The Relationship between Anion Exchange and Net Anion Flow across the Human Red Blood Cell Membrane

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ABSTRACT The conductive (net) anion permeability of human red blood cells was determined from net KCl or K_2SO_4 effluxes into low K^+ media at high valinomycin concentrations, conditions under which the salt efflux is limited primarily by the net anion permeability. Disulfonic stilbenes, inhibitors of anion exchange, also inhibited KCl or K_2SO_4 efflux under these conditions, but were less effective at lower valinomycin concentrations where K^+ permeability is the primary limiting factor. Various concentrations of 4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS) had similar inhibitory effects on net and exchange sulfate fluxes, both of which were almost completely DIDS sensitive. In the case of Cl^-, a high correlation was also found between inhibition of net and exchange fluxes, but in this case about 35% of the net flux was insensitive to DIDS. The net and exchange transport processes differed strikingly in their anion selectivity. Net chloride permeability was only four times as high as net sulfate permeability, whereas chloride exchange is over 10,000 times faster than sulfate exchange. Net OH^- permeability, determined by an analogous method, was over four orders of magnitude larger than that of Cl^-, but was also sensitive to DIDS. These data and others are discussed in terms of the possibility that a common element may be involved in both net and exchange anion transport.

INTRODUCTION

Small anions such as Cl^- and HCO_3^- pass very rapidly across the red blood cell membrane, with the half-time for Cl^- being only about 50 ms at 38°C (Brahm, 1975). Direct electrical measurements (Hoffman and Lassen, 1971; Lassen, 1972) as well as indirect techniques (Harris and Pressman, 1967; Henderson et al., 1969; Scarpa et al., 1968, 1970; Hunter, 1971; Tosteson et al., 1973) demonstrate that the anion flux consists almost entirely of an electroneutral one-for-one anion exchange. Net flow of anions accompanied by cations occurs at a much slower rate. It is only this relatively small net or conductive anion flow which responds to and is a determinant of the membrane potential.

The properties of the net anion flow have not been extensively investigated primarily because of technical limitations. The flow must be measured under conditions where known anion concentration gradients and membrane poten-

tials are imposed. Because direct electrical measurements in human red blood cells are not feasible (Lassen et al., 1971), indirect methods must be used. The chosen procedure involves the use of valinomycin to increase the potassium permeability of the membrane to such an extent that the net permeability to anions becomes the rate-limiting factor in the net K^+-anion efflux. Under such circumstances, it has been possible to determine values for the net permeabilities to chloride and sulfate, P_{Cl} and P_S, and thereby to compare the chloride-sulfate selectivity of the net anion transport process with that of the anion exchange system. Modifications of the technique also allow estimation of P_{OH} and P_{HCO_3}.

The relationship of the two modes of anion permeation, exchange and net flow, has also been evaluated by a detailed comparison of the effects of the disulfonic stilbenes. These compounds are potent and specific inhibitors of the anion exchange system (Knauf and Rothstein, 1971; Cabantchik and Rothstein, 1972, 1974). The comparative data strongly suggest that at least a large portion of the net anion flow in the case of Cl^-, and almost all of it in the case of SO_4^{--} and OH^-, share some common element with the anion exchange system. A brief report of some of these results has been previously presented (Knauf and Fuhrmann, 1974).[1]

MATERIALS AND METHODS

Cell Preparation

Fresh blood was drawn by venipuncture from adult volunteers into either citrate or heparin, and was stored overnight at 4°–6°C. The blood was centrifuged, the white cells and supernate were discarded, and the cells were washed three times in 10 vol of isotonic Tris-NaCl buffer (20 mM Tris [tris(hydroxymethyl)amino-methane], 146 mM NaCl, pH 7.1 at 37°C). A stock suspension of 50% hematocrit was prepared (using a microhematocrit centrifuge for determining the packed cell volume).

For chloride experiments, the suspension was diluted 1 in 5 with a chloride buffer (C) containing 20 mM Tris, 145 mM NaCl, and 1 mM KCl, titrated to pH 7.1 at 37°C with HCl. To load cells with sulfate, the 50% suspension was diluted 1 in 5 in a sulfate buffer solution (S) containing 20 mM Tris, 100 mM Na_2SO_4, and 0.5 mM K_2SO_4, brought to pH 7.1 at 37° C with H_2SO_4. The cells were incubated for 0.5–1 h, then centrifuged and resuspended in fresh medium. This was repeated for a total incubation time of 3.5–4 h, after which the cell chloride was less than 1 mM. Sulfate medium (S) was finally added to bring the cells to the same concentration as in the original 50% suspension.

Treatment with Inhibitors

Cells were treated with 0.11 mM SITS (4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonate) at 10% hematocrit for 10 min at 37°C. The cells were then washed twice with sulfate or chloride buffer and restored to 10% hematocrit. Cells were treated with DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonate) at 20% hematocrit for 30 min at 4°–5°C. In experiments at low DIDS concentrations, to avoid possible reactions between DIDS and Tris, the cells were washed three times with sulfate or chloride buffer containing HEPES (*N*-2-hydroxyethyl piperazine-*N*′-2-ethane sulfonic acid) in place of Tris, before DIDS treatment. At high DIDS concentrations (0.125 mM), however, substitution of Tris for HEPES

[1] The values of P_{Cl} and P_S reported earlier were overestimated due to a systematic error in calculating the net fluxes from the light-scattering data.

did not diminish the effect of DIDS. In all cases, after DIDS treatment cells were washed at least twice in S or C buffer and brought to 10% hematocrit.

Measurement of Volume Change by 90° Light Scattering

Net loss of ions from the cells was determined from the attendant loss of water volume measured by 90° light scattering (Fuhrmann et al., 1971). At the beginning and end of the experiment the relationship of light scattering to cell volume was determined as follows.

A 0.1-ml sample of the 10% cell suspension was added to 14.9 ml C buffer in the measuring cuvette at 37°C. Portions of 5 M NaCl were added to the cuvette and the resulting changes in light scattering were recorded. The osmolarity of the original medium and of the medium after NaCl addition were determined on an Advanced osmometer (Advanced Instruments, Inc., Needham Heights, Mass.). The intensity of scattered light from untreated cells in 300 mOsmol chloride buffer was arbitrarily considered to indicate a cell water volume (V_{H_2O}) of 1 liter. By assuming ideal osmotic behavior of the cells, the V_{H_2O} resulting from the addition of NaCl was calculated. After making a small correction for the dilution of the suspension by the addition of the small portions of NaCl, a calibration curve relating light scattering to cell water volume was constructed (Fig. 1*a*).

For experiments with valinomycin, 0.2 ml of absolute ethanol was first added to 14.9 ml of S or C buffer at 37°C. Ethanol was added both to increase the solubility of valinomycin in water and to render insignificant the possible effects of the small amounts of ethanol added with valinomycin. Next, 0.1 ml of the 10% red cell suspension was added. When the light-scattering trace had reached a constant value (1–2 min), a portion (20 μl or less) of a stock solution of valinomycin (A grade, Calbiochem, San Diego, Calif.) in ethanol was added to give the indicated final concentration. The change in light scattering with time was measured for 10 min after valinomycin addition. Typical records are shown for chloride cells in Fig. 2 and for sulfate cells in Fig. 3. For DIDS-treated cells in sulfate (Fig. 3*b*) there was practically no change in light scattering. In order to ensure that the light scattering was still sensitive to volume changes in these cells, samples of 5 M NaCl were added at the end of the experiment. These produced as large an increase in light scattering as with control cells, thus demonstrating that DIDS has no direct effect on the light-scattering response of the cells. After each measurement, the cuvette was rinsed twice with ethanol and twice with distilled water. This completely removed valinomycin since, when fresh cells were put into the cuvette, no change in light scattering occurred.

Determination of the Initial Rate of Cell Water Volume Change

Two methods were used. In the first, the V_{H_2O} was determined from the light-scattering trace at various times after valinomycin addition by means of the calibration curve (Fig. 1*a*). Since the initial loss of KCl is accompanied by an equivalent cell shrinkage so that the KCl concentration inside the cells remains nearly constant, the initial portion of the curve (Fig. 1*b*) is linear. From this initial slope the rate of change of cell volume was determined. In the case of sulfate cells, there was sometimes a small but rapid initial change in volume of undetermined origin. The values during this period were therefore disregarded. This does not seriously affect the determination of the slope, since in sulfate cells the volume change has a much slower time course than in chloride cells, so the use of later time points is permissible.

In the second method, the initial rate was determined directly from the light-scattering traces. The change in cell volume with time (Fig. 1*b*) gradually decreases, while the change in light scattering with change in cell volume increases as the cells shrink (Fig. 1*a*). Because these effects almost exactly compensate, the increase of light scattering is almost linear with respect to time (Figs. 2 and 3). It was therefore possible to determine the initial

rate of volume change directly from the slope of the curves. Volume changes determined in this way were highly correlated with volume changes determined by the first method described above, with a correlation coefficient (r^2) of 0.97.

Calculation of Net KCl or K_2SO_4 Efflux

Since the water permeability of the red cell is very high, it is possible to calculate the rate of KCl or K_2SO_4 efflux from the change in cell volume on the basis of the assumption that the osmolarity of the solution leaving the cells is equal to that of the medium and that K^+ is always accompanied by Cl^- (SO_4^{--} in sulfate-loaded cells). The limitations of this latter

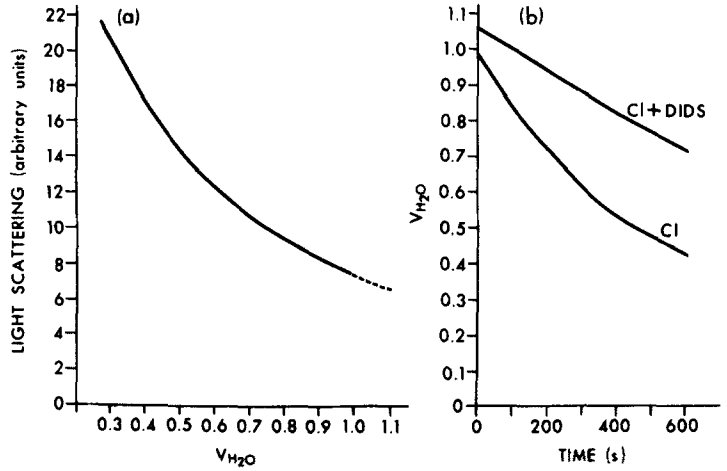

FIGURE 1. (*a*) Relationship of 90° light scattering to cell water volume (V_{H_2O}). The cell water volume was changed by addition of aliquots of 5.2 M NaCl as described in Materials and Methods. Results represent the average of four determinations. The broken line represents an extrapolation to volumes greater than one. (*b*) Changes in cell water volume with time after addition of valinomycin to cells in chloride medium. Cell water volume was determined from light-scattering traces (Fig. 2), by using the calibration curve depicted in Fig. 1*a*. The slightly greater initial volume for the DIDS-treated cells is not a consistent observation and is probably due to swelling of the cells during the course of the experiment. Such small changes in initial volume do not appear significantly to affect the net flux, since measurements of control rates at the beginning and end of the experiment were similar.

assumption are discussed below. Over the region of KCl or K_2SO_4 concentrations corresponding to the medium osmolarity, the relationship between concentration (mM) and osmolarity was determined to be:

$$(K_2SO_4)_e = (\text{mOsmol} - 23.23)/2.095,$$

$$(KCl)_e = (\text{mOsmol} - 9.9)/1.75.$$

The flux of KCl (or K_2SO_4) was then determined as follows:

$$J_{KCl} = \frac{dV_{H_2O}}{dt} \times (KCl)_e. \quad (1)$$

Since the V_{H_2O} is expressed as volume of cell water per liter of cell water originally present

in cells in a 300 mOsmol Cl medium, the flux has units of millimoles KCl per liter of cell water per minute.

That the light-scattering method provides an accurate determination of the rate of efflux of intracellular solutes is demonstrated by the close correlation between measurements of glucose flux by the light-scattering method and by osmotic methods (Fuhrmann,

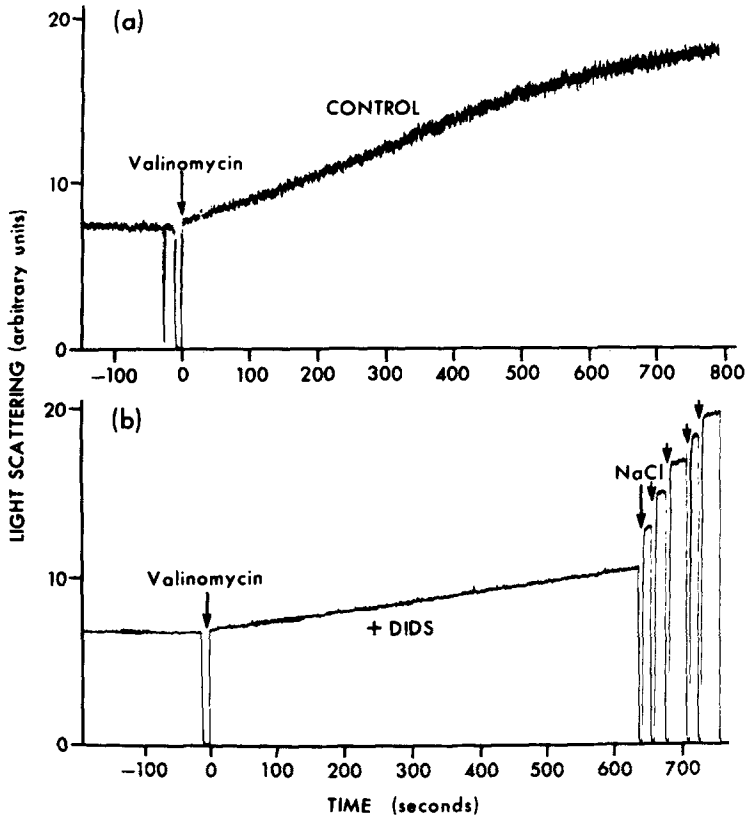

FIGURE 2. Change in 90° light scattering after addition of valinomycin to cells suspended in chloride medium (C). At zero time, with the shutter of the photomultiplier tube closed, valinomycin was added and the shutter was immediately opened. (*a*) Control cells. The dip in the trace at −30 s was caused by briefly closing the shutter. (*b*) Cells pretreated with 30 μM DIDS as described in Materials and Methods. At the points labeled NaCl, the shutter was closed and 0.1 ml of 5.2 M NaCl was added to the suspension, after which the shutter was reopened. Note that a considerable fraction of the net chloride efflux is not inhibited by DIDS.

unpublished data; Fuhrmann et al., 1971). Determinations of net K^+ efflux by flame photometry also gave flux values comparable to those determined by light scattering.

Cell Water, Cell Potassium, and Chloride and Sulfate Ratios

Cell water content was determined by a gravimetric method taking into account the hematocrit, extracellular volume, and density of the cells and medium. The cells were weighed, then dried (15 h at 105°C or 24 h at 80°C), allowed to cool in a dessicator, and

weighed again. From the cell water determination, the factor d = kilograms cell water/kilogram cell solids was calculated, so that the fluxes could also be expressed in millimoles/kilogram dry weight per hour. The average value of d was 1.780 ± 0.032 SEM.

Cell potassium was determined by flame photometry with an Li^+ internal standard. The average value was 125.7 ± 3.7 meq/liter cell water.

Chloride in cells and medium was determined coulometrically by use of a Marius

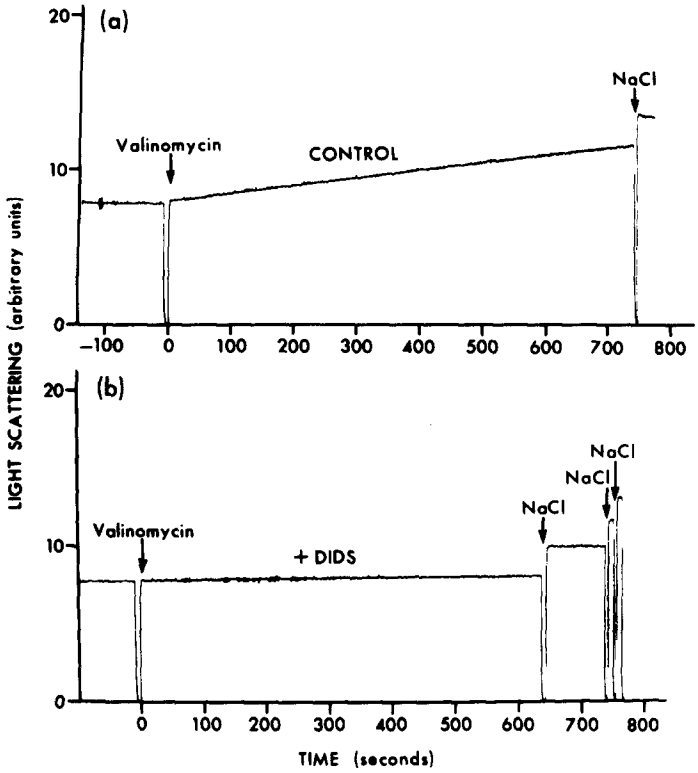

FIGURE 3. Changes in light scattering after addition of valinomycin to cells preequilibrated and suspended in sulfate medium (S). Valinomycin (1.33 μM) was added at zero time. (*a*) Control cells. Note that after NaCl addition the volume decreases transiently and then begins to increase as Cl^- enters the cells in exchange for SO_4^{--}. (*b*) Cells pretreated with 30 μM DIDS. Note that in this case the light-scattering trace is steady after addition of NaCl, since Cl^-/SO_4^{--} exchange has been inhibited by DIDS.

Chlor-o-counter. Alternatively, the chloride ratio between cells and medium was determined by measuring the distribution of ^{36}Cl (Amersham/Searle Corp., Arlington Heights, Ill.). The sulfate ratio was calculated from the distribution of $^{35}SO_4$ (New England Nuclear, Boston, Mass.) between cells and medium at equilibrium. The average value of Cl_i/Cl_o was 0.762 ± .027, while the value of $\sqrt{S_i/S_o}$ was 0.762 ± 0.033.

Calculation of P_{Cl} and P_S

To determine numerical values for the permeabilities from the fluxes measured by light scattering, it was assumed first that the Goldman constant-field equation (Goldman, 1943;

Hodgkin and Katz, 1949) holds for all permeant ions, and second that the Na^+ permeability is negligible in comparison to the K^+ permeability induced by valinomycin (Lev and Buzhinsky, 1967; Mueller and Rudin, 1967; Andreoli et al., 1967; Tosteson et al., 1967; Eisenman et al., 1973; Kaplan and Passow, 1974). The permeabilities of SO_4^{--} and Cl^- were determined from the Goldman-Hodgkin-Katz equations for the fluxes of the two ions which are, respectively:

$$J_{Cl} = P_{Cl} \ln B \frac{[Cl_i B - Cl_o]}{B - 1}, \tag{2}$$

$$J_S = 2 P_S \ln B \frac{[S_i B^2 - S_o]}{B^2 - 1}, \tag{3}$$

where

$$B = \exp[-FE_m/RT]. \tag{4}$$

For conditions of zero current flow, in chloride media:

$$B = \frac{P_K K_i + P_{Cl} Cl_o}{P_K K_o + P_{Cl} Cl_i} \tag{5}$$

In sulfate media:

$$B = \frac{P_K K_i - P_K K_o + \sqrt{P_K^2 K_i^2 - 2P_K^2 K_i K_o + 4[P_K K_o + 4P_S S_i][P_K K_i + 4P_S S_o]}}{2[P_K K_o + 4P_S S_i]} \tag{6}$$

To convert the permeabilities in min^{-1} to cm/s, they must be multiplied by the ratio of the volume of cell water per red cell to the surface area per cell. The data were calculated by using a cell water volume of 0.7 liters/liter packed cell volume, cell volume of 87 μm^3 (Ponder, 1948) and cell surface area of 133 μm^2 (Hoffman et al., 1958). Thus, P(cm/s) = 7.63×10^{-7} P (min^{-1}). Values for the volume to area ratio of red cells in the literature (Ponder, 1948; Houchin et al., 1958; Hoffman et al., 1958; Westerman et al., 1961; Canham and Burton, 1968) vary from 0.561 μm to 0.778 μm; the values chosen give a ratio of 0.654 μm, near the mean of the literature values.

Determination of Net OH^- and HCO_3^- Flux

Red blood cells collected as described above were washed three times in a solution (A) containing 161 mM NaCl and 1 mM KCl and a stock suspension of 10% hematocrit was prepared. The cells were then titrated to the indicated pH with solution A containing 10 mM NaOH or HCl. Treatment with 125 μM DIDS was performed as described above except that the cells were washed in unbuffered media. Except where indicated the CO_2 content of the medium was reduced by bubbling 99.999% pure N_2 through 14.9 ml of solution A. After 0.2 ml of ethanol was added, and after the medium was brought to a pH slightly (about 0.1 unit) below that of the cells, 0.1 ml of the 10% cell suspension was added to the medium. The pH was maintained by addition of 1 mM HCl in solution A, using a pH stat. The rate of efflux of OH^- (or HCO_3^-) from the cells was calculated from the rate of addition of HCl. In experiments with valinomycin it was added immediately after the cells, and the net OH^- flux was determined as the difference between the rate of H^+ addition in the presence and absence of valinomycin.

In this method, some CO_2 and bicarbonate are added with the cell suspension. The total CO_2 in the 10% suspension, determined with a Natelson Microgasometer model 650, was about 1.2 mM. In certain experiments, the cell suspension was bubbled with N_2 for 10 min, after which no CO_2 could be detected.

The hydroxyl ion permeability (including contributions from HCO_3^-) was calculated on the assumption that initially the cells are near Donnan equilibrium, that is:

$$r = \frac{Cl_i}{Cl_o} = \frac{OH_i}{OH_o}, \tag{7}$$

and further,

$$s = \frac{OH_i}{Cl_i} = \frac{OH_o}{Cl_o}. \tag{8}$$

From the Goldman equation,

$$J_{OH} = P_{OH} \ln B \frac{[OH_i B - OH_o]}{B - 1} = s P_{OH} \ln B \frac{[Cl_i B - Cl_o]}{B - 1}, \tag{9}$$

and, therefore, from Eq. (2) and (9):

$$P_{OH} = \frac{J_{OH} P_{Cl}}{s J_{Cl}}. \tag{10}$$

J_{Cl}, J_{OH}, and P_{Cl} were determined experimentally and s was calculated from the external pH, tabulated values for the dissociation constant for water, K_w (Handbook of Chemistry and Physics. 53rd edition. 1972–73. p.D122), and the measured Cl_o. P_{HCO_3} was determined in an analogous manner.

Effects of Net OH^- Flux on P_{Cl} and P_S Measurements

Under the described experimental conditions at high valinomycin concentrations variations in P_{OH} have little effect on the membrane potential and hence on the net Cl^- or SO_4^{--} flux. It was therefore assumed that if KOH efflux occurs, its primary effect would be to cause an overestimate of the volume change due to KCl efflux, and therefore an overestimate of P_{Cl}. Because of intra- and extracellular buffering, the volume change expected for KOH efflux would be approximately one-half that expected for an equal KCl or K_2SO_4 efflux. By comparing the volume change expected for the KOH efflux (measured by pH stat under identical conditions) with the total volume change, it was possible to calculate the fraction of the apparent KCl or K_2SO_4 efflux which could result from KOH efflux, and therefore the amount by which P_{Cl} and P_S might be overestimated. This provides a maximum estimate of the error, since the small potential change due to OH^- flow introduces an error in the opposite direction, but of smaller magnitude.

$^{35}SO_4^{--}$ Exchange

Rate constants for $^{35}SO_4$ efflux under equilibrium exchange conditions were determined as previously described (Knauf and Rothstein, 1971), except that S medium was used. For experiments with valinomycin, K_2SO_4 (56–70 mM) was substituted for some of the Na_2SO_4 in the S medium so as to minimize the difference between E_K and E_{SO_4}, thus minimizing possible membrane potential changes due to valinomycin.

$^{42}K^+$ Exchange

Cells were loaded with $^{42}K^+$ at 50–65% hematocrit in S or C medium containing 50–60 mM K^+ in place of Na^+ and 2 mg/ml glucose for 4 h at 37°C. The cells were washed five times in ice-cold S or C medium containing sufficient potassium so that E_K would be close to E_{Cl} or E_{SO_4}. After 29.4 ml of the same medium had equilibrated in a thermostated chamber at 37°C, ethanol was added, followed by various amounts of valinomycin dissolved in ethanol, so that the total volume of ethanol was 0.4 ml. Exactly 1 min later, 0.2 ml of a

10% cell suspension was added, and samples were taken by syringe according to a modification of the technique of Dalmark and Wieth (1972). At the end of the experiment, a sample of the suspension was taken. ^{42}K radioactivity was determined on a Picker Nuclear Gamma counter (Picker Corp., Cleveland, Ohio) and the results were calculated as described previously for sulfate (Knauf and Rothstein, 1971). Since E_K was not precisely equal to E_{Cl} or E_{SO_4}, the exact potential was determined by using the value of P_{Cl} or P_S obtained from light-scattering measurements. P_K was then calculated from the observed efflux rate constant (^0k_K) as follows (Hunter, 1971):

$$P_K = \frac{B - 1}{\ln B} {}^0k_K. \tag{11}$$

RESULTS

Net Cl^- and SO_4^{--} Fluxes and Calculations of P_{Cl} or P_S

The light-scattering traces of Figs. 2 and 3 indicate that valinomycin induces a substantial shrinkage of red cells equilibrated with either chloride or sulfate medium, and that the shrinkage is inhibited by the disulfonic stilbene, DIDS. The rate of shrinkage is dependent on the valinomycin concentration. This relationship is illustrated in Fig. 4*a* and *b*, in which the net Cl^- and SO_4^{--} fluxes determined from volume loss are plotted against valinomycin concentration. The lower sets of data in each case represent the fluxes in the presence of the anion exchange inhibitors, DIDS in the case of Cl^- and SITS in the case of SO_4^{--}.

Certain general features of the data are apparent: (*a*) in each case the flux tends to reach a limiting value at high concentrations of valinomycin; (*b*) the limiting value is considerably lower in the presence of the inhibitors; and (*c*) the maximum flux for SO_4^{--} is considerably lower than that for Cl^-. The tendency of the fluxes to reach limiting values independent of the valinomycin concentration would be expected if the valinomycin were to increase the K^+ permeability sufficiently so that the anion permeability becomes the limiting factor in the loss of salt and water from the cell. Under these circumstances the net salt efflux should be sensitive to anion transport inhibitors and should be affected by substitution of sulfate for chloride.

In order to permit a quantitative evaluation of the data, the effect of valinomycin on K^+ permeability was determined. To accomplish this, cells were loaded with $^{42}K^+$ and suspended in chloride or sulfate media containing sufficient K^+ so that the equilibrium potentials for K^+ and Cl^- or SO_4^{--} would be nearly the same. Under these circumstances, variations in the P_K:P_{Cl} or P_K:P_S ratio will cause little change in the membrane potential. Thus the membrane potential is approximately known regardless of the values assigned to P_{Cl} and P_S. The potassium permeability, P_K, can then be calculated from the observed potassium efflux rate constant and the membrane potential, on the assumption that the valinomycin-mediated K^+ efflux obeys the Goldman equation (Hunter, 1974).

The results for cells in chloride medium are shown in Fig. 5. The data for cells suspended in sulfate medium were not significantly different. With increasing concentrations of valinomycin the K^+ fluxes increase substantially, but there is a tendency toward saturation at high valinomycin concentrations. When the reciprocal of P_K is plotted against the reciprocal of the valinomycin concentration, a

straight line is obtained with a $K_{1/2}$ of 1.82 μM. The solid line in Fig. 5 is calculated from this value of $K_{1/2}$. Hunter (1974) has also observed saturation of P_K with increasing valinomycin concentrations, but with a somewhat lower $K_{1/2}$ (0.9 μM), possibly due to the lower ethanol concentration used in his experi-

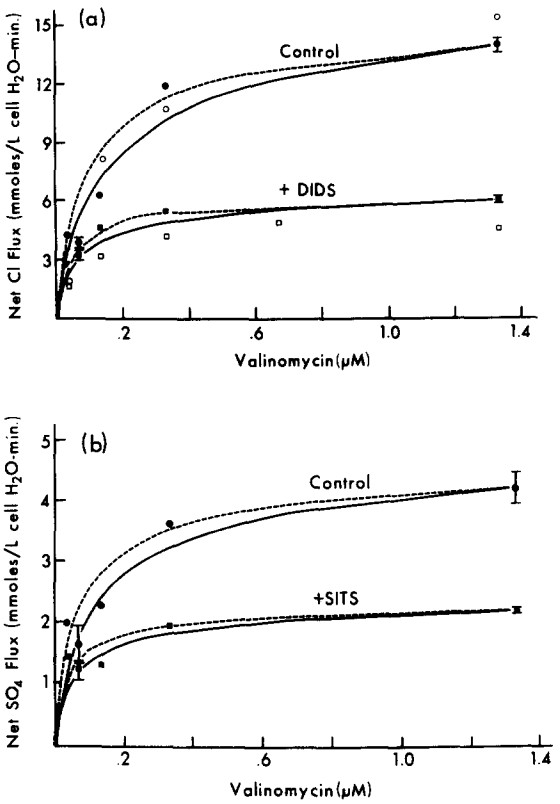

FIGURE 4. (*a*) Net KCl efflux as a function of valinomycin concentration. The upper points (circles) are for control cells; the lower points (squares) for cells treated with 0.125 mM DIDS as described in Materials and Methods. The bars indicate the standard error of the mean for five determinations. The open symbols are results from a separate experiment on cells from the same donor. In these experiments, K_i was 125.4 mM, K_o 1.0 mM, Cl_i 111.7 mM, Cl_o 160 mM, and d was 1.65 kg cell water/kg cell solids. P_{Cl} was determined from the flux at 1.33 μM valinomycin to be 0.033 min^{-1} for the control cells and 0.012 min^{-1} for the DIDS-treated cells. The broken curves were calculated by using the P_K values from Fig. 5; the solid curves with $P_K = 1.5 \times$ valinomycin in μM (see text). (*b*) Net K_2SO_4 efflux as a function of valinomycin concentration. The upper points (circles) are data for control cells; the lower points (squares) for cells treated with 0.11 mM SITS as described in Materials and Methods. The bars indicate the standard error for five determinations. In these experiments, K_i was 99.1 mM, K_o1 mM, S_i 82.2 mM, S_o 112.5 mM, d = 1.65. P_S was determined from the flux at 1.33 μM valinomycin to be 0.0066 min^{-1} for the control cells and 0.0032 min^{-1} for the SITS-treated cells. The broken and solid curves were calculated as described in (*a*).

ments. Kaplan et al. (1976) observed a P_K value of 1.58 min^{-1} at 1 μM valinomycin in red cell ghosts, in good agreement with the data in Fig. 5.

Although a tendency of P_K to saturate with increasing valinomycin concentration was observed by Hunter (1974) and in our experiments (Fig. 5), the data of Hoffman and Laris (1974) suggest a linear relationship. They measured the membrane potential in low K^+ human red cells as a function of valinomycin concentration at 22°C. From the membrane potential and the internal and external concentrations of ions, it is possible to calculate the value of P_K:P_{Cl},

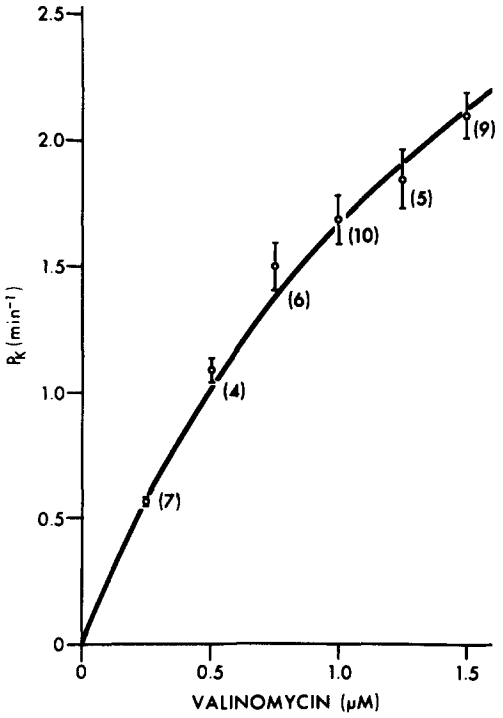

FIGURE 5. P_K as a function of valinomycin concentration. P_K was determined from the ^{42}K efflux as described in Materials and Methods. The bars indicate the standard error and the numbers in parentheses are the number of experiments. The solid curve was calculated by assuming saturation behavior with a K_i of 1.82 μM (determined from a plot of $1/P_K$ vs. 1/valinomycin).

assuming that only K^+ and Cl^- contribute appreciably to the membrane potential. The results of such a calculation (Fig. 6) provide no evidence for saturation behavior of P_K. Unless such behavior were compensated by an exactly reciprocal change in P_{Cl}, the potential measurements favor a linear variation of P_K with increasing valinomycin. A linear relationship is also found in model bilayers (Andreoli et al., 1967; Stark and Benz, 1971; Eisenman et al., 1973).

It is not clear why some experiments suggest a linear relationship between valinomycin concentration and P_K whereas others (ours and those of Hunter [1974]) show a tendency to saturate. It is clear, however, that the potassium

permeability saturates at much higher valinomycin concentrations than does the net KCl or K_2SO_4 efflux (Fig. 4), and thus saturation of P_K cannot be primarily responsible for the plateau in net KCl or K_2SO_4 efflux. The net fluxes presented in Fig. 4 can be predicted in either case (assuming a straight line or the saturation curve of Fig. 5) by using the following assumptions: (*a*) the Goldman constant field equation applies to each ion, and the net anion permeability can be expressed in terms of this equation; (*b*) the membrane potential is primarily determined by the flows of Cl^- and K^+. In particular, since valinomycin specifically increases potassium permeability, the Na^+ permeability is negligible by comparison; and (*c*) valinomycin does not significantly affect the net anion permeability.

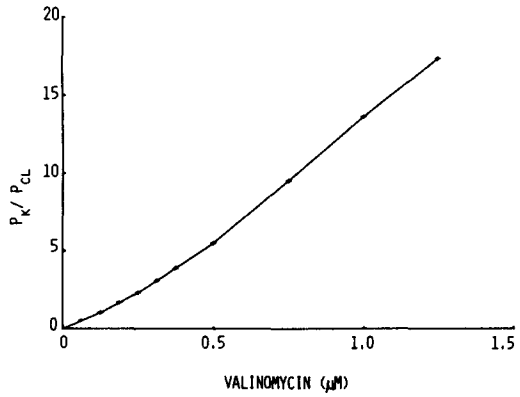

FIGURE 6. P_K/P_{Cl} as a function of valinomycin concentration (from Hoffman and Laris, 1974). The membrane potentials were calculated from the curve of fluorescence of the potential-sensitive cyanine dye, C-6, vs. valinomycin concentration (Fig. 7 of Hoffman and Laris, 1974) by using Fig. 4 to calibrate the fluorescence in terms of potential. From the membrane potential and the internal and external K^+ and Cl^- concentrations, the ratio P_K:P_{Cl} was calculated by means of Eq. (4) and (5). In the experiments of Hoffman and Laris, the internal K was decreased to 1–2 mM by pretreatment with *p*-chloromercuribenzene sulfonate and the temperature was 22–23°C.

The theoretical curves for Cl^- and SO_4^{--} in Fig. 4 indicated by the broken lines were calculated by using values for P_K from Fig. 5 and values for net anion permeability determined from the flux data at the highest valinomycin concentration (1.33 μM), where the net flux should be determined primarily by the net anion permeability. The validity of the assumptions and of the values for net anion permeability is reinforced by the relatively good fit of the theoretical lines and data points in each case. The numerical value for P_{Cl} was 0.033 min^{-1} (or 2.5 $\times$ 10^{-8} cm/s) and for P_S, 0.0066 min^{-1} (or 5 $\times$ 10^{-9} cm/s). After treatment with 0.125 mM DIDS, P_{Cl} was 0.012 min^{-1}, corresponding to 64% inhibition. After SITS treatment, P_S was 0.0032 min^{-1}, corresponding to 52% inhibition. It should also be noted in Fig. 4 that at low valinomycin concentrations, where the net KCl or K_2SO_4 efflux is increasing rapidly and where anion permeability is not the primary limiting factor, DIDS and SITS have small effects, whereas at higher

valinomycin concentrations, where anion permeability limits the net flow, they have a much larger effect. SITS and DIDS thus cause the net salt flux to plateau at lower valinomycin concentrations than in the control, consistent with the expectation that they should affect P_{Cl} and P_S but not P_K. If SITS and DIDS were to decrease net salt flux by reducing P_K, the flux would necessarily be strongly dependent on P_K and therefore on valinomycin concentration, even at high concentrations, contrary to the observations.

Theoretical curves were also calculated by assuming a linear relationship of P_K with valinomycin concentration (solid lines of Fig. 4), taking the value of P_K at 1.33 μM valinomycin as 2.0 min^{-1}. This assumption does not result in a substantially better fit to the data, nor does it alter the basic conclusions that can be inferred. Note that for both the experimental data and the theoretical curves the net salt flux is strongly dependent on P_K even when P_K is equal to or somewhat larger than P_{Cl}. Because K_o is low in comparison to Cl_i, it is only when P_K is much greater than P_{Cl} (such as at 1.33 μM valinomycin) that the K^+-dependent terms dominate both numerator and denominator of Eq. (5), so that the membrane potential and therefore the net chloride flux become nearly independent of P_K.

The fit of the calculated lines and the actual data points in Fig. 4 indicates that the net anion permeability can be determined directly from the net salt efflux at the highest valinomycin concentration (1.33 μM). Under these conditions it can be calculated from the assumptions discussed above that the net salt efflux is almost linearly related to P_{Cl} or P_S (Fig. 7). On the other hand, the net flux reaches a limiting rate with respect to P_K. Even if P_K were increased to infinity the net flux of Cl^- would only increase by 32% over its value at 1.33 μM valinomycin.

A summary of the values of net KCl and net K_2SO_4 efflux at 1.33 μM valinomycin for red cells from different donors is presented in Table I. The average value of P_{Cl} calculated from these data by using Eq. (2) and (5), 2.8×10^{-8} cm/s, agrees well with values obtained by other techniques (Hunter, 1971; Cass and Dalmark, personal communication; Sachs et al., 1975; Kaplan et al., 1976). The value of P_S (6.3×10^{-9} cm/s) is also in good agreement with other estimates (Gunn et al., 1974; Passow et al., 1974; Sachs et al., 1975).

Effect of Valinomycin on Sulfate Permeability

One of the assumptions on which the reported technique is based is that valinomycin affects only K^+ permeability with little or no effect on anion permeability. The possible effect of valinomycin on sulfate permeability was determined by measuring $^{35}SO_4^{--}$ fluxes under equilibrium conditions. To preclude effects of valinomycin resulting from alterations of the membrane potential, experiments were carried out in high potassium media so that the potassium and sulfate equilibrium potentials were nearly the same. Although the measured fluxes include a large component that is attributable to the electrically silent anion exchange system, the net SO_4^{--} flux as estimated from Table I is an appreciable fraction of the total flux (19.4 ± 1.2%), so that any substantial effects of valinomycin should be detectable.

Although there was some random variation, no significant effect of valinomycin on sulfate fluxes was found. In experiments with blood from two different donors, the correlation of sulfate flux with valinomycin concentration was low

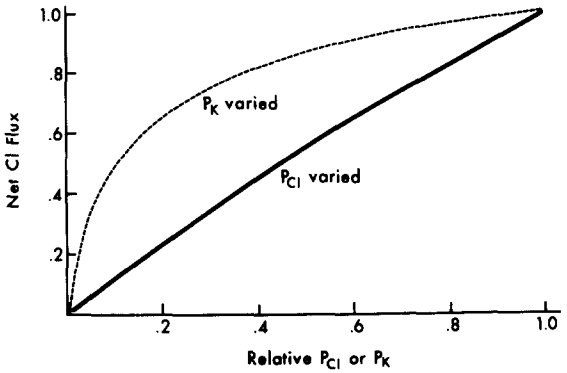

FIGURE 7. Calculation of the variation of net Cl^- flux with P_K and P_{Cl} under conditions prevailing at 1.33 μM valinomycin, 37°C. The ordinate depicts the net efflux of KCl relative to that observed at 1.33 μM valinomycin in control cells. On the abscissa, P_K or P_{Cl} is plotted as a fraction of its measured value in control cells at 1.33 μM valinomycin, 2.0 min^{-1} for P_K (Fig. 5) and 0.0369 min^{-1} for P_{Cl} (Table I). For the solid curve, P_K was held constant at 2.0 min^{-1} and P_{Cl} was set at the fraction of its control value indicated on the abscissa. This corresponds to the experimental situation in the presence of anion permeability inhibitors such as DIDS and SITS. Even when P_{Cl} is increased rather than decreased, the variation of net KCl efflux with P_{Cl} is large: net flux increases 38% with a 50% increase of P_{Cl}. The relationship of net SO_4^{--} flux to P_S (not shown) is almost identical to the relationship shown by the solid line for Cl^-. In the case of the broken line, P_{Cl} was held constant at 0.0369 min^{-1}, P_K was set at some fraction of its value at 1.33 μM valinomycin, and the net KCl efflux was calculated. The plot of net SO_4^{--} flux against P_K (not shown) is similar, except that the plateau is reached at lower values of P_K. This corresponds to the experiments in Fig. 4*a* and *b* where the valinomycin concentration (and hence P_K) was reduced. If P_K is increased above the values shown in this figure, the net Cl^- efflux becomes almost independent of P_K: a 10^{50}-fold increase of P_K causes only a 32% increase in net Cl^- flux. Calculations were performed on the assumption that $P_{Na} \approx 0$ and by using the following mean values for the other parameters: K_i, 125.4 mM; K_o, 1 mM; Cl_i, 111.7 mM; Cl_o, 160 mM.

TABLE I

VALUES OF Cl^- AND SO_4^{--} NET FLUX IN RED CELLS FROM DIFFERENT DONORS

Donor no.	J_{Cl}	J_S
1	14.2±0.4 (6)	4.46±0.37 (6)
2	12.8±1.2 (4)	–
3	18.2±0.7 (4)	4.84±0.26 (3)
4	17.0±1.1 (3)	3.46±0.08 (2)
5	14.4±1.2 (2)	–
6	18.5	5.75
7	19.5	–
8	–	3.23±0.32 (3)
Mean	16.4±1.1 (7)	4.35±0.52 (5)

Fluxes are in millimoles/liter cell water/min.
All values ± SEM. The number in parentheses is the number of determinations.

(r^2 = 0.0003, 0.1367). In both cases, the slope of the regression line describing the sulfate outward rate constant in min^{-1} as a function of the valinomycin concentration in μM was not significantly different from zero at the 90% confidence level. The intercepts at zero valinomycin were 0.0453 and 0.0559 min^{-1}, while the slopes were 0.000054 ± 0.0029 and 0.000689 ± 0.0017. Passow (personal communication) has obtained similar results. Although it would be difficult to detect a small effect of valinomycin on net sulfate permeability by this method, the data do argue against a major effect, particularly against a large increase in permeability. The results shown in Fig. 6 further suggest that valinomycin does not significantly increase P_{Cl}, since this would be reflected in a flattening of the curve of P_K:P_{Cl} vs. valinomycin concentration. Tosteson et al. (1973) have also failed to observe any large effects of valinomycin on net chloride permeability in sheep red cells. All of these results are thus consistent with the assumption that net anion permeability is unaffected by valinomycin.

Comparison of the Effects of DIDS on Net and Exchange Anion Fluxes

From the data presented in Figs. 2–4 it is clear that SITS and DIDS, which are potent and specific inhibitors of anion exchange (Knauf and Rothstein, 1971; Cabantchik and Rothstein, 1972, 1974), also inhibit net anion fluxes. Common sites might, therefore, be involved in both processes. To provide a more stringent test of this hypothesis, the effects of a series of DIDS concentrations on exchange and net fluxes were directly compared.

The results for sulfate-loaded cells are shown in Fig. 8 where net sulfate efflux at 1.33 μM valinomycin, expressed as percent of the control value, is plotted against $^{35}SO_4$ exchange, also expressed as percent of control. The relationship between net and exchange flux in the DIDS-inhibited cells is linear, with a correlation coefficient (r^2) of 0.96, providing strong evidence that DIDS has parallel effects on net and exchange sulfate flow. The intercept is close to zero, indicating that very little if any net or exchange flux is insensitive to DIDS. The slight deviation of the intercept on the ordinate from zero may be due to the slight nonlinearity in the relationship between net sulfate flux at 1.33 μM valinomycin and P_S (see Fig. 7).

When both sulfate and chloride net fluxes were measured on blood from the same donor, the results in Fig. 9 were obtained. The $SO_4^{=}$ results are comparable to those reported in Fig. 8. The chloride results are somewhat different. A high correlation (r^2 = 0.94) was found between the exchange and net fluxes but the intercept was considerably higher. A component of chloride net flow amounting to 36% of the total is insensitive to DIDS, so that some net flow occurs even after virtually all of the exchange has been inhibited. It is uncertain whether this DIDS-insensitive component represents a separate pathway, or whether DIDS binding to each site causes virtually complete inhibition of chloride exchange, but only a partial inhibition of net flow. The former alternative would appear more likely, however, since it would seem improbable that DIDS could cause a partial inhibition of net chloride flow, but a complete inhibition of net sulfate flow if all of the chloride and sulfate net fluxes were mediated by a common, DIDS-sensitive system.

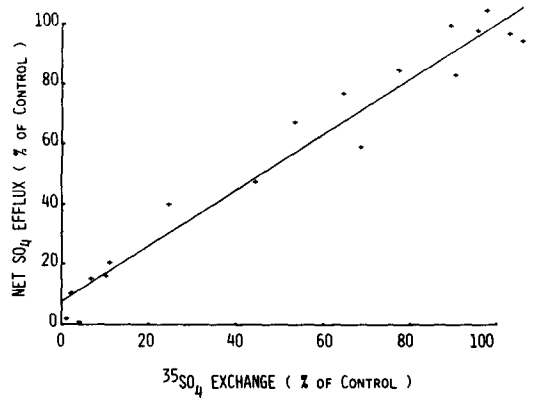

FIGURE 8. Effects of various DIDS concentrations on net SO_4 efflux and on sulfate exchange. For each point, net SO_4^{--} efflux and $^{35}SO_4$ exchange were measured on the same sample of cells which had been treated with DIDS concentrations ranging from 0.5 to 30 μM. Since the net SO_4^{--} efflux at 1.33 μM valinomycin is almost linearly related to P_S (Fig. 7), these data can be used to compare the effects of DIDS on net and exchange anion flow. The data represent results on blood from three donors, using two separate DIDS preparations. The straight line representing the least-squares best fit to the data is given by $y = 0.900x + 8.05$. The effects of DIDS on net and exchange fluxes are highly correlated ($r^2 = 0.96$) and the intercept is not significantly different from zero.

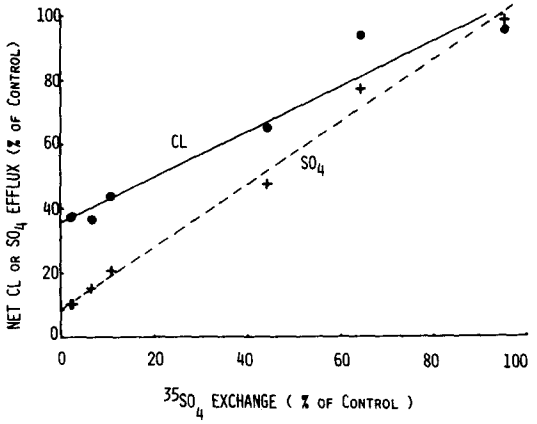

FIGURE 9. Comparison of the effects of DIDS on net Cl^- and SO_4^{--} fluxes and on sulfate exchange. Net Cl^- or SO_4^{--} fluxes at 1.33 μM valinomycin are used as a measure of the effects of DIDS on P_{Cl} or P_S, on the basis of the linear relationship between these quantities shown in Fig. 7. In each case, chloride and sulfate net fluxes and sulfate exchange were measured in samples of cells from the same donor treated with DIDS concentrations ranging from 0.5 to 30 μM. The x-axis can be taken to indicate the effects on Cl^- exchange (at 0°C) as well as on SO_4^{--} exchange, since for DIDS-treated cells the change in Cl^- exchange is 0.96 times the change in SO_4^{--} exchange ($r^2 = 0.99$). Both for SO_4^{--} and for Cl^- the net and exchange fluxes are highly correlated ($r^2 = 0.99$ and 0.94, respectively). For Cl^-, however, the y intercept is significantly different from zero and corresponds to 36% of the total flux.

Net OH^- and HCO_3^- Permeabilities

In the treatment of the data so far, the assumption has been made that when potassium ions flow out of valinomycin-treated cells, they are accompanied by either chloride or sulfate ions. That is, it is assumed that only potassium, chloride, and sulfate ions contribute appreciably to the total membrane conductance. It is possible, however, that potassium efflux could be accompanied in part by hydroxyl or bicarbonate ion efflux or by hydrogen ion influx. Since the concentrations of each of these ions is very low, this could occur only if their net permeability were very large compared to that of chloride and sulfate. The flow of OH^- or HCO_3^- would result in net transfer of acid or base across the membrane. Accordingly, in order to test for possible contributions of these ions to the membrane conductance, cells were suspended in unbuffered media and the net rate of appearance of OH^- upon addition of valinomycin was measured by using a pH stat. Since the red cell membrane in general is far more permeable to anions than to cations, it has been assumed that the pH changes are due to flow of OH^- rather than H^+ ions.

When cells which had been washed in NaCl were added to media at pH 7.5 which had been thoroughly flushed with N_2 to remove CO_2, the results presented in Fig. 10 were obtained. The net OH^- fluxes are large in light of the very low OH^- concentration (7.8×10^{-7} M) and, like the net Cl^- and SO_4^{--} fluxes (Fig. 4), appear to saturate with increasing valinomycin concentration. Theoretical curves calculated as in Fig. 4, by using the value of P_{OH} determined from the flux at 1.33 μM valinomycin as described in Materials and Methods, fit the experimental points reasonably well.

At pH 7.5, when cells and media were gassed with N_2, the apparent rate of net OH^- flow was about 20% lower (statistically significant, at $P < 0.05$) than when the cells and media were exposed to air, presumably because the small amount of HCO_3^- present can contribute significantly to the total flow of base from the cells. At pH 7.1, although the mean OH^- flux was lower when cells and media were gassed with N_2, there was no statistically significant decrease in paired experiments, perhaps due to the lower HCO_3^- concentration at pH 7.1 compared to pH 7.5.

At pH 7.1, where the Cl^- and OH^- fluxes could be directly compared, the value for P_{OH} in the nitrogen-gassed system was 1.30×10^{-3} cm/s, almost 5×10^4 times the value of P_{Cl}. This surprisingly high value of P_{OH} is in good agreement with the observations of Tosteson et al. (1973) on sheep red cells, where P_{OH} is also about five orders of magnitude larger than P_{Cl}.

Because the net HCO_3^- flux is small relative to the net OH^- flux under our experimental conditions, and because the small amounts of HCO_3^- in the system were not directly measured, it is difficult to estimate P_{HCO_3}. Nevertheless, from the observed flux of 0.28 mmol/l cells-min at pH 7.5, P_{HCO_3} would appear to be about 5.5×10^{-7} cm/s.[2] It is thus larger than P_{Cl} but much smaller than P_{OH}.

The observation of such a large OH^- net flux raises the question of the degree

[2] The CO_2 content of air was assumed to be 0.033% (CRC Handbook, Chemical Rubber Co., Cleveland, Ohio) and the α for CO_2 in plasma at 37°C, 0.0308 mmol/liter = mm Hg (Severinghaus et al., 1956). The calculated concentration of HCO_3^- at pH 7.5, if one assumes that the red cell suspension was equilibrated with air, was 0.2 mM.

to which this might influence the calculations of Cl^- and SO_4^{--} permeabilities. In the experiments of Figs. 2–4 and Table I, the medium was exposed to air at pH 7.1. From the apparent OH^- flux for cells under these conditions (including the possible contribution of HCO_3), 1.20 ± 0.07 (n = 28) mmol/liter cells/min, it can be calculated that about 10% of the potassium that leaves the cells might be accompanied by OH^- in chloride medium, and about 19% in sulfate medium. Because of the buffers in both intracellular and extracellular media, the OH^- flow itself should cause no volume change other than that resulting from the change in intracellular pH which, during the first 5 min after valinomycin addition, should be less than 0.2 unit. On this basis, OH^- (and HCO_3^-) flow would lead to a 5% overestimate of P_{Cl} and a 15% overestimate of P_S. In DIDS-treated cells flushed with N_2 the net OH^- flux was inhibited by over 99% at pH

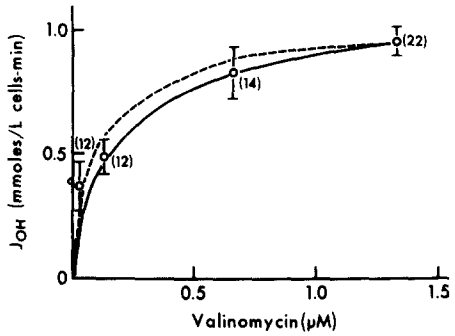

FIGURE 10. Net OH^- flux as a function of valinomycin concentration. Net OH^- flux was measured as described in Materials and Methods at pH 7.5. The medium was gassed with N_2, but the cell suspension was not. The bars represent the standard error and the numbers in parentheses are the number of determinations. The theoretical curves were calculated by using the method and parameters described in the legend to Fig. 7. For the broken line, P_K was taken from Fig. 5, while for the solid line, P_K was assumed to increase linearly (P_K = 1.5 × valinomycin in μM).

7.5 and by 75% at pH 7.1. Thus, net OH^- flow may involve the same DIDS-sensitive system which mediates SO_4^{--} and Cl^- exchange as well as most of the net SO_4^{--} and Cl^- fluxes. From these data it is also clear that OH^- or HCO_3 net flow cannot account for the DIDS-insensitive component of net Cl^- flux.

DISCUSSION

The value of the net chloride permeability reported here, 2.8×10^{-8} cm/s, is in excellent agreement with the first quantitative estimate of P_{Cl}, reported by Hunter (1971). Although Hunter also used valinomycin to increase P_K, in his experiments $^{42}K^+$ unidirectional flux (efflux) was measured as a function of the external K^+ concentration. In K^+-free media, the $^{42}K^+$ efflux is, of course, equivalent to the net flux, and is sensitive to the value of P_{Cl}. Even under these circumstances, however, as can be seen from Eq. (2) and (5) and as has been discussed by Kaplan and Passow (1974), the net flux does not reach a plateau which is independent of P_K, but is rather a function of both P_K and P_{Cl}. At higher

values of external K, where the membrane potential approaches E_K more closely, the ^{42}K efflux also fails to reach a plateau, since much of the isotope efflux consists of K^+-K^+ exchange, which depends almost linearly on P_K but which is insensitive to P_{Cl}. For example, even at 1 mM K_o^+, to effect a 50% decrease in ^{42}K efflux, P_K must be lowered to about 43% of its initial value; the same change in unidirectional efflux requires that P_{Cl} be lowered to 14% of its initial value.

In contrast, the net K^+ flux, as measured here, is almost linearly related to P_{Cl} (Fig. 7) and is quite insensitive to changes in P_K, at least in the range of high valinomycin concentrations (Figs. 4 and 7). Thus, the net flux method provides a more direct measurement of P_{Cl} (or P_S). It can further be calculated that the linearity with P_{Cl} and insensitivity to P_K should further improve with increasing K_o^+. An extension of the method to higher values of external K^+ to test this prediction is currently in progress.

Although the Goldman equation provides a useful formalism with which to calculate P_{Cl}, and although the good agreement of calculated and experimental data provides evidence for its usefulness in the experimental circumstances so far studied, its validity over a wider range of conditions is still uncertain. An advantage of the net flux method presented here is that the almost linear relationship between the parameter measured, net KCl flux, and the value of P_{Cl} requires only that the potassium permeability be high relative to P_{Cl}, and does not require that the Goldman equation exactly describe the valinomycin-mediated K^+ flux. Thus the net K^+ efflux provides a useful empirical measurement of net chloride permeability which can be used, for example, to test the effects of inhibitors, even though the Goldman equation may not provide a completely adequate theoretical description of the system.

Our measurements and those of others (Hunter, 1971; Sachs et al., 1975; Cass and Dalmark, personal communication; Kaplan et al., 1976) demonstrate that the net Cl^- permeability is about four orders of magnitude less than that which would be predicted from measurements of $^{36}Cl^-$ exchange (Brahm, 1975). This virtually rules out the possibility that anion exchange proceeds via a diffusional mechanism, and suggests instead that anions must interact with some specific membrane mechanism which is capable almost exclusively of one-for-one anion exchange. The carrier models of anion exchange (Wieth, 1972; Gunn, 1972; Gunn et al., 1973; Dalmark, 1975) would provide such a mechanism.

Although the Cl^- exchange flux in the red cell is exceptionally high compared to that of other cells, the Cl^- conductance (net flux) is not. In fact, it is similar to that observed in rat liver cells (Claret and Mazet, 1972) and taenia coli smooth muscle (Brading, 1971), and rather low in comparison with systems such as frog skeletal muscle (Hodgkin and Horowicz, 1959; Moore, 1969), bullfrog gastric mucosa (Forte, 1969), and toad bladder (Leaf and Hays, 1962), where P_{Cl} is in the range of 10^{-6} cm/s. The agreement of the red cell membrane potential with the chloride equilibrium potential under most circumstances must therefore be attributed to the very low cation permeability of the red cell ($\sim 2 \times 10^{-10}$ cm/s [Knauf, unpublished data] or only about 1% of the net anion permeability) rather than to an unusually high Cl^- conductance.

For HCO_3^- as well as Cl^-, the net permeability is much lower than that which

would be estimated from exchange measurements, although the net HCO_3^- permeability seems to be somewhat higher than the net Cl^- permeability. For SO_4^{--}, on the other hand, the flux calculated from the net permeability, P_S, amounts to about 20% of the exchange flux under our experimental conditions. The conductive and exchange processes, therefore, appear to differ significantly in their anion selectivity: although Cl^- exchange is 10^4–10^5 times faster than SO_4^{--} exchange (Schnell et al., 1973), P_{Cl} is only four times as large as P_S. The selectivity of the exchange system for Cl^- vs. SO_4^{--} is thus much larger than that of the net system. In the case of OH^-, comparison of the value of P_{OH} at pH 7.1 (1.3×10^{-3} cm/s) with that reported by Crandall et al. (1971) for Cl/OH exchange (3.75×10^{-3} cm/s) suggests that the net OH^- permeability is about one-third of that predicted from exchange measurements. In this case, the net flux exhibits the greater selectivity: P_{OH} is more than four orders of magnitude larger than P_{Cl}.

In addition to the anion selectivity, other properties of net and exchange anion transfer differ. Measurements of net chloride and sulfate fluxes at pH 6.8 indicate that P_{Cl} is almost twice the value at pH 7.1, while P_S is not significantly increased (data not shown). The finding of differences in the effects of pH on P_{Cl} and P_S suggests that these results are not due to alterations in OH^- or HCO_3^- fluxes, but rather reflect a true change in net Cl^- or SO_4^{--} permeability. The increase in net chloride permeability with decreasing pH is in agreement with the data of Scarpa et al. (1970) and is the opposite of the behavior observed for chloride exchange (Gunn et al., 1973). The response of net sulfate flow to pH also differs from that of the corresponding sulfate exchange (Schnell, 1972), in agreement with results of Passow et al. (1974).

The activation energy for the exchange flux of Cl^- or I^- is 31–33 Cal/mol in the range of 0°–15°C (Dalmark and Wieth, 1972; Brahm, 1975), much higher than the value of 13.5 Cal/mol for net flux measured between 10° and 25°C by Cass and Dalmark (personal communication). At higher temperatures, however, the activation energies for both processes appear to be about 22–25 Cal/mol (on the basis of net fluxes from this paper, Hunter, 1971, and Kaplan and Passow, 1974, and of exchange fluxes from Brahm, 1975).

Certain inhibitors seem to affect net and exchange permeabilities differently. Phlorizin, for example, causes a 10-fold increase in P_{Cl} (Kaplan and Passow, 1974) but results in a 90–95% inhibition of chloride exchange (Schnell et al., 1973). In both cases, however, the effects of phlorizin are only observed when it is present at the outside of the membrane. Pronase, which inhibits anion exchange (Passow, 1971) increases net KCl efflux at high valinomycin concentrations (unpublished data).

The differences in the properties of net and exchange anion flow could most easily be explained if completely separate and distinct membrane mechanisms were involved in each process. On the other hand, net and exchange transport exhibit many similarities. The highly specific inhibitors of anion exchange, SITS and DIDS, also affect net chloride and sulfate fluxes. Another inhibitor, dipyridamole, whose mode of action seems to be different from that of the stilbenes, has similar effects on net and exchange chloride and sulfate fluxes (Hoffman and Knauf, 1973; Cotterrell, 1975). Recently, Kaplan et al. (1976) have found

that two inhibitors of anion exchange, one of which is effective only at the outer membrane surface and the other of which inhibits at either surface, inhibit net chloride movements in a similar fashion. Even more strikingly, in the case of the irreversible inhibitor DIDS, where quantitative comparisons can be made, virtually all of the sulfate net flux and the majority of the chloride net flux are inhibited in parallel with sulfate and chloride exchange (Figs. 8 and 9). This provides strong evidence that both net and exchange fluxes involve a common element. This common element is probably the 95,000 dalton protein which, from studies of the binding of DIDS (Cabantchik and Rothstein, 1974) and other inhibitors (Cabantchik et al., 1975; Cabantchik et al., 1976; Ho and Guidotti, 1975; Zaki et al., 1975), appears to be involved in anion exchange. Further evidence for the participation of the 95,000 dalton protein in both net and exchange processes is provided by the recent demonstration (Wolosin et al., 1976) that membrane vesicles from which almost all of the red cell membrane proteins except the 95,000 dalton protein have been extracted still retain DIDS-sensitive net and exchange sulfate fluxes.

If indeed the same underlying mechanism accounts for the major portion of net and exchange fluxes, then models for anion exchange must be able to account for net anion transfer. In terms of the models for anion exchange proposed by Wieth (1972), Gunn (1972), and Dalmark (1975), the one-for-one exchange is explained by the inability of the unloaded carrier to cross the membrane. A net flow would result if a small component of "slippage" or backflow of the unloaded carrier were to occur. Even if this particular model is not tenable, any model in which the rate-limiting step is different for exchange and net flow might permit a reconciliation of the differences in anion selectivity and in response to pH, temperature, and some inhibitors with the involvement of a common mechanism in both net and exchange anion flow.

The technical assistance of Constance Chik, Magdalena Rásko, and Judith Ellen is gratefully acknowledged.

This work was supported in part by the Medical Research Council of Canada, grants MT4665 and MA5149.

Received for publication 30 August 1976

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