Voltage Dependence of DIDS-insensitive Chloride Conductance in Human Red Blood Cells Treated with Valinomycin or Gramicidin

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ABSTRACT   Net K and Cl effluxes induced by valinomycin or by gramicidin have been determined directly at varied external K, denoted by $[K]_o$, in the presence and absence of the anion transport inhibitors DIDS (4,4'-diiso-thiocyano-2,2'-disulfonic acid stilbene), and its less potent analogue SITS (4-acetamido-4'-isothiocyano-stilbene-2,2'-disulfonic acid). The results confirm that pretreatment with 10 μM DIDS, or 100 μM SITS, for 30 min at 23°C inhibits conductive Cl efflux, measured in the continued presence of the inhibitors at 1 mM $[K]_o$, by only 59–67%. This partial inhibition by 10 μM DIDS at 1 mM $[K]_o$ remains constant when the concentration of DIDS, or when the temperature or pH during pretreatment with DIDS, are increased. Observations of such partial inhibition previously prompted the postulation of two Cl conductance pathways in human red blood cells: a DIDS-sensitive pathway mediated by capnophorin (band 3 protein), and a DIDS-insensitive pathway. The present experiments demonstrate that at $[K]_o$ corresponding to values of E_K between −35 and 0 mV the DIDS-insensitive component of net Cl efflux is negligible, being ≤0.1 μMol/g Hb/min, both with valinomycin (1 μM) and with gramicidin (0.06 μg/ml). At lower $[K]_o$, where E_K is below ~ −35 mV, the DIDS-insensitive fraction of net Cl efflux increases to 2.6 μMol/g Hb/min with valinomycin (1 μM), and to 4.8 μMol/g Hb/min with gramicidin (0.06 μg/ml). With net fluxes determined from changes in mean cell volume, and with membrane potentials measured from changes in the external pH of unbuffered red cell suspensions, a current-voltage curve for DIDS-insensitive Cl conductance has been deduced. While specific effects of varied $[K]_o$ on net Cl efflux are unlikely but cannot

strictly be ruled out, the results are consistent with the hypothesis that DIDS-insensitive Cl conductance turns on at an E_m of $\sim$ -40 mV.

INTRODUCTION

Chloride transport in human red blood cells is predominantly mediated by capnophorin, the anion exchange protein (AE1) located in band 3 of SDS polyacrylamide gels, and consists of an electrically silent exchange plus at least one conductive pathway (see Cabantchik, Knauf, and Rothstein, 1978; Knauf, 1979; Fröhlich and Gunn, 1986; Passow, 1986; Jennings, 1992, for reviews). Experiments in which the K permeability of red cells was increased by means of the K-selective ionophore valinomycin, or the cation-selective pore-forming antibiotic gramicidin, established that conductive Cl fluxes are a minor fraction of total red cell Cl transport (Chappell and Crofts, 1966; Harris and Pressman, 1967; Scarpa, Cecchetto, and Azzone, 1968, 1970; Pressman and Heeb, 1972). Kinetic studies of anion exchange in human red cells have supported a ping-pong mechanism in which the transport sites alternately face the intracellular and extracellular solutions, and translocate only when binding an anion (Gunn and Fröhlich, 1979; Jennings, 1982). The results of other kinetic studies are inconsistent with a slippage mechanism for anion conductance in which a conformational change, or back-diffusion, of an unloaded site facilitates unidirectional translocation of Cl (Knauf, Law, and Marchant, 1983; Fröhlich, Leibson, and Gunn, 1983; Kaplan, Pring, and Passow, 1983; Fröhlich, 1984). These experiments gave rise to the concept that DIDS-sensitive conductive transport of Cl occurs through a channel within capnophorin by a process variously denoted as barrier-transit (Knauf et al., 1983), tunneling (Fröhlich et al., 1983), or simply "ionic diffusion" (Kaplan et al., 1983).

Conductive Cl fluxes have been studied in valinomycin-treated red cells from sheep (Tosteson, Gunn, and Wieth, 1973), and from humans (Hunter, 1971, 1974, 1977; Knauf, Fuhrmann, Rothstein and Rothstein, 1977; Knauf et al., 1983; Fröhlich et al., 1983; Fröhlich, 1984; Bennekou, 1984; Bennekou and Christophersen, 1986; Bennekou and Stampe, 1988). By elevating the permeability of K over that of Cl, valinomycin induces a net efflux of K and Cl, associated with cell shrinkage and membrane hyperpolarization (Hoffman and Laris, 1974; Freedman and Hoffman, 1979b), and thus provides a convenient method for the study of Cl conductance. The net anion fluxes induced by cation-selective ionophores differ from anion exchange fluxes with regard to their dependence on temperature and pH, their anion selectivity, and their sensitivity to certain noncompetitive inhibitors (see Knauf et al., 1983, and Passow, 1986, for discussion and references). Parallel inhibition of net and exchange fluxes by DIDS (Knauf et al., 1977) were interpreted in support of the view that both fluxes are mediated by capnophorin. The differing properties of exchange and net fluxes were attributed to their having different rate-limiting steps.

In previous studies, DIDS was found to inhibit only partially the net K efflux induced by valinomycin at physiological $[K]_o$, thus prompting suggestions that the human red cell membrane contains two Cl-conductance pathways: one DIDS-sensitive and the other DIDS-insensitive (Knauf et al., 1977; Hoffman, Kaplan, Callahan, and Freedman, 1980; Kaplan et al., 1983). The 65% inhibition of net efflux of Cl by DIDS, found in studies with valinomycin, contrasts with the 99.999%

inhibition of unidirectional efflux of Cl found with DIDS in the absence of ionophores (Gasbjerg, Funder, and Brahm, 1993). The experiments described in this paper demonstrate that DIDS-insensitive Cl conductance in human red blood cells turns on reversibly when the driving force E_m-E_{Cl} is below $\sim$ -30 mV, and are consistent with this fraction of Cl conductance being dependent on the transmembrane voltage. Preliminary reports of parts of this work were presented previously (Freedman and Novak, 1984, 1987; Freedman, Bifano, Crespo, Pratap, Wallenga, Bailey, Zuk, and Novak, 1988).

MATERIALS AND METHODS

Preparation of Red Cells

Blood from healthy human donors was drawn by venipuncture into heparinized tubes and immediately centrifuged at 13,800 g for 3 min at 4°C. The plasma and buffy coat were aspirated and discarded, and the packed cells were then washed three or four times by centrifugation, each time resuspending in ~5 vol of chilled medium containing 5 mM KCl, 145 mM NaCl, and 5 mM HEPES buffer, pH 7.4 at 23°C. The cells were then adjusted to 50% hematocrit (HCT) in the cold wash solution and kept on ice for use on the same day.

Net Effluxes of K and Cl

Erlenmeyer flasks with flux media consisting of x mM KCl, $(150 - x)$ mM NaCl (for experiments with valinomycin), or $(150 - x)$ mM choline Cl (for experiments with gramicidin), and 5 mM HEPES buffer, pH 7.4 at 23°C, were equilibrated at 23°C on a shaking waterbath (model 6250, Eberbach Corp., Ann Arbor, MI) at 100 oscillations per minute. At staggered times, sufficient 50% HCT red cells were added to the flasks to make the final HCT 1.2%. After another 5 min, triplicate 10-ml samples were taken to determine initial values of cell K and Cl, as described below. After 3 min, 1 μM valinomycin, or 0.06 μg/ml gramicidin, was added to the flask. Triplicate 10 ml samples were then removed at desired times for analyses of cell K, Na, and Cl. In this cold-quench method, samples were taken via a 10-ml Manostat syringe with cannula and transferred to ice-cold 50-ml glass tubes and were rapidly cooled by contact for at least 1 min with glass cold-finger condensers connected to a Lauda RM3 circulator set at 1.5°C. It was unnecessary to cool the initial samples (before addition of valinomycin or gramicidin). The samples were then poured into 10-ml syringe tubes attached to 0.4-ml microcentrifuge tubes as previously described (Freedman and Hoffman, 1979a). After centrifugation for 5 min at 2,000 rpm (model TJ-6, Beckmann Instruments, Palo Alto, CA), the microcentrifuge tubes containing the cells were detached from the syringe tubes and immediately recentrifuged at 4°C for 10 min at 29,000 g (No. J2-21, Beckman Instruments). After analysis of cell K and Cl (see below), the net effluxes of K and Cl, denoted as ^oM_K and $^oM_{Cl}$, were computed as the difference in K or Cl contents before and 10 min after addition of valinomycin, or at longer times after valinomycin for slower fluxes. The final concentration of ethanol added with valinomycin was 0.04%; raising the ethanol to 0.3% did not affect the ion contents for at least 1 h. In preliminary experiments, washing the sampled cells in ice-cold isotonic sucrose (method of Tosteson et al., 1973) resulted in decreased cell Cl contents (not shown).

Initial net K effluxes were also estimated (see Appendix) from the ionophore-induced initial rate of change of mean cell volume (MCV). MCV was determined using the Technicon H-1 Hematology Analyzer which utilizes differential laser light scattering based on Mie scattering theory (Tycko, Metz, Epstein, and Grinbaum, 1985; Mohandas, Kim, Tycko, Orlik, Wyatt, and Groner, 1986). This method has a time resolution of 1 min per point, and gives a Gaussian

distribution of cell volumes. The use of the Technicon instrument avoids the artifactually skewed red cell volume distributions typically obtained with the Coulter channelyzer (e.g., Wilkins, Frandolig, and Fischer, 1970).

For experiments with DIDS or SITS, the cells were preincubated with the inhibitor for 30 min before addition of valinomycin or gramicidin; all net K and Cl effluxes reported in this paper were then measured in the same suspensions in the continued presence of the inhibitor. If I is the inhibited rate of efflux, and U is the uninhibited rate, then $Y = \log(I/U)$ provides an appropriate transformation for using the t test to determine the significance of differences. The log transformation permits comparison of differences instead of ratios; the ratios I/U were also tested with the t distribution.

Electrolyte Analyses

The triplicate samples of packed cells were hemolyzed in 5 ml distilled water, followed by appropriate dilution for analyses of the cellular contents of K, Cl and Hb. K and Na were measured by flame photometry (model 343, Instrumentation Laboratory Inc., Lexington, MA) using 15 mM $LiNO_3$ as internal standard. Cl was estimated with a Buchler-Cotlove chloridometer (Buchler Instruments, Inc., Fort Lee, NJ). The 2%-systematic elevation of cell Cl due to interference by cellular glutathione (Gunn, Dalmark, Tosteson, and Wieth, 1973) would not affect the measurement of Cl fluxes. The coefficient of variation for Cl standards (0.5–2.0 mM), and for triplicate sets of samples, averaged 4%, while that for K standards and triplicate samples averaged 2%. Ion contents were expressed in $\mu M/g$ Hb. Additional reference samples of weighed packed cells, taken in triplicate before addition of valinomycin or gramicidin, were hemolyzed to determine Hb content (g Hb/g cells). For these samples, the 0.4-ml microcentrifuge tubes containing the packed cells were cut at the bottom and ~1 mm below the cell-supernatant interface. The tubes were then weighed, followed by ejection of the cells by means of tubing connecting the tube to a plastic mouthpiece, and then reweighed. Hb was determined using Drabkin's reagent at 540 nm, and was calibrated with cyanmethemoglobin standards (Boehringer Mannheim Diagnostic Corp., Houston, TX). Other triplicate weighed reference samples were dried in a vacuum oven for 18–20 h at 105°C to determine initial water contents (g H_2O/g cells). The reference samples permitted conversion of cell ion contents ($\mu Mol/g$ Hb) to concentrations ($\mu M/g$ H_2O).

The initial membrane potential, E_m, was estimated from the chloride equilibrium potential, $E_{Cl} = 58.7$ r_{Cl}, where the chloride ratio, r_{Cl}, was determined with ^{36}Cl. Washed cells were suspended at 1.2% HCT in medium containing 1 mM KCl and 149 mM NaCl. After adding 0.1 $\mu Ci/ml$ $H^{36}Cl$, the suspensions were allowed to equilibrate for 15–20 min at 23°C, and the pH was then adjusted to the initial value of 7.5 attained in the nonradioactive unbuffered suspensions. Triplicate samples were then centrifuged as described above. Weighed samples of packed cells (~60 mg), or 0.1 ml samples of supernatants, were added to 1 ml distilled water, followed by addition of 1 ml ice-cold 10% trichloroacetic acid (TCA). After centrifugation for ~5 min at 12,000 g, 1 ml of the clear extracts was added to 10 ml aqueous counting scintillant (ACS, Amersham Corp., Arlington Heights, IL.), and the radioactivity was measured by liquid scintillation. The chloride ratio was computed as follows:

$$r_{Cl} = CPM_c(2 + fw)/21\, fw CPM_o,$$

where CPM_c and CPM_o are the counts per minute (corrected for background) in the TCA extracts of cells and medium, respectively, f is the water content (g H_2O/g cells), w is the weight (g) of cells extracted, the factor 2 is the volume (ml) of extraction fluid, and the factor 21 corrects for dilution of the medium.

Apparent Proton Fluxes

The pH-stat method used for these studies does not distinguish between proton influx and hydroxide efflux; for convenience, the data are referred to as apparent proton fluxes. To determine such fluxes, 25 ml of 1.2% HCT cells in unbuffered x mM KCl and $(150 - x)$ mM NaCl, where $x = 1$, 10, 30, 60, 100, and 150, were added to a closed thermostatted beaker containing a pH and reference electrode, and an autoburette tip. After allowing 20–30 min for pH equilibration, the pH stat end point was set to the value of the pH that the suspension had attained, and the pH-stat was started. Valinomycin (1 μM) was then added, and the volume of acid (0.01 N standardized HCl, Mallinckrodt) that was automatically added to maintain constant pH was recorded versus time. Samples of each suspension were also taken to measure Hb content. Proton fluxes were expressed in μM/g Hb/min. The pH-stat (Radiometer ETS 822, The London Company, Cleveland, Ohio) included a PHM 82 standard pH meter, a TTT80 titrator, an ABU 80 autoburette, a pH-stat interface, and a strip chart recorder. The flexible miniature pH and Ag/AgCl reference electrodes (MI506 and MI402) were from Microelectrodes, Inc. (Londonderry, NH). The suspension was stirred with a teflon-coated magnetic bar driven by a Thermolyne Stir-mate (Model S-7805). The measuring vessel was a modified double-walled glass beaker thermostatted at 23°C by a waterbath circulator (see Bisognano, Dix, Pratap, Novak, and Freedman, 1993, for additional details).

Reagents

Ionophores were added from the following stock solutions in ethanol: 3 mM valinomycin (1111 g/mol, Calbiochem-Behring Corp., La Jolla, CA); 0.33 mg/ml gramicidin D (Sigma Chemical Co., St. Louis, MO). Stock solutions of DIDS (498.5 g/mol, Sigma Chemical Co.) were prepared on the day of use at 5.0 mg/ml, or 10 mM, in 20 mM NaOH. The extinction coefficient of DIDS at 342 nm was 4.5 (10^4) M^{-1} when dissolved in saline, water, or 20 mM NaOH. All other chemicals were reagent grade.

RESULTS

Time Course of Net Electrolyte and Water Shifts after Valinomycin

The intracellular electrolyte concentrations $[K]_c$, $[Cl]_c$, and $[Na]_c$, and the cell water content, during 3 h of incubation of human red blood cells with 1 μM valinomycin at 1 mM $[K]_o$ are shown in Fig. 1 for two donors (*filled and open symbols*). Elevation of the K permeability by valinomycin permits net efflux of K down its concentration gradient (Fig. 1 *A*, *circles*). The associated hyperpolarization drives Cl out of the cell against its concentration gradient but down its electrochemical gradient (Fig. 1 *A*, *squares*). Net K and Cl effluxes are associated with cell shrinkage (Fig. 1 *C*), resulting in increased $[Na]_c$ (Fig. 1 *B*). During cell shrinkage the osmotic coefficient of Hb rises such that sufficient water is retained to result in decreased concentrations of intracellular K and Cl (Fig. 1 *A*), thus yielding a hypertonic effluent. The cellular contents of electrolytes and water before and 3 h after addition of valinomycin are shown in Table I. The results indicate a discrepancy averaging 92 μM/g Hb between cation and anion movements that can be accounted for either by postulating an efflux of OH or an influx of protons (see below). The calculated average osmolarities of the valinomycin-induced effluents were 420 and 437 mosm in the two experiments. A hypertonic effluent of $\sim$350 mosm was also noted in valinomycin-treated cells that had half of their intracellular Cl replaced with SCN anions (Freeman et al., 1987).

The dashed lines in Fig. 1 represent a satisfactory curve fit of the time courses of the water and electrolyte shifts according to an integrated model of red cell equilibria and kinetics (Lew and Bookchin, 1986; Freeman et al., 1987), as applied to valinomycin-treated cells (see Discussion).

Net K and Cl Effluxes Induced by Valinomycin and Gramicidin

The results in Fig. 2 are from a representative experiment in which valinomycin-induced net effluxes of K (left) and Cl (right) were determined with [K]$_o$ varied between 1 and 150 mM, either with pretreatment and then incubation with 10 μM
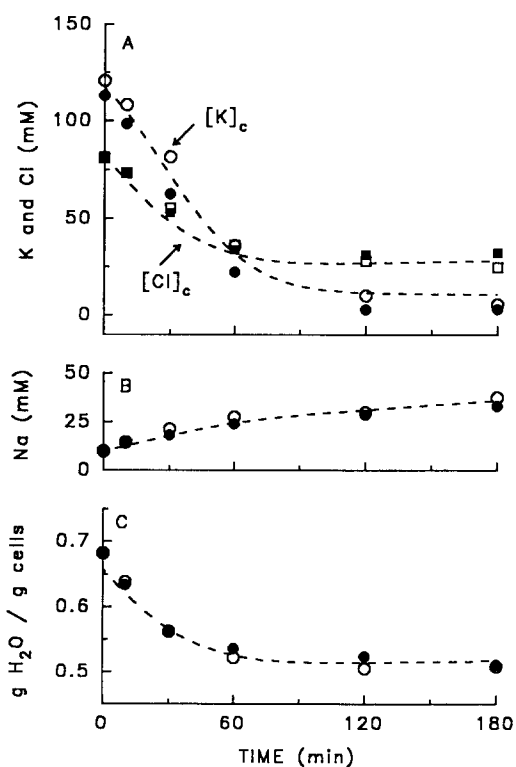

FIGURE 1. Time courses of (A) [K]$_c$ and [Cl]$_c$, (B) [Na]$_c$, and (C) cell water content after addition of 1 μM valinomycin at 1 mM K$_o$. Cells were washed and suspended at 1.2% HCT in 1 mM KCl and 149 mM NaCl at 23°C. Samples were taken before and then 10, 30, 60, 120, and 180 min after addition of valinomycin (see Materials and Methods). Data are from experiments with two donors (filled and open symbols). The initial water and Hb contents of the washed cells were 0.682 and 0.683 g H$_2$O/g packed cells, and 0.283 and 0.287 g Hb/g packed cells. Hemolysis reached 1.6% at 2 h and 1.8% at 3 h. The values plotted were corrected for 0.025 ml/g packed cells of trapped extracellular medium, as determined with ^{14}C-sucrose. The dashed lines are theoretical fits (see Discussion), obtained by minimizing the sum of the squared differences between the predicted and experimental values.

DIDS (bottom), or with no exposure to DIDS (top). With the cold-quench method, the net effluxes are clearly linear in the presence or absence of DIDS for [K]$_o$ ≥ 30 mM, and also at 10 mM [K]$_o$ in the presence of DIDS (Fig. 2). In two other experiments at 1 mM [K]$_o$ without DIDS (Fig. 3), in which samples were taken at 4 and 10 min after the addition of ionophore, the initial rates of net efflux of K and Cl were linear for up to 10 min with 3 μM valinomycin (circles), but not with 60 ng/ml gramicidin (squares).

To improve the time resolution of the measured fluxes, the ionophore-induced decreases in mean cell volume (MCV) were determined using a clinical instrument: the Technicon H-1 Hematology Analyzer which utilizes isovolumetric sphering of the cells and differential laser light scattering (Tycko et al., 1985; Mohandas et al., 1986).

TABLE I

Cell Electrolyte Content and Mean Osmolarity of Valinomycin-induced Effluent

Donor	K_c	Cl_c	Na_c	H_c	Water		Osmolarity of effluent
	$\mu Mol/g\ Hb$				$g/g\ cells$	$g/g\ Hb$	$mosm$
A							
Initial	280	188	23		0.657	2.322	
Final	6	23	37		0.483	1.132	
Change	−274	−165	+14	(+95)		−1.190	437
B							
Initial	259	188	21		0.658	2.293	
Final	3	31	32		0.486	1.127	
Change	−256	−157	+11	(+88)		−1.166	420

Shown above are intracellular contents of K, Cl, Na, and water measured before and 3 h after addition of 1 μM valinomycin for the experiment described in Fig. 1. Also shown is the gain in H, equivalent to a loss of OH, needed for electroneutrality. The water content, w' (g/g Hb), were calculated from the measured water contents, w (g/g cells), as follows:

$$w' = [w/(1 - w)] [(1 - w^0)/Hb],$$

where w^0 is the initial water content (g/g cells), and Hb is the initial Hb content. Hb was 0.283 g/g cells for donor A and 0.287 g/g cells for donor B. All values were corrected for trapped medium of 0.025 ml/g cells, as determined with ^{14}C-sucrose. The mean osmolarity of the effluent was calculated as the sum of the changes in K, Na, Cl, and H (μMol/g Hb) divided by the change in w (g/g Hb).
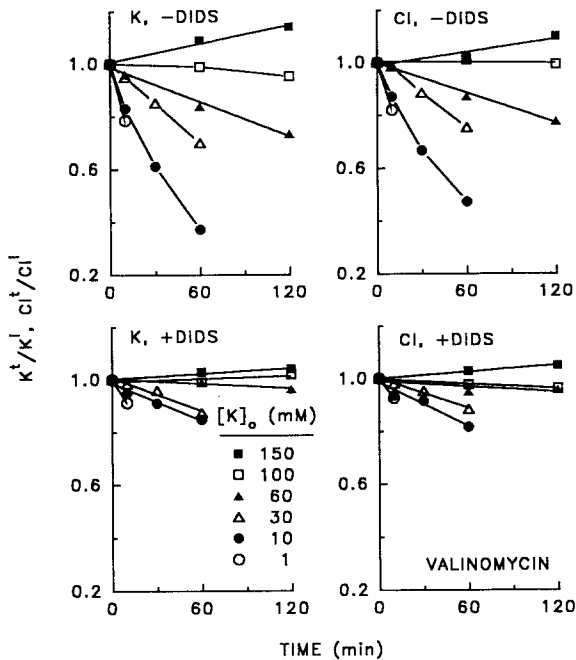

FIGURE 2. Valinomycin-induced net effluxes of K (*left*) and Cl (*right*), measured by the cold-quench method, at varied $[K]_o$ with (*bottom*) and without (*top*) DIDS. Two sets of Erlenmeyer flasks with flux media containing x mM KCl, 150-x mM NaCl, where x = 1, 10, 30, 60, 100, and 150, and 5 mM HEPES buffer, pH 7.4 were equilibrated at 23°C. After adding washed red cells to a final HCT of 1.2%, DIDS was added to 10 μM to one set of flasks and these were incubated for 30 min before adding iono-phore. K and Cl were determined (see Materials and Methods) before and 10 min after, or 10, 30, and 60 min after, or 60 and 120 min after addition of 1 μM valinomycin, the longer times being used for the slower rates of efflux. The ratios K^t/K^i and Cl^t/Cl^i refer to the values of K_c and Cl_c (μMol/g Hb) determined t min after adding valinomycin, as normalized to the initial values (indicated by superscript i) before valinomycin.

The initial rate of net K efflux, $^{o}M_{K}$, is proportional to the initial rate of change of mean cell volume, dV''_{w}/dt, by a factor which includes the virial coefficients of the osmotic coefficient of hemoglobin and also the osmotic coefficient of the salts (see Appendix). In a preliminary experiment to check the range of the instrument, the hematocrit was varied by dilution from 50 to 0.5%. The measured hematocrits agreed precisely with those expected (not shown); the measured mean cell volume was independent of hematocrit over the range tested, thus verifying that the clinical instrument gives accurate results with the 1.2% hematocrit suspensions routinely used for determining the fluxes by the cold-quench method. At 1 mM $[K]_{o}$, the ionophore-induced decreases in mean cell volume are linear, within experimental error, for up to 10 min with valinomycin (Fig. 4 A), and for up to 4 min with gramicidin (Fig. 4 B). The solid lines (Fig. 4, A and B) are linear regressions which fall within the remarkably small experimental errors. The average coefficient of variation for the measurements of mean cell volume was $\pm 1\%$. Importantly, $^{o}M_{K}$ calculated from the rate of decrease of cell volume in Fig. 4A is 8.0 μM/g Hb/min with 3 μM
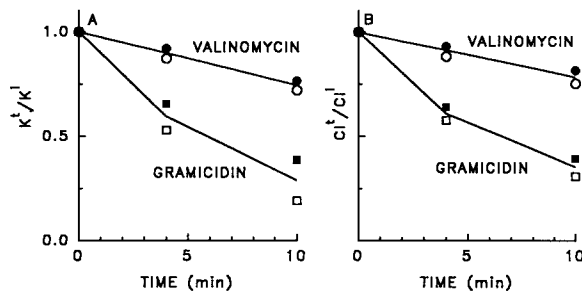

FIGURE 3. Net efflux of (A) K and (B) Cl induced by 1 μM valinomycin (*circles*) or 60 ng/ml gramicidin (squares) at 1 mM $[K]_{o}$ in the absence of DIDS. The protocol was as described in Fig. 2 except that 149 mM choline chloride instead of NaCl was used with gramicidin. Samples were taken at 4 and 10 min in order to examine the initial linearity of the rate of efflux. Filled and open symbols are from two experiments.

valinomycin, in precise agreement with the same value estimated by the cold-quench method during the first 10 min after addition of ionophore (see Fig. 5 A, *open circles and triangles*). Net K effluxes induced by gramicidin also agree when determined by the two methods (compare *empty circles* with *triangles* in Fig. 5 B).

As $[K]_{o}$ is increased from 1 to 100 mM, the net efflux of K decreases considerably, as measured directly by the cold-quench method (Fig. 2, *left*), or by the rate of decrease of cell volume (Fig. 4 C, *open circles and squares*). The dashed line in Fig. 4 C represents the results of an experiment with 10 μM DIDS in which the rate of decrease of mean cell volume is found to be reversible upon exposure to 1 mM $[K]_{o}$ for 10 min, and then subsequent exposure to 100 mM $[K]_{o}$.

Effect of Varied [Valinomycin] and [Gramicidin]

Initial net effluxes of K were measured by the cold-quench method after adding varied concentrations of valinomycin (Fig. 5 A, *open circles*) or gramicidin (Fig. 5 B, *open circles*). Experiments with valinomycin were conducted in Na media whereas

those with gramicidin were in choline media. The half-maximally effective [valinomycin] of 1 μM (Fig. 5 A) agrees with the results of Fröhlich et al. (1983), obtained at a similar HCT but at low [Cl]$_o$. This apparent potency of valinomycin at 1 μM is 10 × lower than reported by Knauf et al. (1983), partly because we used a 10-fold higher HCT. The maximal flux of 10 μM/g Hb/min is close to the value obtained at 0.3 mM [K]$_o$, pH 7.8 at 25°C, with 11 μM valinomycin at about 1.4% HCT by Fröhlich et al.
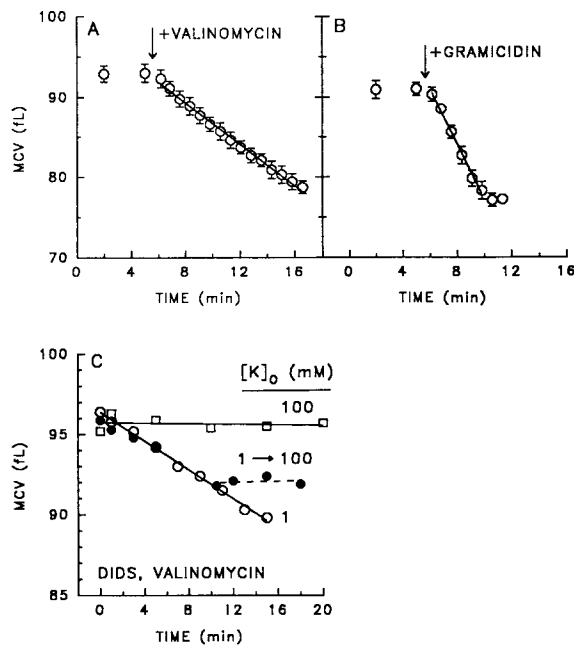

FIGURE 4. (A and B) Effect of valinomycin (A) or gramicidin (B) at 1 mM K$_o$ in the absence of DIDS on the rate of decrease of mean cell volume (MCV), as measured with the Technicon H-1 Hematology Analyzer. Cells were washed, and then suspended at 1.2% % HCT in 5 ml of medium containing 1 mM KCl, 149 mM NaCl (for valinomycin) or 149 mM choline chloride (for gramicidin), and 5 mM HEPES buffer, pH 7.4 at 23°C. At 2 and 5 min after adding cells, 200 μl samples were removed, and initial values of MCV were determined. 1 min later (*arrows*), 3 μM valinomycin or 60 ng/ml gramicidin was added, and samples were removed after 10 s, and then every 40–45 s, for ~10 min.

Each sample was immediately aspirated at designated times into the H-1 analyzer for measurement of the time course of the decrease in MCV. The net efflux of KCl was computed from the slope of the time course (see Appendix). For each ionophore, data represent averages of six time courses determined on the same day. Error bars represent ± 1 SD. Solid lines are linear regressions. (*C*) Reversibility of the rate of decrease in MCV upon switching the cells from media containing 1 mM [K]$_o$ to 100 mM [K]$_o$ (*filled circles, dashed line*). Solid lines show the time courses of MCV at 1 mM [K]$_o$ (empty circles) and at 100 mM [K]$_o$ (*open* squares). The decrease in MCV was induced with 1 μM valinomycin after preincubation for 30 min with 10 μM DIDS in medium containing 1 mM KCl and 149 mM NaCl, or 100 mM KCl and 50 mM NaCl.

(1983), and is also close to the result obtained at 10 mM [K]$_o$, pH 7.04 at 37°C, with 1.3 μM valinomycin at 0.1% HCT by Knauf et al. (1983). °M_K was significantly greater at ≥5 μM valinomycin than at 3 μM ($P < 0.025$), and a small but significant ($P < 0.001$) net influx of sodium was detected (Fig. 5 A, *filled symbols*), suggesting that too much valinomycin perturbs the red cell membrane nonspecifically. For subsequent experiments valinomycin was used at 1 or 3 μM, and gramicidin at 0.06 μg/ml.

Effects of DIDS and SITS on ^oM_K at varied $[K]_o$

Net effluxes of K, obtained by the cold-quench method from the initial slopes in two to six experiments with 1 μM valinomycin, were determined at varied $[K]_o$ (Fig. 6) in the presence (*circles*) and absence (*triangles*) of 10 μM DIDS (Fig. 6 A) and 100 μM SITS (Fig. 6 B), and are plotted vs $[K]_o$ (*lower axes*) and also vs E_K (*upper axes*). At 1 mM $[K]_o$, corresponding to maximal hyperpolarization with E_K at −122 mV, the inhibition of net K efflux by DIDS was 59 ± 11% (Table II). This degree of inhibition
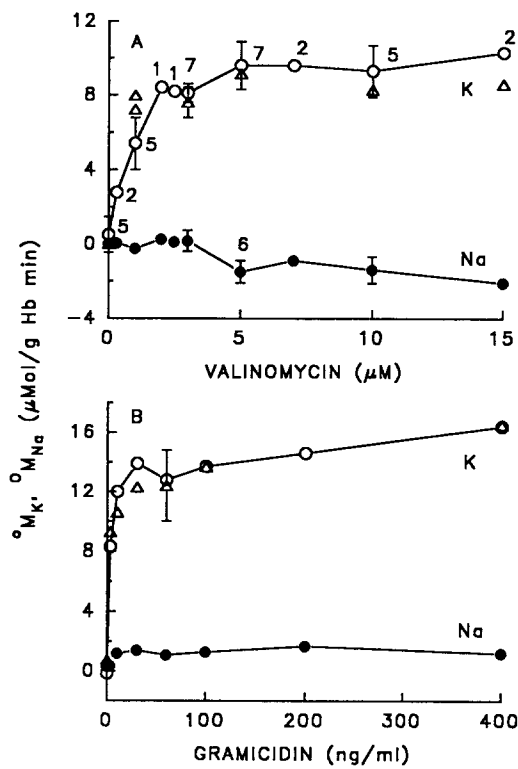

FIGURE 5. Effect of varied [valinomycin] and [gramicidin] on net K and Na fluxes, ^oM_K (*open symbols*) and $^oM_{Na}$ (*filled circles*). (A) Valinomycin. Human red blood cells were washed and then suspended at 1.2% HCT in 1 mM KCl, 149 mM NaCl, and 0, 5, or 10 mM HEPES buffer, pH 7.4. at 23°C. Fluxes of K and Na were measured by the cold-quench method (*circles*) with time points taken before and 10 min after the addition of valinomycin at 0–15 μM [valinomycin] (see Materials and Methods). Data are pooled from six experiments with red blood cells from nine donors. Points represent mean ± SD with the number of donors indicated by the points. (B) Gramicidin. Data by the cold-quench method (*circles*) are averages from two donors. The red cells were washed and incubated in 1 mM KCl, 149 mM choline Cl, and 5 mM HEPES, pH 7.4 at 23°C. Fluxes were measured at 0–0.4 μg/ml [gramicidin D] (see Materials and Methods). In A and B, triangles represent comparable fluxes determined from the time course of decrease of MCV, as described in the legend to Fig. 4.

is in satisfactory agreement with previous reports of 64% irreversible inhibition by DIDS (Knauf et al., 1977; Kaplan et al., 1983), and 65% reversible inhibition by DNDS (4,4′-dinitrostilbene-2,2′-disulfonate) (Fröhlich et al., 1983). As $[K]_o$ was increased by isotonic substitution for $[Na]_o$ in experiments with valinomycin (Fig. 6 A), and similarly for $[choline]_o$ in experiments with gramicidin (not shown), the DIDS-insensitive fluxes decreased above 60 mM $[K]_o$ to ≤0.1 μM/g Hb/min. The same pattern of inhibition at varied $[K]_o$ was also evident with 1 μM valinomycin and 100 μM SITS (Fig. 6 B).

From the results in Fig. 6A, it is evident that the DIDS-insensitive net efflux of K increases at low $[K]_o$, corresponding to values of E_K below ~ -35 mV, but is ≤ 0.1 $\mu M/g$ Hb/min between -35 and 0 mV. To examine quantitatively how the extent of inhibition by DIDS depends on $[K]_o$, 60 mM $[K]_o$ was selected as a convenient condition such that net K efflux is still sufficiently large to measure the degree of inhibition. ^oM_K and $^oM_{Cl}$ were determined in six experiments with valinomycin and in four experiments with gramicidin at 1 and 60 mM $[K]_o$. The results, summarized in Table II, show that with 1 μM valinomycin the inhibition by DIDS increased from 59% at 1 mM $[K]_o$ to 87% at 60 mM $[K]_o$ ($P < 0.01$ comparing paired I/U; $P < 0.01$ comparing paired log I/U, where I is the inhibited rate of efflux and U is the uninhibited rate). With 60 ng/ml gramicidin the inhibition by DIDS increased from
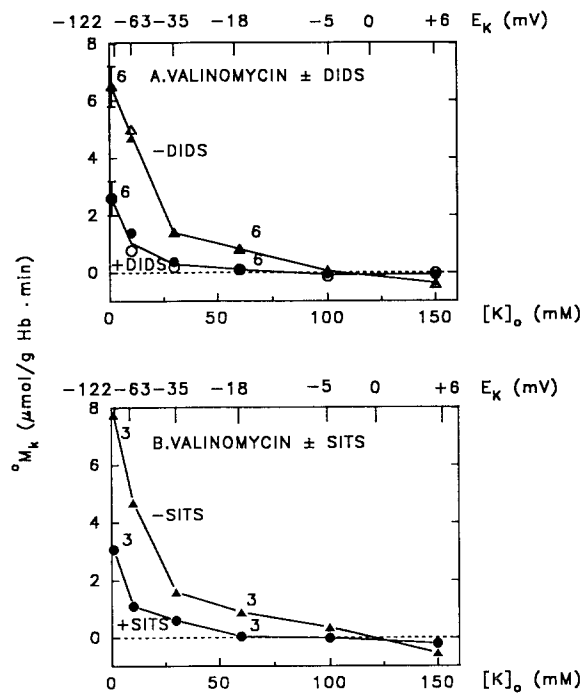

FIGURE 6. Effect of (A) DIDS and (B) SITS on valinomycin-induced net K efflux at varied $[K]_o$. ^oM_K was estimated from initial slopes (see Fig. 2). (A) DIDS. Points at 10, 30, 100, and 150 mM $[K]_o$ are from two experiments (*filled and open symbols*) with (*circles*) and without (*triangles*) pretreatment with 10 μM DIDS for 30 min; points with error bars at 1 and 60 mM $[K]_o$ are means $\pm$ SD for six experiments (at 60 mM $[K]_o$ the error bars are less than the diameter of the symbols). (B) SITS. Fluxes were determined with and without pretreatment with 100 μM SITS for 30 min in one or three experiments as indicated. E_K was calculated as described in Table II and is shown on the upper abscissa of each panel.

67% at 1 mM $[K]_o$ to 87% at 60 mM $[K]_o$ ($P < 0.025$ comparing paired I/U; $P < 0.05$ comparing paired log I/U).

As the concentration of DIDS was increased, the extent of inhibition increased to a maximal extent at 1 μM inhibitor; between 10 and 100 μM [DIDS], the inhibition at 1 mM $[K]_o$ remained partial and averaged 65% in two experiments (Fig. 7, *circles*). In the same experiments at 60 mM $[K]_o$, inhibition by DIDS was virtually complete at 1–100 μM inhibitor (Fig. 7, *triangles*). In another experiment, the temperature at which the cells were pretreated and then incubated with 0–100 μM DIDS was increased to 37°C; under this condition, the degree of inhibition at 1 mM $[K]_o$ also remained partial at 65% (not shown). In two other experiments, treating the cells

with 10 μM DIDS for 1 h at 37°C at pH 9.5 instead of pH 7.4, and then measuring ^0M_K and $^0M_{Cl}$ as usual, resulted in inhibition averaging 54% (not shown). When SITS was varied from 100 μM to 10 mM, the inhibition at 1 mM $[K]_o$ remained constant at 50% (not shown). These results all imply that the partial inhibition by DIDS or SITS of net efflux of K and Cl at 1 mM $[K]_o$ is maximal.

A control experiment tested whether or not the inhibition by DIDS is affected by the cell shrinkage that occurs after adding 1 μM valinomycin (see Fig. 1 C). In the absence of valinomycin, addition of 50 mM sucrose shrunk the cells by an extent comparable to that which occurred during the first 10 min after addition of valinomycin at 1 mM $[K]_o$. Inclusion of 50 mM sucrose in the flux media with valinomycin had no effect on the pattern of apparent inhibition by DIDS at either 1 mM $[K]_o$ or at 60 mM $[K]_o$ (not shown). The only effect noted with 50 mM sucrose was a slight stimulation of valinomycin-induced net efflux of K due to increased $[K]_c$, as expected.

TABLE II

Percent Inhibition of ^0M_K by 10 μM DIDS at 1 and 60 mM $[K]_0$

Ionophore	$[K]_0$	E_K	DIDS	^0M_K	$^0M_{Cl}$	Inhibition of ^0M_K
	mM	*mV*		*μM/g Hb/min*		*%*
Valinomycin	1	−122	−	6.5 ± 0.7	5.3 ± 0.9	59 ± 11
			+	2.6 ± 0.6	2.5 ± 0.7	
	60	−18	−	0.8 ± 0.2	0.5 ± 0.2	87 ± 8
			+	0.1 ± 0.04	0.1 ± 0.05	
Gramicidin	1	−122	−	14.5 ± 1.1	11.6 ± 1.3	67 ± 2
			+	4.8 ± 0.1	3.2 ± 0.6	
	60	−18	+	0.8 ± 0.2	0.7 ± 0.1	87 ± 10
			+	0.1 ± 0.1	0.1 ± 0.08	

^0M_K and $^0M_{Cl}$ were determined using 1 μM valinomycin and 0.06 μg/ml gramicidin. Values above are the means ± SD for 6 or 4, respectively, separate experiments, performed as described in the legends to Fig. 2 for valinomycin, or in Fig. 4 B for gramicidin, and in Materials and Methods. E_K was calculated from $-(RT/F)\ln([K]_c/[K]_0)$ where $[K]_c$ was measured to be 287 ± 19 μM/g Hb (SD, $n = 6$), water content was taken as 2.395 g/g Hb (see Table I), and $RT/F = 25.5$ mV at 23°C. The increased percent inhibition with increased $[K]_0$ is significant for valinomycin ($P < 0.01$) and for gramicidin ($P < 0.05$).

Current-Voltage Analysis of DIDS-insensitive Cl Transport

To determine the dependence on the driving force (E_m–E_{Cl}, as distinct from E_K) of DIDS-insensitive net Cl efflux, the fluxes induced by valinomycin or gramicidin were determined from the changes in mean cell volume in the presence of 10 μM DIDS at varied $[K]_o$. In parallel unbuffered suspensions of the same cells, E_m was measured from the change in external pH that occurs upon addition of the proton ionophore FCCP (Macey, Adorante, and Orme, 1976; Freedman and Novak, 1983; Bifano, Novak, and Freedman, 1984). The resultant current-voltage curves at 1 μM valinomycin or 60 ng/ml gramicidin (Fig. 8), as well as at 3 μM valinomycin (not shown),

are consistent with DIDS-insensitive Cl conductance increasing markedly when E_m-E_{Cl} is below ~ -30 mV.

At 1 mM $[K]_o$ with 10 μM DIDS, E_m differed from E_K by 19 and 26 mV in two experiments with 1 μM valinomycin, by 13 mV in another experiment with 3 μM valinomycin, and by 20 and 22 mV, respectively, in two other experiments with 60 ng/ml gramicidin. An increase in Cl conductance at hyperpolarizing voltages would contribute to the disparity between E_m and E_K. Thus, for quantitative current-voltage analysis of human red blood cells treated with potassium ionophores, E_m approaches E_K, but cannot be assumed to be equal to the potassium equilibrium potential, even in the presence of DIDS (cf Kracke and Dunham, 1987; Fröhlich and King, 1988; Kaji, 1993). The difference between E_m and E_K is expected to be even larger in the absence of DIDS.

Proton and Sodium Fluxes

In two experiments with DIDS-treated cells at 1 mM $[K]_o$, the net gain of Na during 10 min after addition of 1 μM valinomycin was <0.1 μM/g Hb/min, either in the
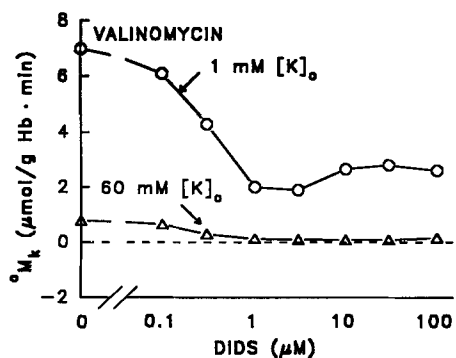

FIGURE 7. Effect of varied [DIDS] on valinomycin-induced net K efflux at 1 and 60 mM $[K]_o$. Cells were washed and then suspended in 1 mM KCl, 149 mM NaCl, 5 mM HEPES (*circles*), or 60 mM KCl, 90 mM NaCl, 5 mM HEPES (*triangles*), both at pH 7.4 at 23°C. DIDS (0–100 μM) was added, and the suspensions were incubated for 30 min, followed by determination of ^oM_K as described for Fig. 6. Data are averages from experiments with two donors.

presence or absence of 50 μM ouabain. In the same experiments, Na flux was <0.3 μM/g Hb/min at 1 and 60 mM $[K]_o$ in the presence and absence of 0.5 mM vanadate, which inhibits the red cell Na and Ca pumps. A gain of Na comparable to the DIDS-insensitive efflux of K and Cl could easily have been detected. The absence of significant Na fluxes argues against the possibility that nonspecific leakage pathways are present under the experimental conditions employed.

At 1 μM valinomycin and 1 mM $[K]_o$, ^oM_K exceeded $^oM_{Cl}$ by 1.3 ± 0.2 μM/g Hb/min (SD, n = 6), and by a comparable extent with 60 ng/ml gramicidin (Table II). In addition to Cl/HCO$_3$ exchange and Cl conductance, capnophorin has also been postulated to mediate OH conductance (Knauf et al., 1977), electroneutral DIDS-sensitive H:Cl cotransport, or, indistinguishably, Cl/OH exchange (Jennings, 1978; Critz and Crandall, 1980; Bisognano et al., 1993). The decreased $[Cl]_c$ after addition of valinomycin (Fig. 1A) results in an increased inward Cl concentration gradient, which could serve as a driving force for proton entry via H:Cl cotransport or Cl/OH exchange. Alternatively, membrane hyperpolarization could drive conductive OH efflux or proton influx. To attempt to account for the small yet significant

disparity between the measured net effluxes of K and Cl (Table II), apparent proton fluxes were determined by a pH-stat method after addition of 1 μM valinomycin at varied $[K]_o$ (Fig. 9) in the presence (squares) and absence (circles) of DIDS. Above 60 mM $[K]_o$, the DIDS-insensitive proton fluxes were ≤ 0.01 μM/g Hb/min; below 30 mM $[K]_o$, corresponding to an E_K of -35 mV, these fluxes increased to 0.1–0.2 μMol/g Hb/min.
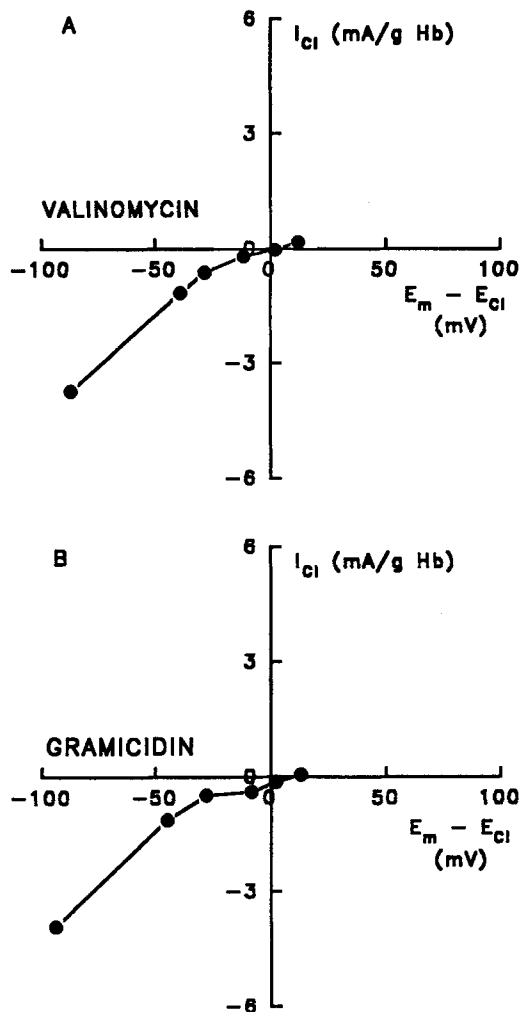

FIGURE 8. Current-voltage plot of DIDS-insensitive fraction of net chloride efflux induced by (A) 1 μM valinomycin, or (B) 60 ng/ml gramicidin, at varied $[K]_o$. Cells were washed and suspended at 1.2% HCT in media containing x mM KCl and $(150 - x)$ mM NaCl for valinomycin, or $(150 - x)$ mM choline chloride for gramicidin, where x = 1, 10, 30, 60, 100, and 150, and then incubated with 10 μM DIDS for 30 min. Net effluxes of chloride, $^oM_{Cl}$, were determined from the time courses of decrease of MCV as described in Fig. 4. The current, i_{Cl}, was computed from $i_{Cl} = -F^oM_{Cl}$, where F is the Faraday constant ($9.65\cdot10^4$ coul/mol). $E_{Cl} = 58.7\log r_{Cl}$, where the chloride ratio, $r_{Cl} = [Cl]_i/[Cl]_o$ was determined with ^{36}Cl (see Methods). E_{Cl} was -10 mV. The membrane potential, E_m was determined at varied K_o in parallel suspensions from the change in pH that occurred when 1 μM FCCP was added 1 min after addition of valinomycin. The pH was recorded for ~ 5 min until a new steady value was attained. The change in membrane potential was computed as $\Delta E_m = -58.7\Delta pH$, where ΔpH is the steady pH reached after FCCP, minus the initial pH before addition of DIDS. The results shown are each representative of two identical experiments.

At 1 mM $[K]_o$ the magnitude of the DIDS-insensitive apparent proton flux (Fig. 9, squares) is less than 10% of the DIDS-insensitive Cl flux (Fig. 6A, Table II). The apparent proton fluxes thus account for most of the small disparity between the measured K and Cl fluxes (Table II), and also substantiate the assumption concerning the approximate equivalency of the K and Cl fluxes that is made when estimating the fluxes by means of changes in mean cell volume (see Appendix, Eq. A5). The

apparent proton fluxes are also sufficiently small as to have a negligible effect on the curvature of the current-voltage plots (Fig. 8).

In a control experiment (not shown) with 10 μM DIDS, net K efflux, as determined from the rate of decrease of mean cell volume, was 2.3 ± 0.8 μMol/g Hb/min (SD, n = 3) in the presence of 5 μM ethoxzolamide, and was 2.4 ± 0.5 μMol/g Hb/min (SD, n = 3) in the absence of the inhibitor. The lack of effect of the permeant carbonic anhydrase inhibitor ethoxzolamide implies that the DIDS-insensitive K and Cl effluxes are independent of any cyclic flux of protons that might be coupled to an exchange of K for H mediated by valinomycin. Such a process would be expected to contribute negligibly to the net efflux of K in bicarbonate-free suspensions.

DISCUSSION

The results in this paper extend characterization of the ionophore-induced DIDS-insensitive fraction of conductive Cl transport in human red blood cells at varied [K]$_o$, corresponding to varied extents of membrane hyperpolarization (Figs. 6 and 8, Table II). The pattern of partial inhibition at 1–10 mM [K]$_o$, with DIDS-insensitive fluxes
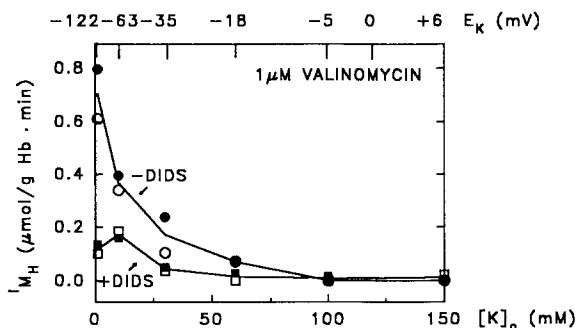

FIGURE 9. Effect of 10 μM DIDS on valinomycin-induced net proton influx (or hydroxide efflux) at varied [K]$_o$. Proton influxes, ^iM_H, were determined by a pH-stat method (see Materials and Methods) for suspensions equilibrated with air. Data are from two donors (*filled and open symbols*).

being ≤0.1 μM/g Hb/min at higher [K]$_o$, is independent of the specific inhibitor employed (DIDS or SITS), and of the ionophore used to induce the fluxes (valinomycin or gramicidin). The inhibition at 1 mM [K]$_o$ was maximal, and remained partial with increased [DIDS] (Fig. 7), [SITS], temperature, and the pH during treatment with DIDS, and was also unaffected by cell shrinkage. The degree of inhibition by DIDS reached 87% at 60 mM [K]$_o$ (Table II) and appeared virtually complete at higher [K]$_o$. The stimulation of ionophore-induced DIDS-insensitive net efflux of K and Cl at low [K]$_o$, corresponding to hyperpolarizing voltages, is reversible (Fig. 4 *C*), and is consistent with a direct effect of voltage on Cl conductance (Fig. 8), although specific effects of varied extracellular K, while unlikely, cannot strictly be ruled out by the present results.

With resealed ghosts at low [K]$_o$, partial inhibition of net Cl efflux by external 2 mM DAS (4,4'-diacetamidostilbene-2,2'-disulfonic acid), and by either internal or external 10 mM APMB (2-(4'-aminophenyl)-6-methylbenzene-thiazole-3',7-disulfonic acid), was reported by Kaplan, Scorah, Fasold, and Passow (1976). With intact red cells, partial inhibition by DIDS at low [K]$_o$ was described by Knauf et al. (1977) and

by Kaplan et al. (1983) who suggested that the human red blood cell membrane contains two Cl conducting pathways: one DIDS-sensitive, and the other DIDS-insensitive. In contrast, Bennekou and Stampe (1988) more recently concluded that DIDS inhibits the Cl conductance of human red blood cells almost entirely, and inferred that only one major transport pathway exists for net anion flow. In their study, however, the fluxes were followed for only 90 s; during this short time interval the changes in $[K]_c$ are too small for accurate measurement by their techniques, and the DIDS-insensitive net efflux of Cl that we have observed and characterized would not have been detected.

The results in Fig. 6 demonstrate that the DIDS-insensitive flux increases at $[K]_o$ corresponding to values of E_K below -35 mV; at $[K]_o$ corresponding to values of E_K between -35 and 0 mV, the DIDS-insensitive net effluxes of K and Cl are ≤ 0.1 $\mu Mol/g$ Hb/min. The results in Fig. 8 are consistent with DIDS-insensitive Cl conductance being dependent on voltage, with an increase when the driving force, E_m-E_{Cl}, is below ~ -30 mV. This conclusion is qualified by the unlikely possibility that varied $[K]_o$ might specifically affect red cell Cl conductance. In extensive previous studies of fluxes mediated by capnophorin no K-specific sites have been identified (Cabantchik et al., 1978; Knauf, 1979; Fröhlich and Gunn, 1986; Passow, 1986). DIDS-sensitive cation fluxes have, however, been found under nonphysiological conditions at low ionic strength (Jones and Knauf, 1985).

In our study, care was taken to sample the suspensions at times that ensured that the initial rates of net efflux of K and Cl were linear within the limits imposed by centrifuging the cells using the cold-quench method (Figs. 2 and 3), and within the 1% experimental uncertainty afforded by use of the Technicon H-1 Hematology Analyzer to measure the rate of decrease of mean cell volume (Fig. 4). These rates would decrease at longer times as the ion gradients dissipate to new levels. Any slight underestimation of the net effluxes in this study would only make the stimulating effect of low $[K]_o$ (Fig. 6), or of hyperpolarizing voltage (Fig. 8), even greater than estimated.

Our results raise the question of whether the DIDS-insensitive fluxes increase at decreased $[K]_o$ due to the increased gradient of K, or respond purely to the effect of the increased driving force (E_m-E_{Cl}), as suggested in Fig. 8. Dependence of Cl conductance on the gradient of K seems unlikely in that this explanation would likewise require a specific K binding site on capnophorin, or a mediated cotransport capability. The valinomycin-induced, DIDS-insensitive, net efflux of K and Cl seen at 1 mM $[K]_o$ (Fig. 6A) is more than an order of magnitude greater than the KCl cotransport fluxes found in mature human red blood cells over a range of pH from 6 to 8 (Brugnara and Tosteson, 1987), and some 50 times greater than the furosemide-sensitive K/Na/Cl cotransport fluxes (Brugnara, Canessa, Cusi, and Tosteson, 1986). Moreover, the similar curvature in the current-voltage plots obtained with gramicidin and valinomycin (Fig. 8) argues against attributing the apparent dependence on voltage to a specific interaction of K with valinomycin.

One way of testing for a specific effect of $[K]_o$ would be to measure the net fluxes in cells in which $[K]_o$ is varied by using PCMBS (p-chloromercuribenzenesulfonate) or nystatin. Even with this approach it would be impractical to set a ratio of 125 for internal to external [K] with 60 mM $[K]_o$, and thus to determine if partial inhibition

would be seen with cells hyperpolarized at high $[K]_o$. It would seem desirable in further studies to measure E_m in a series of experiments with varied $[K]_o$ at altered $[K]_c$.

The most likely explanation for the results is suggested by plotting the net Cl efflux, ${}^oM_{Cl}$, vs the driving force, (E_m-E_{Cl}), in accordance with the relation ${}^oM_{Cl} = g_{Cl}(E_m-E_{Cl})/F$. Our experimental current-voltage curves for DIDS-insensitive Cl conductance (Fig. 8) are consistent with a marked increase in Cl conductance, g_{Cl}, at a value of E_m-E_{Cl} around -30 mV, when the flux is induced either with 1–3 μM valinomycin or 60 ng/ml gramicidin. The flux turns on when E_m itself is around -40 mV. In the framework of this interpretation, the results do allow some evaluation of whether hyperpolarizing voltages increase the DIDS-insensitive Cl conductance itself, or, alternatively, decrease the potency of DIDS in inhibiting Cl conductance. It would seem unlikely that the results could be explained by a hypothetical mechanism in which hyperpolarizing voltages reduce the affinity of DIDS because our experimental design included pretreatment of the cells with the irreversible, covalent inhibitor before addition of the ionophore, and then measurement of the fluxes in the continued presence of the inhibitor. A mechanism involving a voltage-dependent affinity of DIDS would predict results that contradict the finding that raising the concentration of DIDS by two orders of magnitude from 1–100 μM failed to increase the extent of inhibition at 1 mM $[K]_o$ (Fig. 7).

Anion exchange is unaffected when E_m is altered with valinomycin (Gunn and Fröhlich, 1979; Wieth et al., 1980), or with gramicidin (Fröhlich et al., 1983). At low $[Cl]_o$, the fraction of valinomycin-induced net efflux of Cl that was inhibited by DNDS was interpreted to be dependent on voltage, while the DNDS-insensitive fraction was reported to be independent of voltage (Fröhlich et al., 1983). In related studies (Freedman et al., 1988), DIDS-insensitive Cl conductance appears also to depend on voltage in the presence of the fluorescent potentiometric indicator WW781. The present experiments verify that the same pattern of inhibition by DIDS is evident in the absence of WW781 as was previously found in its presence. The nonlinear current-voltage curve for DIDS-insensitive Cl transport is inconsistent with simple electrodiffusion. Further experiments will be necessary in order to clarify the reason for the different results obtained with DNDS and DIDS, and to understand in more depth the mechanism of the apparent voltage dependence of DIDS-insensitive net Cl transport.

The finding that DIDS inhibits net efflux of Cl by as much as 87% at low membrane potentials (Table II) further implicates capnophorin as the mediator of net Cl fluxes. The past practice of computing the apparent percent inhibition at a particular $[K]_o$ is somewhat misleading because the fluxes in the presence and absence of DIDS have different driving forces, further underscoring the desirability of measuring E_m. Because the percentage of DIDS that binds specifically to band 3 protein was estimated to be 90–95% (Cabantchik and Rothstein, 1974; Ship, Shami, Breuer, and Rothstein, 1977), or as low as 70% (Lepke, Fasold, Pring, and Passow, 1976), some possibility remains open that another protein also reacts with DIDS and contributes to Cl conductance.

In previous studies of nystatin-treated human red blood cells, a nonideal thermo-dynamic model, and a computer program denoted IONIC, described equilibrium

water contents at varied tonicity and pH with an accuracy of 2.4% (Freedman and Hoffman, 1979a), and also predicted E_{Cl} to within 3 mV (Freedman and Hoffman, 1979b). Nonideal osmotic behavior of valinomycin-treated red cells is indicated by the hypertonic effluent (Table I, see also Freeman et al., 1987), contrasting with the simplifying assumption of an ideal isotonic effluent made in past analyses (Knauf et al., 1977). An integrated red cell model using an equilibrium calculation similar to that of IONIC, along with a set of kinetic equations, has also been developed (Lew and Bookchin, 1986). Among the simulated experiments discussed with this model was the time course of water and electrolyte shifts after an increase in P_K, such as occurs with valinomycin. Subsequently, the model was reported to describe the alkaline hypertonic effluent observed after adding valinomycin to red cells that had half of their intracellular chloride replaced with SCN anions, a condition that hastens ionic re-equilibration by short-circuiting Cl conductance (Freeman et al., 1987). We have rewritten the integrated model in Turbo-Pascal for the IBM-AT and PS/2 microcomputers and have successfully predicted the time courses of electrolyte concentration and water shifts shown in Fig. 1. The model includes the nonideal osmotic coefficient of Hb, and was modified to include nonideal osmotic coefficients of the salts (0.93), and the temperature dependencies of the dissociation constant of HEPES buffer (-0.014 $\Delta pK_a/°C$, Good et al., 1976), and of the isoelectric point of Hb (Freedman and Hoffman, 1979a). The model was first checked by reproducing the derived reference state parameters, and the predicted time course of water and electrolyte contents for the simulated experiments with valinomycin (Lew and Bookchin, 1986, their Table 2 and their Fig. 5). To fit the time courses of $[K]_c$, $[Cl]_c$, $[Na]_c$, and water content (Fig. 1), values for P_{Cl} and for the ratio $P_{K\text{-}VAL}/P_{Cl}$ were adjusted (the turnover rate constant for Na:K:2A cotransport, or k_{CO}, was set at 10^{-6} h^{-1}). For any values of $P_{K\text{-}VAL}/P_{Cl} \geq 20$, the model fit the data equally well. At $P_{K\text{-}VAL}/P_{Cl} = 20$, the derived best-fit value of $P_{Cl} = 1.2$ h^{-1} is comparable with estimates by others (Knauf et al., 1977; Hunter, 1971, 1977). The root mean square difference between experimental and predicted values was 4 mM for K_c, 5 mM for Cl_c, 1.5 mM for Na_c, and 0.02 g H_2O/g cells for cell water content. It is also noteworthy that the model predicts linear net fluxes for the first 10 min after adding valinomycin at all K_o between 1 and 150 mM, in accordance with our results (Figs. 2–4). A good fit of the time course does not necessarily imply that the adjusted parameters are precisely determined, or that the model is complete in all respects. For example, in further theoretical work, the dependence of DIDS-insensitive Cl conductance on voltage suggested in Fig. 8 should be incorporated into the integrated model for refined predictions.

A dramatic but irreversible and nonspecific increase in ouabain- and furosemide-insensitive Na and K influxes has been reported to occur at an E_K of $\sim$ +10 mV (Kracke and Dunham, 1987; Halperin et al., 1989, 1990). Human red blood cells are not known to be depolarized under physiological conditions; nor do they contain depolarizing Na channels. In contrast, membrane hyperpolarization due to Ca-induced K channels does occur with elevated $[Ca]_c$. Such changes in E_m were monitored continuously and characterized with the fluorescent potentiometric indicator WW781 (Freedman and Novak, 1983). Although the physiological and pathophysiological significance of red cell Cl conductance and its regulation are not well

understood, any increase in Cl conductance at hyperpolarizing voltages would increase the rate of loss of KCl, and consequently increase the rate of cell shrinkage after hyperpolarization with elevated $[Ca]_c$. Such an effect could occur during the formation of dehydrated, irreversibly sickled cells, or in other pathophysiological conditions involving membrane disorders.

APPENDIX

To compute the net efflux of KCl from the measured rate of change of red cell volume, the effect of an increasing osmotic coefficient of hemoglobin during cell shrinkage should be taken into account. The nonideal expression for osmotic equilibrium is as follows:

$$[\varphi_s(K_c + Cl_c) + \varphi_{Hb}Hb]/V_w = \Phi_o C_o, \tag{A1}$$

where K_c, Cl_c, and Hb are intracellular solute contents (μM/cell), V_w is the cell water content (ml/cell), C_o (mmolal) is the total concentration of extracellular salts, φ_{Hb} and φ_s are the osmotic coefficients of hemoglobin and of the salts, and Φ_o is the osmotic coefficient of the extracellular solution (Freedman and Hoffman, 1979, Eq. A7). Multiplying by V_w and differentiating with respect to time gives

$$\varphi_s d(K_c + Cl_c)/dt + Hbd\varphi_{Hb}/dt = \Phi_o C_o dV_w/dt. \tag{A2}$$

The osmotic coefficient of Hb is given by $\varphi_{Hb} = a + bHb/V_w + cHb^2/V_w^2$ (Freedman and Hoffman, 1979, Eq. A6). Differentiating φ_{Hb} with respect to time, then substituting into Eq. A2, and rearranging, yields

$$d(K_c + Cl_c)/dt = [\Phi_o C_o + bHb^2/V_w^2 + 2cHb^3/V_w^3)/\varphi_s]dV_w/dt. \tag{A3}$$

If ideal osmotic behavior is assumed, then $\Phi_o = \varphi_s = a = 1$, and $b = c = 0$, and Eq. A3 reduces to the expression used by Knauf et al. (1977). Relaxing these assumptions by taking $a = 1$, $b = 0.0645$, $c = 0.0258$, and $\Phi_o = \varphi_s = 0.93$ (Freedman and Hoffman, 1979), and evaluating Eq. A3 at time zero where $Hb/V_w = 7.3$ mmolal gives for the initial flux,

$$d(K_c + Cl_c)/dt = [(C_o + 25.3)]dV_w/dt. \tag{A4}$$

The measured K and Cl effluxes are equivalent to within 10% (Table II); the small disparity is mostly accounted for by an apparent proton influx (Fig. 9). Approximating that $d(K_c)/dt = d(Cl_c)/dt$, the expression for the net efflux, ^oM_K (μM/g Hb/min) is given by

$$^oM_K = [(C_o + 25.3)/2MCH]dV_w/dt \tag{A5}$$

where the mean corpuscular hemoglobin, MCH, is 32 (10^{-12}) g/cell. Applying Eq. A5 to the present experimental conditions where $C_o = 305$ mM, and converting to V'_w (= $10^{12}V_w$) given in fl/cell we obtain the desired expression permitting estimation of the initial net K efflux from the measured rate of change of cell volume,

$$^oM_k = 5.16dV'_w/dt. \tag{A6}$$

REFERENCES

Bennekou, P. 1984. K$^+$-Valinomycin and chloride conductance of the human red cell membrane. Influence of the membrane protonophore carbonylcyanide m-chlorophenylhydrazone. *Biochimica et Biophysica Acta.* 776:1–9.

Bennekou, P., and P. Christophersen. 1986. Flux ratio of valinomycin-mediated K$^+$ fluxes across the human red cell membrane in the presence of the protonophore CCCP. *Journal of Membrane Biology.* 93:221–227.

Bennekou, P., and P. Stampe. 1988. The effect of ATP, intracellular calcium and the anion exchange inhibitor DIDS on conductive anion fluxes across the human red cell membrane. *Biochimica et Biophysica Acta.* 942:179–185.

Bifano, E. M., T. S. Novak, and J. C. Freedman. 1984. The relationship between the shape and the membrane potential of human red blood cells. *Journal of Membrane Biology.* 82:1–13.

Bisognano, J. D., J. A. Dix, P. R. Pratap, T. S. Novak, and J. C. Freedman. 1993. Proton (or hydroxide) fluxes and the biphasic osmotic response of human red blood cells. *Journal of General Physiology.* 102:99–103.

Brugnara, C., and D. C. Tosteson. 1987. Cell volume, K transport, and cell density in human erythrocytes. *American Journal of Physiology.* 252(Cell Physiology 21):C269–C276.

Brugnara, C., M. Canessa, D. Cusi, and D. C. Tosteson. 1986. Furosemide-sensitive Na and K fluxes in human red cells. Net uphill Na extrusion and equilibrium properties. *Journal of General Physiology.* 87:91–112.

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

Cabantchik, Z. I., P. A. Knauf, and A. Rothstein. 1978. The anion transport system of the red blood cell. The role of membrane protein evaluated by the use of "probes'. *Biochimica et Biophysica Acta.* 515:239–302.

Chappell, J. B., and A. R. Crofts. 1966. Ion transport and reversible volume changes of isolated mitochondria. *In* Regulation of Metabolic Processes in Mitochondria. J. M. Tager, S. Papa, E. Quagliariello, and E. C. Slater, editors. Elsevier, Amsterdam. 293–314.

Critz, A. E. and E. D. Crandall. 1980. pH Equilibration in human erythrocyte suspensions. *Journal of Membrane Biology.* 54:81–88.

Freedman, J. C., and J. F. Hoffman. 1979a. Ionic and osmotic equilibria of human red blood cells treated with nystatin. *Journal of General Physiology.* 74:157–185.

Freedman, J. C., and J. F. Hoffman. 1979b. The relation between dicarbocyanine dye fluorescence and the membrane potential of human red blood cells set at varying Donnan equilibria. *Journal of General Physiology.* 174:187–212.

Freedman, J. C., and T. S. Novak. 1983. Membrane potentials associated with Ca-induced K conductance in human red blood cells. Studies with a fluorescent oxonol dye, WW781. *Journal of Membrane Biology.* 72:59–74.

Freedman, J. C., and T. S. Novak. 1984. K and Cl conductance of valinomycin treated human red blood cells, as determined with the fluorescent potentiometric indicator WW781. *Journal of General Physiology.* 184:18a. (Abstr.)

Freedman, J. C., and T. S. Novak. 1987. Chloride conductance of human red blood cells at varied E_K. *Biophysical Journal.* 51:565a. (Abstr.)

Freedman, J. C., E. M. Bifano, L. M. Crespo, P. R. Pratap, R. Wallenga, R. E. Bailey, S. Zuk, and T. S. Novak. 1988. Membrane potential and the cytotoxic Ca cascade of human red blood cells. *In* Cell Physiology of Blood. R. B. Gunn and J. C. Parker, editors. Society of General Physiology Series, Rockefeller University Press, New York. 218–231.

Freeman, C. J., R. M. Bookchin, O. E. Ortiz, and V. L. Lew. 1987. K-permeabilized human red cells lose an alkaline, hypertonic fluid containing excess K over diffusible anions. *Journal of Membrane Biology.* 196:235–241.

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

Fröhlich, O., and R. B. Gunn. 1986. Erythrocyte anion transport: the kinetics of a single-site obligatory exchange system. *Biochimica et Biophysica Acta.* 864:169–194.

Fröhlich, O., and P. A. King. 1988. Mechanisms of anion net transport in the human erythrocyte. *In* Cell Physiology of Blood. R. B. Gunn and J. C. Parker, editors. Society of General Physiology Series, Rockefeller University Press, New York. 181–192.

Fröhlich, O., C. Leibson, and R. B. Gunn. 1983. Chloride net efflux from intact erythrocytes under slippage conditions. Evidence for a positive charge on the anion binding/transport site. *Journal of General Physiology.* 81:127–152.

Gasbjerg, P. K., J. Funder, and J. Brahm. 1993. Kinetics of residual chloride transport in human red blood cells after maximum covalent 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid binding. *Journal of General Physiology.* 101:715–732.

Good, N. E., G. D. Winget, W. Winter, T. N. Connolly, S. Izawa, and R. M. M. Singh. 1966. Hydrogen ion buffers for biological research. *Biochemistry.* 5:467–477.

Gunn, R. B., and O. Fröhlich. 1979. Asymmetry in the mechanism for anion exchange in human red blood cell membranes. Evidence for reciprocating sites that react with one transported anion at a time. *Journal of General Physiology.* 74:351–374.

Gunn, R. B., M. Dalmark, D. C. Tosteson, and J. O. Wieth. 1973. Characteristics of chloride transport in human red blood cells. *Journal of General Physiology.* 61:185–206.

Halperin, J. A., C. Brugnara, M. T. Tosteson, T. Van Ha, and D. C. Tosteson. 1989. Voltage-activated cation transport in human erythrocytes. *American Journal of Physiology. (Cell Physiology,* 26).257: C986–C996.

Halperin, J. A., C. Brugnara, T. Van Ha, and D. C. Tosteson. 1990. Voltage-activated cation permeability in high-potassium but not low potassium red blood cells. *American Journal of Physiology. (Cell Physiology* 27). 258:C1169–1172.

Harris, E. J., and B. C. Pressman. 1967. Obligate cation exchanges in red cells. *Nature.* 216:918–920.

Hoffman, J. F., and P. C. Laris. 1974. Determination of membrane potential in human and amphiuma red blood cells by means of a fluorescent probe. *Journal of Physiology.* 239:519–552.

Hoffman, J. F., J. H. Kaplan, T. J. Callahan, and J. C. Freedman. 1980. Electrical resistance of the red cell membrane and the relation between net anion transport and the anion exchange mechanism. *Annals of the New York Academy of Sciences.* 341:357–360.

Hunter, M. J. 1971. A quantitative estimate of the non-exchange-restricted chloride permeability of the human red cell. *Journal of Physiology.* 218:49–50P. (Abstr.)

Hunter, M. J. 1974. The use of lipid bilayers as cell membrane models: an experimental test using the ionophore, valinomycin. *In* Drugs and Transport Process. B. A. Callingham, editor. Macmillan, London. 227–240.

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

Jennings, M. L. 1978. Characteristics of CO_2-independent pH equilibration in human red blood cells. *Journal of Membrane Biology.* 40:365–391.

Jennings, M. L. 1982. Stoichiometry of a half-turnover of band 3, the chloride transport protein of human erythrocytes. *Journal of General Physiology.* 79:169–185.

Jennings, M. L. 1992. Inorganic anion transport. *In* The Structure of Biological Membranes. P. Yeagle, editor. CRC Press, Boca Raton, FL. 781–832.

Jones, G. S., and P. A. Knauf. 1985. Mechanism of the increase in cation permeability of human erythrocytes in low-chloride media. *Journal of General Physiology.* 86:721–738.

Kaji, D. M. 1993. Effect of membrane potential on K-Cl transport in human erythrocytes. *American Journal of Physiology.* 264: (*Cell Physiology,* 33):C376–C382.

Kaplan, J. H., K. Scorah, H. Fasold, and H. Passow. 1976. Sidedness of the inhibitory action of disulfonic acids on chloride equilibrium exchange and net transport across the human erythrocyte membrane. *FEBS Letters.* 62:182–185.

Kaplan, J. H., M. Pring, and H. Passow. 1983. Band-3 protein mediated anion conductance of the red cell membrane. Slippage vs ionic diffusion. *FEBS Letters.* 156:175–179.

Knauf, P. A. 1979. Erythrocyte anion exchange and the band 3 protein: transport kinetics and molecular structure. *Current Topics in Membranes and Transport.* 12:249–363.

Knauf, P. A., G. F. Fuhrmann, S. Rothstein, and A. Rothstein. 1977. The relationship between anion exchange and net anion flow across the human red blood cell membrane. *Journal of General Physiology.* 69:363–386.

Knauf, P. A., F-Y. Law, and P. J. Marchant. 1983. Relationship of net chloride flow across the human erythrocyte membrane to the anion exchange mechanism. *Journal of General Physiology.* 81:95–126.

Kracke, G., and P. Dunham. 1987. Effect of membrane potential on furosemide-inhibitable sodium influxes in human red blood cells. *Journal of Membrane Biology.* 98:117–124.

Lepke, S., H. Fasold, M. Pring, and H. Passow. 1976. A study of the relationship between inhibition of anion exchange and binding to the red blood cell membrane of 4,4'-diisothiocyano stilbene-2,2'-disulfonic acid (DIDS) and its dihydro derivative (H2 DIDS). *Journal of Membrane Biology.* 29:147– 177.

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

Macey, R. I., J. S. Adorante, and F. W. Orme. 1978. Erythrocyte membrane potentials determined by hydrogen ion distribution. *Biochimica et Biophysica Acta.* 512:284–295.

Mohandas, N., Y. R. Kim, D. H. Tycko, J. Orlik, J. Wyatt, and W. Groner. 1986. Accurate and independent measurement of volume and hemoglobin concentration of individual red cells by laser light scattering. *Blood.* 68:506–513.

Passow, H. 1986. Molecular aspects of band 3 protein-mediated anion transport across the red blood cell membrane. *Reviews in Physiology, Biochemistry and Pharmacology.* 103:61–203.

Pressman, B. C., and M. J. Heeb. 1972. Permeability studies on erythrocyte ghosts with ionophorous antibiotics. *In* Molecular mechanisms of antibiotic action on protein biosynthesis and membranes. E. Muñoz, F. García-Ferrandiz, D. Vazquez, editors. Elsevier, Amsterdam. 603–614.

Scarpa, A., A. Cecchetto, and G. F. Azzone. 1968. Permeability of erythrocytes to anions and the regulation of cell volume. *Nature.* 219:529–531.

Scarpa, A., A. Cecchetto, and G. F. Azzone. 1970. The mechanisms of anion translocation and pH equilibration in erythrocytes. *Biochimica et Biophysica Acta.* 219:179–188.

Ship, S., Y. Shami, W. Breuer, and A. Rothstein. 1977. Synthesis of tritiated 4,4'-diisothiocyano-2,2'-stilbene disulfonic acid ([3H]DIDS) and its covalent reaction with sites related to anion transport in human red blood cells. *Journal of Membrane Biology.* 33:311–324.

Tosteson, D. C., R. B. Gunn, and J. O. Wieth. 1973. Chloride and hydroxyl ion conductance of sheep red cell membrane. *In* Erythrocytes, Thrombocytes, Leukocytes. E. Gerlach, K. Moser, E. Deutsch, W. Wilmans, editors. G. Thieme Publishers, Stuttgart. 62–66.

Tycko, D. H., M. H. Metz, E. A. Epstein, and A. Grinbaum. 1985. Flow cytometric light scattering measurement of red cell volume and hemoglobin concentration. *Journal of Applied Optics.* 24:1355–1365.

Wieth, J. O., J. Brahm, and J. Funder. 1980. Transport and interactions of anions and protons in the red blood cell membrane. *Annals of the New York Academy of Sciences.* 341:394–418.

Wilkins, B., Jr, J. E. Frandolig, and C. L. Fischer. 1970. An interpretation of red cell volume distributions measured by pulse height analysis. *Journal of the Association for the Advancement of Medical Instrumentation.* 4:99–105.