pH = 7.2 at 0°C). After the cells had been washed free of nystatin, they were incubated for 3 h at 37°C in flux medium (millimolar): 80 NaCl, 5 KCl, 10 Na$_2$SO$_4$, 5 NaHEPES, 5 glucose, and 100 sucrose (pH = 7.4). Measurements of $r_{SO_4}$ and $k_{SO_4}$ were made as usual (see Methods). These data are means of two determinations. Variation between determinations is 2% for $r_{SO_4}$ and 3% for $k_{SO_4}$.  

* Calculated from cell water contents as described in Methods. Control % H$_2$O: 71.4.

$\%SO_4 = [(SO_4)_{cell}/(SO_4)_{medium}]^{1/2}$; 37°C.

$E_m = 61 - \log_{10} r_{SO_4}$.

Control $k_{SO_4} = 1.33 h^{-1}$.
TABLE VII
CHLORIDE TRANSPORT (*kCl) IN HUMAN RED CELLS WITH ALTERED CATION CONTENTS: CELLS AT CONSTANT VOLUME WITH DIFFERENT MEMBRANE POTENTIAL (E_m)

| Fraction of control | rCl | E_m | Fraction of control |
|---------------------|-----|-----|---------------------|
| volume*             |     |     |                     |
| 1.00                | 1.03| +0.7| 1.00                |
| 1.01                | 1.28| +5.8| 0.68                |
| 0.99                | 1.71| +12.6| 0.53               |

Human red cells with rCl values of 1.03, 1.28, and 1.71, respectively, were obtained by nystatin pretreatment in media of the following composition (millimolar): 140, 200, or 260 KCl; 10 NaCl; 5 NaHEPES; 5 glucose; and 30 sucrose (pH = 7.2 at 0°C). After the cells had been washed free of nystatin, they were incubated for 2 h at 37°C and then 1 h at 0°C in flux medium (millimolar): 150 NaCl; 5 KCl; 5 NaHEPES, 5 glucose; and 0, 100, or 200 sucrose (pH = 7.4). Measurements of rCl and *kCl were made as usual (see Methods). These data are means of two determinations. Determinations of rCl vary by 1% while those of *kCl vary by 3%.

* Calculated from cell water contents as described in Methods. Control % H2O: 72.0.
† rCl = (Cl)_out/(Cl)_medium; 0°C.
‡ E_m = 54-log10 rCl.
§ Control *kCl at 0°C = 2.61 min⁻¹.

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