Mechanism of the Increase in Cation Permeability of Human Erythrocytes in Low-Chloride Media

Involvement of the Anion Transport Protein Capnophorin

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In Memory of Dr. Jens Otto Wieth

ABSTRACT When human erythrocytes are suspended in low-Cl^- media (with sucrose replacing Cl^-), there is a large increase in both the net efflux and permeability of K^+. A substantial portion (>70% with $Cl^- < 12.5$ mM) of this K^+ efflux is inhibited by the anion exchange inhibitor DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonic acid). This inhibition cannot be explained as an effect of DIDS on net Cl^- permeability (P_{Cl}) and membrane potential, but rather represents a direct effect on the K^+ permeability. When cells are reacted with DIDS for different times, the inhibition of K^+ efflux parallels that of Cl^- exchange, which strongly indicates that the band 3 anion exchange protein (capnophorin) mediates the net K^+ flux. Since a noncompetitive inhibitor of anion exchange, niflumic acid, has no effect on net K^+ efflux, the net K^+ flow does not seem to involve the band 3 conformational change that mediates anion exchange. The data suggest that in low-Cl^- media, the anion selectivity of capnophorin decreases so that it can act as a very low-conductivity channel for cations. Na^+ and Rb^+, as well as K^+, can utilize this pathway.

INTRODUCTION

The human red cell is remarkable because, although its membrane poses a formidable barrier to the passage of cations, anions exchange between intra- and extracellular compartments quite rapidly. Mond (1927) proposed that this selectivity was due to electrostatic effects of fixed cationic charges lining water-filled channels. Since he also noted a decrease in sulfate influx and an increase in

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Volume 86 November 1985 721–738

potassium efflux as the extracellular pH was raised, he concluded that these fixed charges must be dissociable groups. He proposed that as the pH increases in these channels, hydrogen ions are liberated, and finally, at very high pH, the charge barrier is so diminished or reversed as to allow the passage of cations.

Mond (1927), Passow (1964), Wilbrandt and Schatzmann (1960), La Celle and Rothstein (1966), Donlon and Rothstein (1969), and Morel (1973) found that the rates of cation efflux increased at low external anion concentrations. This was not due solely to an increase in the driving force for cation efflux because of the change in membrane potential, but also represented a true increase in the apparent cation permeability. For an aqueous pore lined with positive fixed charges, there will be a Donnan distribution of OH^- and Cl^- ions between the pore and the medium, such that $OH_p/OH_m = Cl_p/Cl_m$, where the subscripts p and m refer to pore and medium, respectively. Thus, when Cl_m is reduced at constant OH_m, OH_p will increase, raising the pH within the pore, titrating the fixed charges, and increasing cation permeability. The effects of external Cl^- on $SO_4^=$ and K^+ fluxes are consistent with this model (Wilbrandt and Schatzmann, 1960; Passow, 1969; Donlon and Rothstein, 1969), according to which the increase in cation permeability in low-Cl^- media results from a breakdown in the selectivity of a fixed-charge pore that normally transports anions.

Over the past several years, however, evidence has steadily accumulated against the concept that a fixed-charge pore is the rate-limiting barrier for anion transport (Passow, 1969; Hunter, 1971, 1977; Knauf, 1979; Passow et al., 1980; Macara and Cantley, 1983). Instead, anions cross the membrane by combining with a specific transport site in the band 3 protein (Cabantchik and Rothstein, 1974; Lepke et al., 1976), which then undergoes a conformational change to release the anion at the other side of the membrane (Gunn and Fröhlich, 1979; Jennings, 1982). The band 3 protein, also known as capnophorin (Wieth and Bjerrum, 1983), catalyzes a very tightly coupled one-for-one exchange of anions, such that net (conductive) flow of anions is only 1/10,000th the rate of anion exchange (Hunter, 1971, 1977; Knauf et al., 1977). The demonstration that the fixed-charge model is no longer valid for anions also eliminates this model as a likely explanation for the low-Cl_o–induced cation permeability increase.

On the surface, it would seem difficult to understand how a carrier-type transport mechanism could permit net flow of cations at low Cl_o. However, recent models of capnophorin suggest that it may form an aqueous channel through the membrane, which is blocked by a gating mechanism that imparts to the system its high anion/cation selectivity and one-for-one exchange properties (Knauf, 1979; Passow et al., 1980; Macara and Cantley, 1983). This model is supported by evidence that DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonate), a very selective inhibitor of anion exchange (Ship et al., 1977), also inhibits net Cl^- and $SO_4^=$ flow (Knauf et al., 1977), which suggests that capnophorin can mediate net anion flow as well as anion exchange. The kinetics of this flux show that it does not involve the same protein conformational change as anion exchange (Knauf et al., 1983*a*, *b*), but rather that anions simply diffuse at a low rate through an aqueous channel formed by capnophorin. The existence of this net anion diffusion through capnophorin suggests that a similar process might explain the cation flux increase at low external Cl^- concentrations.

The possible involvement of capnophorin is suggested by the fact that the anion transport inhibitor SITS (4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid) inhibits the K^+ and Na^+ permeability increase in low-Cl^- media (Knauf, 1970). Many mechanisms could be invoked to explain this inhibition, such as the possibility that SITS affects membrane potential by inhibiting the net chloride permeability, P_{Cl} (Hoffman and Knauf, 1973; Knauf et al., 1977). In the present study, therefore, we have attempted to further characterize the low-Cl_o–induced cation flux, and in particular to examine its possible relation to capnophorin. A brief report of some of these observations has previously appeared in abstract form (Jones et al., 1983).

METHODS

Cell Preparation

Fresh blood was drawn as needed by venipuncture into 10-ml heparinized evacuated glass tubes. Cells were washed at least three times in KCl or NaCl HEPES-buffered saline (150 mM KCl or NaCl, 2.5 mM HEPES, pH 7.6 at room temperature) and white cells were removed by aspiration. Cells were concentrated by centrifugation and diluted to a known hematocrit for treatment with nystatin or DIDS or for flux measurements.

Na^+ and/or K^+ Fluxes

Cells were added to prewarmed medium in a 25-ml Erlenmeyer flask in a 37°C water bath to give a hematocrit of 2–2.5%. Sucrose was substituted for NaCl to maintain tonicity when extracellular Cl^- was lowered. Tetramethylammonium (TMA^+) was used to replace Na^+ in the experiments using equal Na^+ and K^+. To avoid complications arising from the interdependence of OH^- and Cl^- gradients, in all experiments with the exception of that shown in Fig. 1, the pH of the medium was set so that these gradients were equal. The buffer was 2.5 mM HEPES, and, for experiments with 12.5 mM Cl^-, the pH was 6.5. All pH values are given at room temperature. At various times, 1-ml samples were removed and added to either 3 or 4 ml $MgCl_2$, with a tonicity approximately equal to that of the flux medium. The high external Cl^- in this medium effectively stops the cation efflux. Samples were centrifuged, and the Na^+ and/or K^+ concentration of the supernatant was determined by flame photometry (model 143, Instrumentation Laboratories, Boston, MA). Uncentrifuged samples were also taken to determine total Na^+ and/or K^+ and the hemoglobin content in the cell suspension.

In several pilot experiments, 10^{-4} M ouabain was added, but it had no measurable effect on the cation flux, probably both because the low-Cl_o–induced flux is so high and because the Na-K pump rate is so low in the low-K^+ media used for most experiments. Thereafter, ouabain was omitted from all nominally K^+-free experiments. External K^+ in such "K-free" media ranged from ~0.05 to ~0.12 mM.

Flux Calculations

The rate of loss of K^+ or Na^+ from cells was normalized by dividing by the amount of hemoglobin measured. Since the hemoglobin content is fairly constant, the effect is to normalize flux measurements for the number of red cells.

Cytoplasmic Anion Substitution

Cells were loaded with iodide by washing three times in HEPES-buffered saline in which Cl^- was replaced by I^-. Cells were allowed to stand at room temperature for 5–10 min between washes.

Net Cl^- Flux

The procedure for measuring net Cl^- efflux is essentially similar to that used by Knauf et al. (1983*a*). DIDS-treated or control cells were suspended at 0.1% hematocrit in flux medium containing 1.33 μM valinomycin and 1.33% ethanol. For sampling, ~10 ml of cell suspension was poured into a centrifuge tube to which 20 ml ice-cold $MgCl_2$ (~300 mM) or NaCl wash buffer (150 mM NaCl, 20 mM HEPES, pH 7.6 at room temperature) was added. Cells were washed twice with 20 ml ice-cold buffer and then lysed with ~2 ml deionized, distilled H_2O. Samples of lysate were taken for determination of K^+ by flame photometry and for determination of hemoglobin as cyanmethemoglobin using Drabkin's solution (Fisher Scientific Co., Pittsburgh, PA) and cyanmethemoglobin standard (Data Medical Associates, Arlington, TX) (Crosby et al., 1954).

Calculation of Net K^+ and Cl^- Permeabilities

Net KCl efflux was measured in 2.5 mM KCl, 10 mM NaCl, 290 mM sucrose, 2.5 mM HEPES, pH 6.5 at 37°C with 1.33 μM valinomycin present. The apparent chloride permeability (P_{Cl}) was calculated by using the Goldman-Hodgkin-Katz equation (Goldman 1943; Hodgkin and Katz, 1949) as described by Knauf et al. (1983*a*). Cell water and K^+ and Cl^- concentrations were determined as previously described (Knauf et al., 1983*a*), and Na^+ was measured by a flame photometric technique similar to that used for K^+.

For an electrodiffusive process with a constant voltage gradient through the membrane, the net K^+ flux is given by (Goldman, 1943; Hodgkin and Katz, 1949):

$$J_K = P_K \ln B(BK_i - K_o)/(B - 1), \tag{1}$$

where

$$B = (P_K K_o + P_{Na} Na_o + P_{Cl} Cl_i)/(P_K K_i + P_{Na} Na_i + P_{Cl} Cl_o), \tag{2}$$

and where K_i, Na_i, and Cl_i are the intracellular concentrations of K^+, Na^+, and Cl^-, respectively, while K_o, Na_o, and Cl_o are the corresponding extracellular concentrations of these ions. P_{Cl} was determined from measurements of net KCl flux with valinomycin as described in Knauf et al. (1983*a*). The potassium permeability (P_K) was calculated from K^+ efflux in the absence of valinomycin by an iterative technique in which Eq. 1 was solved for P_K. P_{Na} was assumed to be equal to P_K (see Table II); if P_{Na} were set equal to zero, this would not change the reported P_K values. Since driving forces are expressed in millimolar and fluxes in millimoles per kilogram hemoglobin per minute, the permeabilities are given in units of millimoles per kilogram hemoglobin per minute times millimolar (mmol/kg Hb·min·mM) (Knauf et al., 1983*a*).

DIDS Treatment

DIDS is a potent specific inhibitor of the red cell anion exchange system, which binds noncovalently at first but over time forms covalent bonds with band 3 (Lepke et al., 1976; Ship et al., 1977). Both the covalent and noncovalent binding greatly (>99%) inhibit anion exchange. For most experiments showing DIDS inhibition, 10 μM DIDS was in the medium during the flux. However, some experiments required pretreatment with DIDS to form covalent bonds. To accomplish this, red blood cells (25% hematocrit) were treated in the presence of 10 μM DIDS at 5°C in HEPES-buffered NaCl. Samples were withdrawn before the addition of DIDS and at several times during the course of treatment. Each sample was washed three times with ice-cold NaCl HEPES buffer containing 0.5% bovine serum albumin to complex free DIDS, and then washed two times to remove the albumin.

Another sample was removed and stored for at least 30 min at room temperature to allow complete reaction between DIDS and band 3. These cells in which >99% of Cl^- exchange has been eliminated were considered to represent 100% inhibition of DIDS-sensitive processes.

Cl^- Exchange

After DIDS treatment, cells to be used for Cl^- exchange measurements were washed once and suspended until use in a storage buffer (112 mM KCl, 38 mM NaCl, 27 mM sucrose, and 10 mM glucose) to keep internal ion concentrations from changing during storage at ~6°C. The following day, cells were incubated at 25% hematocrit with ^{36}Cl (1.87 μCi/ml) for 15 min at 37°C in Na HEPES wash buffer and then for 10 min on ice, and isotope exchange was measured according to the method of Knauf et al. (1978). Briefly, packed red cells were injected into a chamber containing stirring medium at 0°C. Extracellular $^{36}Cl^-$ was measured by drawing samples through filters. Infinity values were obtained by allowing a sample from each flux to remain at room temperature for at least 30 min, and then centrifuging and sampling the supernatant. The medium used in these Cl^- exchange experiments was 12 mM NaCl, 294 mM sucrose, 2.5 mM HEPES, pH 6.3 at room temperature.

Nystatin Treatment

Nystatin was prepared by dissolving 5 mg/ml in methanol (MeOH). After mixing for 20 min or longer, particulates were removed by repeated centrifugation until no pellet could be observed.

Cells were washed once in 75 mM NaCl, 75 mM KCl, 25 mM sucrose, 2.5 mM HEPES, pH 6.96 at room temperature, and then washed three times at 8% hematocrit in the same solution containing 60 μl of the nystatin-MeOH solution per milliliter of packed cells.

Cells were stored at least 10 min at 0°C between washes to allow ionic equilibration. To remove nystatin, cells were washed eight times at room temperature with 5 min between each wash. They were resuspended in the eighth wash and stored at 0°C overnight. To prepare for flux measurements, cells were washed three times in 150 mM TMA Cl, 25 mM sucrose, 2.5 mM HEPES, pH 6.9 at room temperature. The flux medium was 12.5 mM TMA Cl, 305 mM sucrose, 2.5 mM HEPES, pH 5.9 at room temperature.

Rb^+ Fluxes

Cells were treated with nystatin as described above, except that the medium contained 150 mM KCl and no NaCl. After washing, the cells were resuspended in this medium (without nystatin) and were loaded with ^{86}Rb, 2.6 μCi/ml, at 37°C for 3 h at 25% hematocrit. The cells were washed three times and then resuspended for flux measurement at 1% hematocrit, in 12.5 mM NaCl, 2.5 mM HEPES, 300 mM sucrose, pH 5.9 at room temperature. 0.8 ml supernatant was sampled and counted (LS-150, Beckman Instruments, Inc., Palo Alto, CA) with 8 ml scintillation fluid (Liquiscint, National Diagnostics, Somerville, NJ). For infinite-time samples, 1.0 ml flux suspension was added to 0.2 ml 30% trichloroacetic acid (TCA), vortexed, and centrifuged and then 0.8 ml of supernatant was added to 8 ml scintillation fluid for counting. All counts were corrected for background. Infinity counts were corrected for TCA quench. The flux was expressed as percent ^{86}Rb leaving the cell per time after counts were corrected for the extracellular ^{86}Rb at time zero.

RESULTS

Effects of DIDS on K^+ Flux in Low-Cl^- Media

In order to show that the SITS inhibition of cation permeability (Knauf, 1970) was not peculiar to that compound, DIDS was tested for its effect on K^+ efflux from cells in media with several different concentrations of external Cl^- (Cl_o). Fig. 1 shows that in untreated cells (circles), as Cl_o decreases, the efflux of K^+ increases. The basal K^+ efflux observed in high Cl_o was not affected by 10 μM DIDS (Fig. 1*B*), but DIDS greatly inhibited the enhanced K^+ efflux seen in low-Cl^- media (Fig. 1*A*). At low Cl_o, there was also a DIDS-insensitive flux that varied

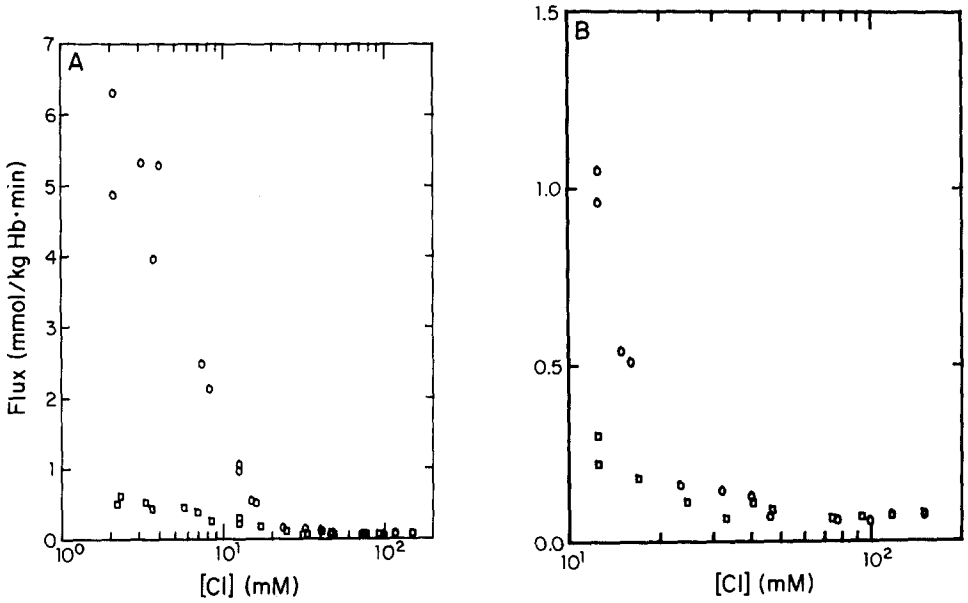

FIGURE 1. Effect of DIDS on K^+ efflux in low-Cl^- media. Net K^+ efflux was measured as described in the Methods. (○) Control cells; (□) plus 10 μM DIDS. Points represent individual flux measurements from separate experiments. (*A*) Entire range of data. Note that the DIDS-sensitive flux increases as external Cl^- decreases. (*B*) Flux with $Cl_o > 10$ mM. At high Cl^- concentrations (>40 mM), there is no significant effect of DIDS.

somewhat from day to day. These results are very similar to those obtained earlier with SITS (Knauf, 1970).

Since DIDS inhibits not only anion exchange (electroneutral) but also net (conductive) anion flux (Knauf et al., 1977, 1983*a*), and since in low-Cl^- media the K^+ permeability is greatly increased, in the presence of DIDS the membrane potential (E_m) might deviate from the Cl^- equilibrium potential in the direction of the K^+ equilibrium potential. This would make E_m less positive (inside with respect to outside) and would therefore reduce the driving force for K^+ efflux. A test of this explanation for the effect of DIDS is shown in Table I. Valinomycin,

a K^+ ionophore, can be used to increase the K^+ permeability (P_K) manyfold, thus leaving the Cl^- permeability (P_{Cl}) as the limiting factor for KCl efflux (Hunter, 1977; Knauf et al., 1977). P_{Cl} can then be calculated from the K^+ flux and the concentrations of K^+ and Cl^-. Table I shows P_{Cl} calculated for normal and DIDS-inhibited cells in low-Cl media. These P_{Cl} values were then used to calculate P_K from measurements of K^+ efflux in the absence of valinomycin.

Several conclusions are readily apparent from the values shown in Table I. The Cl^- permeability in control cells is much greater than the K^+ permeability (without valinomycin), so E_m is dominated by the Cl^- ratio. Even after DIDS treatment, ~16% of P_{Cl} remains, so E_m would still be largely controlled by the Cl^- ratio even if the K^+ permeability were unchanged (compare control P_K with DIDS P_{Cl}). The K^+ flux after DIDS treatment, however, is much lower than expected ("Expected J_K"), assuming that DIDS only affects K^+ efflux by changing E_m. This suggests that, in addition to its possible effects on E_m, DIDS has a direct inhibitory effect on P_K that accounts for most or all of the decrease in K^+ efflux.

TABLE I

K^+ and Cl^- Permeabilities in Low-Cl^- Media

	Control	10 μM DIDS	Percent inhibition
P_{Cl} (mmol/kg Hb·min·mM)	0.16622 (0.0072)	0.02734 (0.0005)	83.6
P_K (mmol/kg Hb·min·mM)	0.00355	0.00100	71.8
J_K (mmol/kg Hb·min)	0.989 (0.075)	0.267 (0.022)	73.0
Expected J_K	0.989	0.779	21.2
E_m (mV)	50.6	47.4	

Cells were prepared and fluxes were measured in media without (control) or with 10 μM DIDS as described in the Methods. P_{Cl} was calculated from net KCl efflux in 12.5 mM Cl^- medium with valinomycin present as described in the Methods. Numbers in parentheses are SEMs for four determinations. Net K^+ efflux (J_K) was measured without valinomycin in 12.5 mM NaCl, 295 mM sucrose, 2.5 mM HEPES, pH 6.5 ($n = 24$). The expected J_K is the K^+ flux expected assuming that DIDS changes P_{Cl} but not P_K. P_K is the value of K^+ permeability calculated from the observed values of J_K and P_{Cl}.

This conclusion can be tested by using a counterion for K^+ whose permeability is high and is unaffected by DIDS. I^- freely traverses the red cell membrane via the anion exchange system (Dalmark and Wieth, 1972; Wieth et al., 1973; Dalmark, 1976) and therefore cells can be loaded with I^- by repeated washings in Cl^--free I^- media. The net I^- permeability is much larger than P_{Cl} and seems primarily to represent diffusion across the membrane unrelated to the band 3 protein, since Knauf et al. (1983*a*) found that DIDS had no significant effect on net I^- (electrogenic) flux. Any DIDS effect on K^+ efflux in I^--loaded cells, therefore, cannot be due to a change in E_m resulting from inhibition of net anion flow, but must be due to DIDS directly affecting P_K. Fig. 2 shows that DIDS still strongly inhibits K^+ efflux in I^--loaded cells. The action of DIDS on K^+ efflux is therefore not due to inibition of net anion permeability, but is probably due to direct action on a K^+ permeability pathway.

Since DIDS is highly specific for band 3, with ~90% DIDS bound to capnophorin (Ship et al., 1977), these data strongly suggest that the K^+ permeability

increase is mediated by capnophorin. However, the possibility still remains that a few DIDS molecules could bind to a minor, not yet identified, protein of the red cell that controls P_K. Since the DIDS reaction characteristics of such a minor protein would probably be different from those of capnophorin, the existence of a minor P_K-controlling protein could be detected by using cells carefully pretreated with DIDS. Accordingly, suspensions of cells were treated with DIDS at 5°C for various time periods. At 5°C, the formation of covalent DIDS–band 3 bonds is slow enough (Lepke et al., 1976; Ship et al., 1977) that samples taken at different incubation times will have anion transport (measured as Cl^- exchange) inhibited to predictably different extents. Fig. 3 shows the percent inhibition of Cl^- exchange for different times of incubation at 5°C. K^+ efflux in

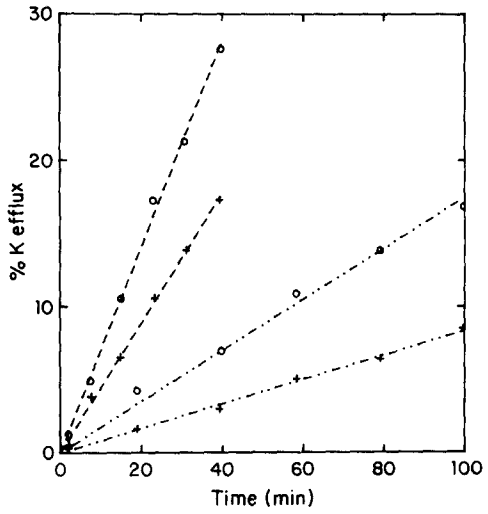

FIGURE 2. DIDS inhibition of net K^+ flux in Cl^-- and I^--loaded cells. Cells were washed three times in 5 mM HEPES, pH 7.4, containing either 150 mM NaI (○) or 150 mM NaCl (+) and were suspended in either 12.5 mM NaI (○) or 12.5 mM NaCl (+), with sucrose replacing I^- or Cl^-. In I^- media, the cells exhibited an even larger rate of K^+ efflux than in Cl^- (broken lines), and this flux, like that in Cl^-, was inhibited by 10 μM DIDS (dash-dotted lines). DIDS inhibition for this experiment was 76% for I^- and 84% for Cl^-.

12.5 mM Cl^- medium was also measured for samples of these cells. Fig. 4 is the graph obtained when the DIDS-sensitive fraction of K^+ efflux is plotted against Cl^- exchange in cells treated with DIDS for various times. The lower curve (dotted) shows the results expected if the only action of DIDS were inhibition of net Cl^- flux. The obvious lack of correspondence of the data points to this curve provides further proof that DIDS does not act simply by decreasing P_{Cl}, but rather exerts direct inhibition on a cation conductive pathway. The identity line (dashed) marks the results expected assuming that DIDS directly inhibits K^+ flux by reacting with capnophorin. The experimental results fall quite near this line, in agreement with the hypothesis that band 3 allows the passage of cations under these conditions.

Ion Selectivity

To further characterize this cation flux pathway, the permeability of other alkali metal ions was investigated. Knauf (1970) had observed SITS inhibition of both Na^+ and K^+ permeabilities and Donlon and Rothstein (1969) had reported that Na^+ and K^+ had similar exit rates (with a ratio ranging from 0.74 to 1.31). The strategy chosen here for comparing Na^+ and K^+ permeabilities was to simultaneously measure both Na^+ and K^+ fluxes from cells that had been treated with nystatin so that they contained equal concentrations of each ion. All solutions used for these experiments similarly contained equal Na^+ and K^+ concentrations.

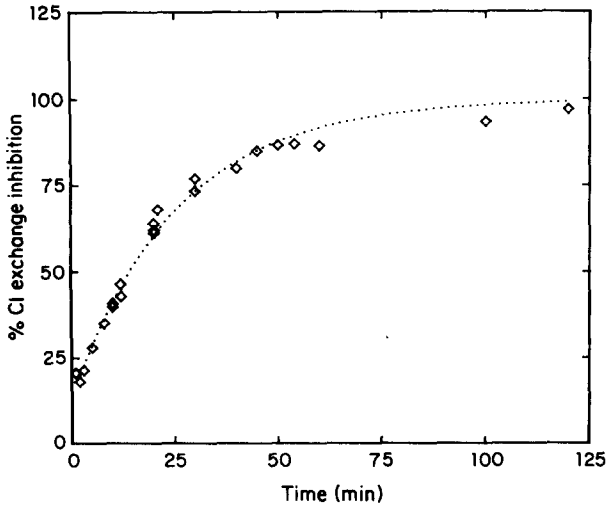

FIGURE 3. Effect of DIDS treatment time on inhibition of Cl^- exchange. Washed red cells were treated with 10 μM DIDS and Cl^- exchange was measured at 0°C in 12 mM NaCl, 294 mM sucrose, 2.5 mM HEPES, pH 6.3, after overnight storage as described in the Methods. Inhibition of Cl^- exchange was calculated by comparing the rate constant for Cl^- exchange with that of a cell sample taken before addition of DIDS. Inhibition was >99% for cells treated with DIDS for >25 min at 37°C. Data are from five separate experiments. The dotted line is calculated according to the equation: percent inhibition = $100\ [1 - e^{-k(t+t_o)}]$, where t is time of incubation at 5°C, and the rate constant, k, and the apparent DIDS wash-out time, t_o, were determined by nonlinear least-squares fit analysis. $k = 3.93 \times 10^{-2}$ min^{-1}. $t_o = 3.66$ min.

Since the driving forces for both ions were the same, any differences in flux should indicate permeability differences. Table II shows that the DIDS-sensitive fluxes for Na^+ and K^+ are almost identical. The free mobility of Na^+ in aqueous solutions, however, is only 65% of the mobility of K^+ (Robinson and Stokes, 1965). Even though both P_{Na} and P_K are greatly increased, therefore, the pathway through the membrane appears to be somewhat Na^+ selective.

Similar experiments were performed using ^{86}Rb to see whether the cation permeability increase is generalized to include this ion. Fig. 5 shows that, like Na^+ and K^+, Rb^+ efflux also increases in low Cl_o. The fraction of Rb^+ leaving

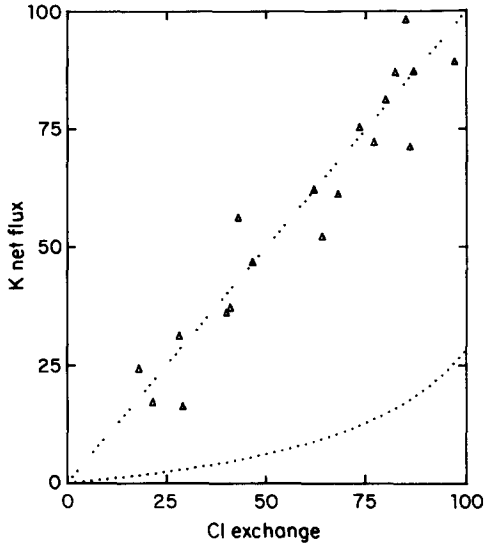

FIGURE 4. Comparison of DIDS inhibition of net K^+ flux and of Cl^- exchange in low-Cl^- medium. Cells were treated with 10 μM DIDS at 5°C for different lengths of time and then net K^+ efflux at 37°C or ^{36}Cl exchange at 0°C was measured in 12 mM Cl^- media as described in the Methods. The inhibition of the DIDS-sensitive portion of net K^+ flux is plotted against the inhibition of Cl^- exchange. The DIDS-sensitive portion of net K^+ efflux was calculated from the difference between the control flux before DIDS treatment and the flux remaining after treatment with 10 μM DIDS for >25 min at 37°C, which inhibited chloride exchange by >99%. The diagonal line of identity represents the result expected if reaction of DIDS with band 3 inhibits both Cl^- exchange and net K^+ efflux in parallel. The curve near the bottom is the result expected if DIDS has no direct effect on P_K, but only acts by changing P_{Cl} and E_m.

TABLE II

Comparison of Na^+ and K^+ Fluxes in Low-Cl^- Media

Ion*	Total flux‡	DIDS-insensitive flux§	DIDS-sensitive flux‖	*P* DIDS vs. Control¶
Na^+	1.59 (0.27)	0.46 (0.06)	1.13 (0.22)	<0.0001
K^+	1.47 (0.26)	0.36 (0.05)	1.11 (0.22)	<0.0001
Na/K	1.11	1.27	1.02	<0.01
P (Na vs. K)**	<0.01	<0.0001	>0.5	

* Cells were loaded with 75 mM each of NaCl and KCl by using nystatin. Values are means of 14 determinations, with SEMs indicated in parentheses.

‡ Fluxes are expressed in millimoles per kilogram hemoglobin per minute.

§ Flux in the presence of 10 μM DIDS.

‖ Total flux − DIDS-insensitive flux.

¶ *P* values were calculated using a paired *t* test pairing DIDS and control fluxes, which were measured on the same day.

** *P* values were calculated using a paired *t* test comparing the Na and K effluxes measured simultaneously from the same samples.

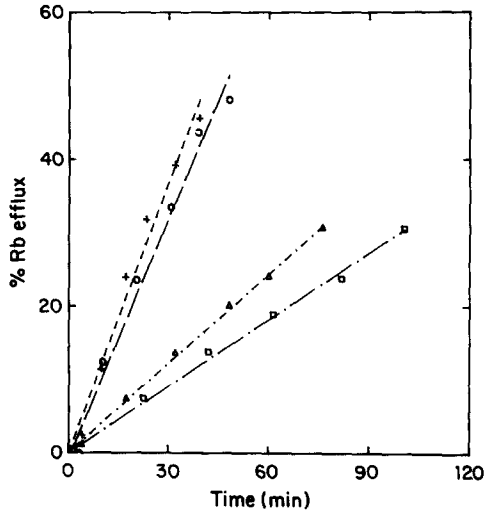

FIGURE 5. Cells were loaded with ^{86}Rb and efflux was measured in 12.5 mM NaCl medium without (+, ○) or with (△, □) 10 μM DIDS as described in the Methods. The amount of ^{86}Rb that had exited from the cell was expressed as a percent of the total ^{86}Rb initially present in the cell, after correction for extracellular ^{86}Rb present at time zero. Two experiments are shown. Fluxes for cells suspended in 150 mM NaCl medium were typically ~10 times lower.

the cell per unit time (0.5–1.2%/min) is in the range of that observed for K^+ or Na^+ under the same conditions in nystatin-treated cells. DIDS also inhibits the low-Cl_o–induced Rb^+ flux, which implies that Rb^+ uses the same pathway as Na^+ and K^+. Therefore, low-Cl_o modification of band 3 induces a cation permeability increase that is generalized to include at least these three ions.

Relation to Anion Exchange Mechanism

Anion exchange requires a conformational change of the anion transport site in the band 3 protein that is not necessary for net anion flow (Knauf et al., 1983*b*). To see whether or not this conformational change is required for net cation flow in low-Cl^- media, niflumic acid was used. Niflumic acid is a noncompetitive

TABLE III

Effect of Niflumic Acid on K^+ Efflux in Low-Cl^- Medium

	Control	50 μM niflumic acid
Mean	1.01	1.09
SEM	0.04	0.03
N	8	7

Net K^+ efflux into 12.5 mM Cl^- medium with or without 50 μM niflumic acid was measured at 37°C as described in the Methods. Values represent results of experiments on two different days, each normalized by dividing by the median flux rate for that day. The *P* value calculated from an unpaired *t* test is >0.1, which indicates no significant difference between the flux with and without niflumic acid. At 37°C, sulfate exchange is 50% inhibited by ≈12 μM niflumic acid.

inhibitor of anion exchange (Cousin and Motais, 1979; Knauf and Mann, 1984), which binds to band 3 preferentially when the transport site faces outward and which prevents the conformational change that mediates anion exchange (Knauf and Mann, 1984). Table III shows that there is no inhibitory effect of niflumic acid on K^+ efflux into 12.5 mM Cl^- media. The anion exchange–related conformational change, therefore, does not appear to be required for the band 3–mediated cation permeability increase.

DISCUSSION

Relationship of the Cation Permeability Increase to Capnophorin

Although DIDS and SITS have been used to inhibit K^+ fluxes from valinomycin-treated cells (Knauf et al., 1977), metabolically depleted cells treated with Ca^{2+} (Hoffman and Knauf, 1973), and vanadate-treated cells (Fuhrmann et al., 1984), in these cases K^+ permeability was increased so that it approached or exceeded Cl^- permeability, altering the membrane potential and making the Cl^- flux rate-determining. Several pieces of evidence argue against this as a possible mechanism for the DIDS inhibition of net K^+ efflux in low-Cl^- media. First, from Table I and Fig. 4, it is clear that the decrease in K^+ driving force resulting from the change in P_{Cl} and hence in membrane potential is insufficient to account for the observed decrease in K^+ flux caused by DIDS. Second, Knauf (1970) observed that both K^+ efflux and Na^+ influx were inhibited by SITS. A change in membrane potential would be expected to have opposite effects on efflux and influx. Finally, and perhaps most significantly, DIDS inhibits K^+ efflux even when I^- is used to replace Cl^- (Fig. 2), conditions under which DIDS should have no effect on E_m.

Another possible trivial explanation for DIDS inhibition of cation flux could be that inhibition of Cl^-/OH^- (or more correctly, Cl^-/HCO_3^-) exchange affects internal pH. This secondary effect must also be ruled out because the internal OH^- concentration (OH_i) will equilibrate according to the equation $OH_i = Cl_iOH_o/Cl_o$. Since for all experiments (Fig. 1 excepted) the external OH^- concentration (OH_o) was lowered to keep the ratio OH_o/Cl_o constant, there should be no change in the internal pH regardless of Cl^-/OH^- inhibition by DIDS. In the case of the experiments shown in Fig. 1, the internal pH should not rise above ~9.3–9.4 even at the lowest Cl_o. La Celle and Rothstein (1966) have shown that the apparent P_K in low-Cl^- media is not affected by increasing the internal pH in the range of 8–9.3. Thus, it is unlikely that changes in the internal pH could explain any part of the effect of DIDS on cation permeability.

On the other hand, there is a very strong correlation between the effects of DIDS on net K^+ efflux in low-Cl^- media and its effects on anion exchange (Fig. 4). This strongly argues that the DIDS effect involves a reaction with the same capnophorin sites that are involved in the inhibition of anion exchange. These results cannot completely rule out the possibility that a protein other than band 3 has very similar DIDS reaction characteristics or that the DIDS–band 3 combination induces secondary changes in some other structure that permits the passage of cations in low-Cl^- media. Nevertheless, the simplest hypothesis compatible with the data would seem to be that in low-Cl^- media capnophorin can

act as a channel to permit net flow of K^+, as well as Na^+ (Table II) and Rb^+ (Fig. 5). Although the cation permeability with low Cl_o is very large in comparison with the normal, very low cation permeability of the red cell (Fig. 1), it would not involve a very large flow of cations through band 3. If all of the band 3 molecules are involved in the flux, one can calculate from the observed flux of 3 mmol/kg Hb·min in 5 mM Cl^- medium that the turnover number would be ~1 K^+/band 3·s. This is several orders of magnitude lower than the turnover number for anion exchange, ~50,000 ions/s (Brahm, 1977) and is even lower than the turnover number for net anion flow, ~5 ions/s. Thus, even under these circumstances, band 3 is a rather inefficient channel for cations, and one that could probably not be observed by electrophysiological techniques.

Mechanism of the Cation Permeability Increase

If the DIDS-sensitive flux component does involve the flow of cations through the band 3 protein, this would imply that some change of structure in the protein has altered its normally very high selectivity for anions over cations. The mechanism of this change in selectivity might be very interesting, not only in terms of band 3 function, but also as an example of general mechanisms whereby specific transport systems may serve other functions under unusual experimental or pathological circumstances, thereby greatly altering the properties of the membrane.

The increased permeability does not appear to be due to a Donnan effect from lower extracellular Cl^-. Assuming Donnan equilibrium, OH^- and Cl^- ions should be distributed between the extracellular medium (m) and any other compartment (x) such that $OH_m/Cl_m = OH_x/Cl_x$. So long as OH_m/Cl_m is held constant, as in most of the experiments reported here, the pH should not change within any hypothetical pore. Thus, the experiments reported here argue against the original hypothesis (Mond, 1927; Passow, 1969) that the effect of low Cl_o is primarily due to titration of fixed charges within the membrane. Other factors must be responsible for the cation permeability increase.

Several possible factors might be involved in the low-Cl^-–induced change in anion/cation selectivity. The decrease in external Cl^- will affect the relative amount of capnophorin with the transport site in the outward-facing form (E_o) (Dalmark, 1975; Knauf, 1979; Furuya et al., 1984). This might be significant, since in the case of net anion flow, it seems that the outward-facing forms of capnophorin (E_o and the corresponding chloride-loaded form, ECl_o) have a higher net anion permeability than do the forms (E_i and ECl_i) with the transport site facing inward (Knauf et al., 1983*a*, *b*; Fröhlich, 1983, 1984). Under the circumstances of the experiment in Table III, however, niflumic acid should increase the fraction of E_o and ECl_o by ~2.8-fold, while decreasing the fraction of E_i and ECl_i by nearly half (calculated assuming symmetric Cl^- dissociation constants at the two sides of the membrane, as discussed in Knauf and Mann, 1984). Since niflumic acid has no significant effect on cation flux under these circumstances, it seems unlikely that the cation permeabilities of E_o and E_i are markedly different and thus unlikely that the shift between these forms is of any importance in eliciting the increase in cation permeability.

A second factor that might be important is the change in external ionic

strength. This could alter the structure of capnophorin, and indeed there is evidence that in low ionic strength media, trypsin can cleave a site of capnophorin at the external surface that is not affected at higher salt concentrations (Jenkins and Tanner, 1977). Cotterrell and Whittam (1971) have observed a similar increase in cation permeability, even when nonpenetrating anions such as EDTA and citrate were used to replace Cl^- in the medium, which suggests that bulk ionic strength is not the sole variable involved in the flux increase. It would seem possible, however, that what is important is the ionic strength within some limited region of capnophorin, which may not be accessible to large anions such as citrate or EDTA. For example, Wieth and Bjerrum (1982) and Wieth et al. (1983) have found effects of chloride on the titration of a guanidino group, which they attribute to local changes in ionic strength in a region that is inaccessible to citrate.

Finally, La Celle and Rothstein (1966) and Donlon and Rothstein (1969) postulated that membrane potential is a critical element in provoking the permeability change. If one considers the permeability barrier of capnophorin for ions to equal the membrane thickness (~45 Å), with a 10-fold anion gradient, the field strength would be >100,000 V/cm. If the permeability barrier is thinner, the field strength would be correspondingly greater. It would not seem improbable that such a high electric field could alter the structure of capnophorin by reorienting dipoles within the protein structure. At present, it is not clear which of these factors (ionic strength or membrane potential) or others are most important for the permeability change. Experiments to investigate this question, however, are in progress.

Comparison with Other Cation Leak Mechanisms

The low-Cl_o–induced cation leak contrasts with other cation leaks inducible in the red cell. The Gardos effect involves Ca^{2+} and specifically increases K^+ and Rb^+ permeabilty (Gardos, 1956; Lew, 1974; Romero, 1976). In contrast, the low-Cl_o leak can be observed in the presence of the Ca^{2+} chelator EDTA (Cotterrell and Whittam, 1971) and allows the passage of Na^+, as well as K^+ and Rb^+.

It has been suggested that the increase in cation flux caused by treatment of red cells with *p*-chloromercuriphenylsulfonic acid (PCMBS) may involve capnophorin on the basis that this permeability increase can be observed in vesicles that are highly enriched in band 3 (Grinstein and Rothstein, 1978; Solomon et al., 1983). Even if it does involve capnophorin, however, this permeability change seems to be different from that caused by low Cl_o. Although PCMBS uptake is greatly inhibited by SITS (Knauf and Rothstein, 1971) or DIDS (Rothstein et al., 1974), the effect of PCMBS on cation permeability is, if anything, enhanced by SITS and DIDS. This contrasts sharply with the inhibition by these agents of the low-Cl_o–induced cation permeability increase, seen in this and a preceding (Knauf, 1970) study.

Funder et al. (1978) reported an increased Li^+ flux when HCO_3^- replaced Cl^- in the extracellular medium. The Li^+ flux was also inhibited by DIDS and appeared to be band 3–mediated. This phenomenon, however, can be distin-

guished from the Na^+, K^+, Rb^+ leak with low Cl_o by a number of criteria. The Li^+ flux involves an $LiCO_3^-$ complex that then crosses the membrane by means of the band 3 anion exchange mechanism. The bicarbonate concentrations required for Li^+ efflux are quite high in contrast with the experiments described in this paper, where no bicarbonate at all was added to cells or media. Na^+ is less likely to form these complexes and K^+ is even less so. The similar flux rates for Na^+ and K^+ (Table II) thus indicate that Na^+ and K^+ are not transported as carbonate complexes. Finally, the lack of any inhibition by the anion exchange inhibitor niflumic acid is further evidence that the low-Cl_o–induced cation flux is not via formation of an anionic complex. In their original paper, Funder et al. (1978) described the $LiCO_3^-$ flux mechanism as a case of cations being smuggled through the membrane "in an anion disguise." The data presented here indicate that in low-Cl^- media, there is another mechanism that permits cations to traverse band 3 without a change of wardrobe.

The assistance of Nancy A. Mann, Jean E. Kalwas, and Matthew C. Severski is gratefully acknowledged.

This work was supported by National Institutes of Health grant AM27495 and by Individual National Research Service Award HL06678 to G.J. This paper is based on work performed under Contract No. DE-AC02-76EV03490 with the U.S. Department of Energy at the University of Rochester Department of Radiation Biology and Biophysics and has been assigned Report No. UR-3490-2451.

Original version received 26 November 1984 and accepted version received 27 June 1985.

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