Chloride Net Efflux from Intact Erythrocytes under Slippage Conditions

Evidence for a Positive Charge on the Anion Binding/Transport Site

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ABSTRACT   Tracer chloride and potassium net efflux from valinomycin-treated human erythrocytes were measured into media of different chloride concentrations, Cl_o, at 25°C and pH 7.8. Net efflux was maximal [45–50 mmol (kg cell solids)$^{-1}$ min^{-1}] at $Cl_o = 0$. It decreased hyperbolically with increasing Cl_o to 14–16 mmol (kg cell solids)$^{-1}$ min^{-1}. Half-maximal inhibition occurred at $Cl_o = 3$ mM. In the presence of the anion exchange inhibitor DNDS, net efflux was reduced to 5 mmol (kg cell solids)$^{-1}$ min^{-1}, independent of Cl_o. Of the three phenomenological components of net efflux, the Cl_o-inhibitable (DNDS-inhibitable) component was tentatively attributed to "slippage," that is, net transport mediated by the occasional return of the empty transporter. The Cl_o-independent (DNDS-inhibitable) component was tentatively attributed to movement of chloride through the anion transporter without the usual conformational change of the transport site on the protein ("tunneling"). These concepts of slippage and tunneling are shown to be compatible with a model that describes the anion transporter as a specialized single-site, two-barrier channel that can undergo conformational changes between two states. Net chloride efflux when the slippage component dominated ($Cl_o = 0.7$ mM) was accelerated by a more negative (inside) membrane potential. It appears that the single anion binding-and-transport site on each transporter has one net positive charge and that is neutralized when a chloride ion is bound.

INTRODUCTION

Anion fluxes across the human red cell membrane can be divided into two phenomenologically different components: an electrically silent exchange of extracellular and intracellular anions and a net transport component (conductance pathway). The exchange pathway has been characterized in several

kinetic studies (e.g., Gunn et al., 1973; Dalmark, 1975, 1976; Brahm, 1977; Gunn and Fröhlich, 1979; for a comprehensive review see Knauf, 1979), but much less is known about the characteristics of anion net transport. Hunter (1971, 1977) demonstrated that the monovalent anion net transport is several orders of magnitude smaller than the tracer exchange transport. This is true even in the presence of valinomycin, which renders the red cell membrane highly permeable to potassium and eliminates most of the restricting effect of an anionic diffusion potential on net anion fluxes. Kaplan and Passow (1974) showed that extracellular phlorizin, which inhibits anion exchange, enhances net efflux of KCl from valinomycin-treated cells. Knauf et al. (1977) provided evidence that most of the anion net permeability arises from the same transport system as exchange transport. They showed for both chloride and sulfate fluxes a linear relationship between the inhibition of the anion exchange mechanism caused by pretreatment with DIDS (4,4'-diisothiocyanostilbene-2,2'-disulfonate) and the inhibition of the net anion transport mechanism. 100% inhibition of sulfate exchange corresponded to >90% inhibition of sulfate net flux, but 100% inhibition of chloride exchange corresponded to only 65% inhibition of chloride net flux. Therefore, ~35% of the chloride conductance appeared not to be due to transport on the anion transporter but may be attributed to additional permeability pathways.

One notion of how net transport is accomplished by a carrier-type transporter is that the unloaded transporter can change its accessibility from one side of the membrane to the other. The "return of the empty carrier" must be 10^3–10^4 times slower than the transport of the loaded transporter for the rapidly exchanged monovalent anions such as chloride.[1] It is this rare occurrence of the presumed conformational change of the unloaded transporter that has led to the use of the term "slippage" to describe this net flux component in the chloride transport across the red cell membrane (Vestergaard-Bogind and Lassen, 1974).

In this paper we test the consistency and applicability of this concept of slippage to the anion conductance across the human red cell membrane. These studies relate to the recent findings of Knauf and Law (1980) and of Kaplan et al. (1980) that at anion concentrations above 50 mM chloride net fluxes increased with increasing chloride concentrations in the presence of a constant driving force. This behavior may be expected when transport sites are infrequently occupied by chloride. But we have previously shown that the chloride transport is half-saturated at ~60 mM on the inside of the cell and at <4 mM on the outside of the cell (Gunn and Fröhlich, 1979). Consequently, the external transport site should be saturated when Cl_o is >50 mM and net chloride efflux by slippage should be negligible since almost no empty transport sites are available to mediate the empty return.

Our immediate purpose in these experiments was threefold. We wanted to determine the conditions under which net transport can be described by a slippage mechanism. We wanted to determine whether the anion conductance

[1] With the slowly exchanged divalent anions such as sulfate the conformational changes of the empty and loaded transporter are of the same order of magnitude (Knauf et al., 1977).

observed by Kaplan, Knauf, and co-workers constitutes primarily a conduct-
ance component independent of slippage. Finally, we also wanted to determine
the charge on the anion binding and transport site by measuring the electrical
potential dependence of net transport under conditions when slippage is the
dominant component.

MATERIALS AND METHODS

Preparation of Cells

We performed all experiments with intact cells from one donor (O.F.) prepared for
each experimental series from fresh whole blood taken into heparin-treated containers.
After removing the white cells and plasma by centrifugation and aspiration, we
washed the cells thoroughly with extensively N_2-bubbled media containing 150 mM
KCl, 27 mM glycylglycine, pH 7.8, at 0°C, to remove as much bicarbonate as
possible. For the chloride tracer flux experiments we preequilibrated the cells with
$^{36}Cl^-$ and then packed them in nylon tubes (Gunn and Fröhlich, 1979). For the
potassium flux experiments, we packed the cells without tracer and stored them on
ice until used (typically several hours).

Efflux Media and Reagents

We prepared the media into which the cells would be dispersed during the efflux
measurements from extensively N_2-bubbled stock solutions: type A, 150 mM KCl (or
NaCl), 27 mM glycylglycine, pH 7.8, at 25°C; and type B, 25 mM Na-citrate (or K-
citrate), 27 mM glycylglycine, 200 mM sucrose, pH 7.8, at 25°C. In one experiment
we also used solutions containing 75 mM Na- or K-citrate, 27 mM glycylglycine, pH
7.8, at 25°C as a stock. We mixed the stock solutions in the proper proportions to
obtain the efflux media of the desired chloride or potassium concentrations. Potassium
was substituted for by an equivalent concentration of sodium; chloride was substituted
iso-osmotically and iso-ionically by citrate/sucrose (Gunn and Fröhlich, 1979). ^{36}Cl as
aqueous NaCl was purchased from ICN (Irvine, CA) and DNDS (4,4'-dinitrostilbene-
2,2'-disulfontate) was purchased from ICN-K & K Rare Chemicals (Irvine, CA). We
stored the DNDS in the dark to ensure that it remained in the *trans*-isomeric form,
which is the inhibitory conformation (Fröhlich and Gunn, 1981). Valinomycin was
purchased from Sigma Chemical Co. (St. Louis, MO) and used from stock solutions
containing 5 mg/ml ethanol, and was added directly to the efflux medium just before
injection of the cells. The final ethanol concentration never exceeded 0.5% (vol/vol).
Gramicidin was a gift from Dr. H.-J. Apell, University of Konstanz, Federal Republic
of Germany.

Net Flux Experiments

The experiments presented here are net efflux measurements of either potassium or
tracer chloride. Except where noted differently, they were all performed at pH 7.8
and 25°C. The packed cells (0.2–0.4 ml) were injected into 20–25 ml well-stirred and
thermostatted influx medium containing the appropriate amounts of valinomycin
and DNDS. We took samples of the extracellular medium either by withdrawing into
syringes through Millipore filters (Gunn and Fröhlich, 1979) or by an inhibitor
stopping technique. In the latter technique we added a 2-ml sample of the efflux
mixture to a test tube containing 50 μl of a 20-mM-DNDS solution, mixed briefly by
vortexing and centrifuged in a desk-top centrifuge. The samples from the control flux
experiments in the presence of DNDS were centrifuged without additional DNDS.

We always measured efflux during the linear phase (initial rate). Consequently, the delay between the time of sample withdrawal and the separation of the cells in the inhibitor stopping technique compared with the more rapid filtration method did not alter the observed rate. Since we found that the filters and prefilters contained small amounts of potassium, we could employ the filtration technique only in the chloride tracer flux measurements and were obliged to use the inhibitor technique in the potassium flux experiments. The concentration of potassium in the efflux medium was determined by atomic absorption flame spectrophotometry (model 460; Perkin-Elmer, Norwalk, CT).

We measured tracer chloride net efflux from preloaded cells into media containing chloride of the same specific activity as the intracellular chloride, but of different concentrations. This was achieved by mixing the appropriate amount of tracer-containing supernatant of the packed cells with the appropriate amount of type B stock to give the desired chloride concentration. We measured radioactivity in a Packard Tricarb (Downers Grove, IL) liquid scintillation counter. We calculated the fluxes from the rate of appearance of potassium or tracer chloride in the extracellular phase and the amount of cells in the efflux suspension, determined by hemoglobino-metry.

Chloride Exchange Flux Experiments

For the chloride tracer exchange experiments we pretreated the washed intact cells with gramicidin in order to render them highly cation permeable. For this we incubated the cells at a hematocrit of 3% for 1 h at 4°C in 150 mM KCl, 27 mM glycylglycine, pH 7.8, plus gramicidin (80 μl of a 0.1% ethanolic stock solution per milliter of packed cells). We then collected the cells by centrifugation, preloaded them with tracer chloride, and packed them in nylon tubes in the same way as for the net flux experiments. The control cells were subjected to the same treatment except for the addition of the gramicidin stock solution.

The efflux media were prepared from stock solutions of 150 mM choline chloride plus 27 mM glycylglycine, and of 25 mM Na_3- or K_3-citrate plus 200 mM sucrose and 27 mM glycylglycine, all of pH 7.8 at 0°C. These solutions were mixed in the proper proportions to obtain media of the desired Cl concentration in which Cl was substituted for by citrate/sucrose and Na or K was kept constant at 75 mM.

We measured the chloride exchange flux as tracer efflux by the syringe filtration method and calculated the flux values from the rate constants of the appearance of tracer in the extracellular media and the cellular chloride content (Gunn and Fröhlich, 1979). In the calculations of the kinetic parameters $K_{1/2\text{-out}}$ and $V_{\text{max-out}}$, the half-saturation constant and the maximal value of the Cl_o-stimulated chloride exchange flux we modified the previously reported procedure. Previously we subtracted the small flux at $Cl_o = 0$ from the other flux values before fitting the kinetic parameters to the data with the nonlinear least-squares method of Wilkinson (1961). Since these background fluxes are largely inhibitable by DNDS (unpublished results), it appears likely that this residual flux is due to contaminations of bicarbonate or chloride in the system. We therefore added a small correcting equivalent concentration of chloride to the nominal value of Cl_o and then proceeded with the curve-fitting procedure. This is justified because chloride and bicarbonate exhibit similar transport kinetics (Lambert and Lowe, 1978; Gunn et al., 1980). This correction was only 50–150 μM.

Estimates of the K and Cl Permeabilities

For comparison purposes we estimated the membrane potential V_m and the K and Cl permeabilities P_K and P_{Cl} under our experimental conditions by using the Goldman

flux equation and the Goldman-Hodgkin-Katz equation in the same way as previously done by Hunter (1977) and Knauf et al. (1977):

$$M_{Cl} = P_{Cl}\,(\ln B)\frac{(Cl_i \cdot B - Cl_o)}{B - 1} \qquad (1)$$

with

$$V_m = (RT/F)\,\ln B \qquad (2)$$

and

$$B = \frac{P_K \cdot K_o + P_{Cl} \cdot Cl_i}{P_K \cdot K_i + P_{Cl} \cdot Cl_o}. \qquad (3)$$

In the conversion of the values of P_K and P_{Cl} between the units of min^{-1} and $cm\ s^{-1}$ we used the conversion factor previously used by Knauf et al. (1977): $P\ (cm\ s^{-1}) = 7.63 \times 10^{-7}\ P\ (min^{-1})$. In the estimates of P_K from the exchange flux of K for Rb (with valinomycin) and for Na (with gramicidin), we assumed that Rb and Na were as well transported as K by valinomycin and gramicidin, respectively. Since this is approximately the case (Läuger, 1972; Hladky and Haydon, 1972), the hetero-exchange permeabilities are close to the values measured if tracer-K equilibrium exchange fluxes had been measured.[2] Since these experiments were performed near equilibrium the value of the time constant of exchange was taken to be the value of the K permeability.

THEORY

A possible mechanism for net anion transport in human red cells is the return of the empty transport site after a normal half-cycle of anion exchange. There is now considerable evidence for a single-site, alternating access transporter with ping-pong kinetics in anion exchange (Gunn and Fröhlich, 1979, 1982; Grinstein et al., 1979; Knauf et al., 1980; Jennings, 1980, 1982). A scheme for this mechanism is presented in Fig. 1. It is kinetically identical to the mobile carrier scheme described previously by several investigators working on other transport systems (e.g., Geck, 1971; Regen and Morgan, 1974; Lieb and Stein, 1974). The analysis of the red cell anion transport system is greatly simplified over these systems because of the tight one-for-one coupling of anion influx. The very low rate of anion net transport compared with the rapid turnover during anion exchange implies that the return of the empty transporter is infrequent and of little importance in the steady state distribution of the several transporter configurations.

The rapid chloride exchange rate, given by the reaction steps in the exchange loop connecting C_i, CX_i, CX_o, C_o, CY_o, CY_i, and C_i, in order, implies that each of these reactions has (at saturating substrate concentrations) a

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[2] In artificial bilayer membranes, the valinomycin-induced Rb conductance is roughly 10 times the K conductance (Läuger, 1972), so K-Rb exchange measurements would overestimate the P_K. However, since in K-Rb exchange the half-cycle of K movement is rate limiting, the rate of K-Rb exchange is probably closer to the rate of K-K exchange than to the rate of Rb-Rb exchange. In view of the other approximations and assumptions involved in the derivation of the relative permeabilities of K and Cl (see footnote 5), the K-Rb and K-Na exchange data are adequate to estimate P_K.

turnover rate of at least $400 \ \text{s}^{-1}$ at 0°C and nearly $10^5 \ \text{s}^{-1}$ at 37°C (Brahm, 1977; Gunn and Fröhlich, 1979). By contrast, any net chloride efflux mediated by the reaction loop C_i, CX_i, CX_o, C_o and directly back to C_i must be at least three orders of magnitude slower. Since the first three reactions are a normal half-cycle of chloride exchange and are therefore very rapid, the conformational change from C_o to C_i must be the rate-limiting step for net chloride efflux by this mechanism, which is called slippage. One consequence of the relative slowness of the inward slippage reaction $C_o \rightarrow C_i$ is that the steady state concentrations of the transport conformations C_o, C_i, CX_i, etc., are
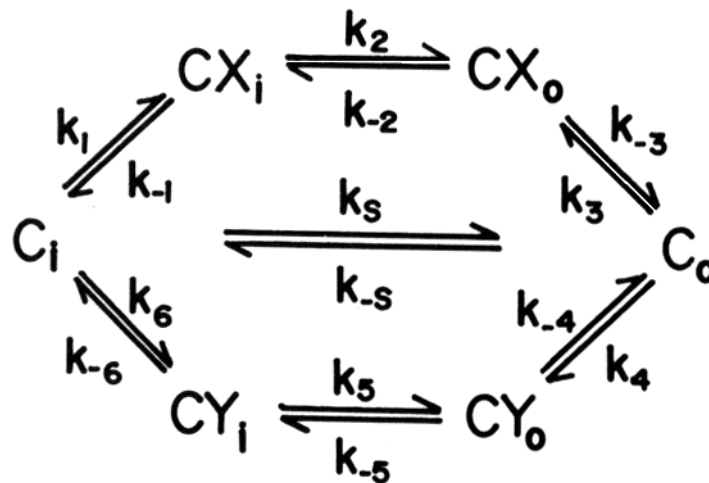

FIGURE 1. Kinetic scheme describing the exchange transport of the anions X and Y and anion net transport by slippage. k_2, k_{-2}, k_5, and k_{-5} are the rate constants for the conformational change (translocation) of the loaded transporter. The k_{+i} ($i = 1, 3, 4,$ and 6) are the association rate constants of the anions X and Y with the conformations C_o or C_i, and the k_{-1} are the dissociation rate constants. If $X = {}^{36}\text{Cl}$ and $Y = {}^{35}\text{Cl}$, the corresponding rate constants of the top and the bottom half-cycle are assumed to be identical (i.e., $k_1 = k_6$, $k_{-1} = k_{-6}$, etc.). k_s and k_{-s} are the rate constants of translocation of the empty transporter (outward slippage and inward slippage, respectively).

practically unaffected by the rate of the slippage reaction. Thus, net transport by the slippage mechanism may be described well by the simpler scheme with which we have modeled anion exchange transport and whose kinetic parameters we have previoiusly determined (Gunn and Fröhlich, 1979).

The conformations of interest in modeling anion net transport are C_o and C_i. The expressions for the steady state concentrations of C_o and C_i, derived from the ping-pong kinetic scheme, are:

$$C_o/C_{\text{tot}} = \left[1 + \frac{\text{Cl}_o}{a} \left(\frac{b}{\text{Cl}_i} + e + f \right) \right]^{-1} ; \tag{4}$$

$$C_i/C_{\text{tot}} = \left[1 + \frac{\text{Cl}_i}{b} \left(\frac{a}{\text{Cl}_o} + e + f \right) \right]^{-1} . \tag{5}$$

C_{tot} is the total concentration of transporter molecules in the system. Cl_i and Cl_o are the intracellular and extracellular chloride concentrations, respectively, and a, b, e, and f are constants that are combinations of the individual rate constants shown in Fig. 1 (see Appendix). C_o/C_{tot} and C_i/C_{tot} can also be expressed in terms of phenomenological transport parameters, namely the half-saturation constants of anion exchange:

$$C_o/C_{tot} = \{1 + Cl_o/K_{1/2\text{-out}}\}^{-1}; \tag{6}$$

$$C_i/C_{tot} = \{1 + Cl_i/K_{1/2\text{-in}}\}^{-1}; \tag{7a}$$

$$= Cl_o\{Cl_o + K_{1/2\text{-out}}\}^{-1} \{1 + Cl_i/K_{1/2\text{-in}}^{max}\}^{-1}. \tag{7b}$$
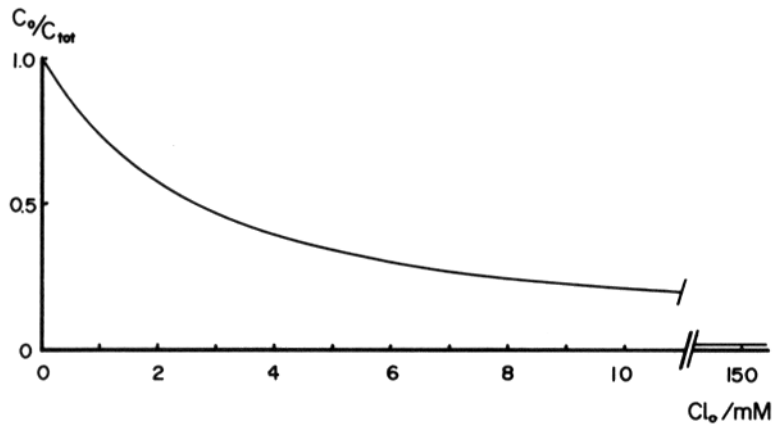

FIGURE 2. Theoretical plot of the fraction of transporter molecules that are in the conformation C_o as a function of the extracellular chloride concentration Cl_o. The curve was calculated from Eqs. 1 and 2 using a value of $K_{1/2}(Cl_o) = 3$ mM.

$K_{1/2\text{-out}}$ and $K_{1/2\text{-in}}$ are the half-saturation constants of Cl_o- and Cl_i-stimulated chloride exchange flux, respectively. They are themselves Michaelis-Menten-type functions of the trans-anion concentrations (Gunn and Fröhlich, 1979; see Appendix). $K_{1/2\text{-in}}^{max}$ is the inside half-saturation constant at saturating extracellular (transmembrane) chloride concentrations. In Eqs. 6 and 7b C_o/C_{tot} and C_i/C_{tot} are given as functions of Cl_o. Using values of $K_{1/2\text{-out}} = 3$ mM and $K_{1/2\text{-in}}^{max} = 65$ mM, obtained at pH 7.8 and 0°C for chloride exchange (Gunn and Fröhlich, 1979), we can show quantitatively the dependence of these ratios on Cl_o (Fig. 2). At $Cl_o = 0$ all transport molecules are in the conformation C_o so that $C_o/C_{tot} = 1$. This preferential accumulation of transporters in a particular conformation has been previously referred to as "recruitment" (Rothstein et al., 1976; Grinstein et al., 1979; Jennings, 1980). Recruitment is most evident in a carrier-type transporter whose slippage reaction rate is much slower than the rate of translocation of the loaded transport site. This is clearly the case with the anion transporter. As Cl_o is increased, more chloride binds to the outward-facing transport site, which subsequently can be translocated and recruited into an inward-facing conformation. Consequently, the proportion of C_o decreases and the proportion of C_i

increases with increasing Cl_o. The concentration of Cl_o that decreases the proportion of C_o to one-half is equal to $K_{1/2\text{-out}}$, the half-saturation constant of Cl_o-stimulated chloride exchange. At high Cl_o almost all outward-facing sites are occupied by chloride, so that C_o/C_{tot} approaches zero and C_i/C_{tot} approaches a limiting value that depends on Cl_i.

Since chloride net transport by the slippage mechanism involves the conformations C_o and C_i, it can be quantitatively expressed by Eq. 8:

$$M_{net}^{efflux} = k_{-s} \cdot C_o/C_{tot} - k_s \cdot C_i/C_{tot}, \tag{8}$$

where k_{-s} and k_s are the rate constants of the conformational change from C_o to C_i and from C_i to C_o, respectively. Net efflux is the difference between the rates of inward translocation of the outward-facing site (inward slippage) and the rate of outward translocation of the inward-facing site (outward slippage).

We believe that the rate of outward slippage is relatively small compared with the rate of inward slippage, for two reasons. First, an intrinsic asymmetry of the anion transporter is also reflected in an asymmetry of the translocation rates of the unloaded conformations. Applying the principle of microscopic reversibility to the scheme in Fig. 1, one obtains

$$k_{-s}/k_s = k_{-1}k_{-2}k_3/k_1k_2k_{-3} = b/a. \tag{9}$$

b/a is the asymmetry of anion exchange transport given by the asymmetry in the transport half-saturation constants: $b/a = K_{1/2\text{-in}}^{max}/K_{1/2\text{-out}}^{max}$. We have previously shown that this ratio equals ~ 15 (Gunn and Fröhlich, 1979), and this was recently confirmed by Knauf et al. (1980). This measured asymmetry could be due to an asymmetric surface concentration of anions on the two sides of the membrane due to an asymmetric charge density on the two membrane surfaces, or it could reflect an intrinsic kinetic property of the transporter (Gunn and Fröhlich, 1979). If this asymmetry is intrinsic to the transporter then one would expect k_s to be ~ 15 times smaller than k_{-s}. In this case, even at extracellular chloride concentrations of 20 mM where C_i/C_{tot} is about three times larger than C_o/C_{tot}, the rate of outward slippage would still be about five times smaller than the rate of inward slippage.

The other factor that governs the relative rates of net transport in the two directions is the membrane potential V_m. Chloride net transport is equivalent to net transport of negative charges that have to be compensated for for reasons of electroneutrality. In our experiments we increased the permeability of the red cell membrane for cations with the potassium-selective ionophore valinomycin so that the (electrogenic) net movement of chloride can be compensated for by the movement of an equivalent amount of potassium ions (Hunter, 1971, 1974, 1977; Kaplan and Passow, 1974; Knauf et al., 1977; Cass and Dalmark, 1979). In fact, a potassium diffusion potential in the presence of high concentrations of valinomycin can be employed to energize net transport of chloride against its concentration gradient. The (inside negative) membrane potential caused by the potassium gradient doubles the rate of net efflux of chloride (which is demonstrated in Fig. 6). This increase in net efflux must be due to an increased difference between inward slippage

and outward slippage of empty transporters. Since at low membrane potentials the outward slippage is probably 15-fold smaller than inward slippage, this increased difference must primarily reflect an increase in inward slippage rather than a further diminution of the outward slippage. This means that the rate of inward slippage, k_{-s}, is enhanced and the rate of outward slippage, k_{-s}, is reduced and thus the back flux component in Eq. 8 is reduced.

It appears therefore from these considerations that the rate of outward slippage is a small fraction of the rate of inward slippage. The second term in Eq. 8 can then be neglected and the rate of chloride net efflux can be approximated by Eq. 10:

$$M_{net}^{efflux} = k_{-s} \times C_o/C_{tot}. \tag{10}$$

Eq. 10 means that net efflux by slippage under our conditions is the product of the inward slippage rate constant (itself a function of voltage) and the number of transport molecules in the conformation C_o (or the probability with which the transporter is in the conformation C_o). We can test Eq. 10 experimentally by measuring chloride net efflux as a function of the extracellular chloride concentration under conditions under which k_{-s} is constant. We would expect k_{-s} to be constant if the membrane potential is kept constant. Then net efflux should be directly proportional to C_o/C_{tot} and should have the same dependence on Cl_o as shown in Fig. 2 for C_o/C_{tot}. Net efflux by the slippage mechanism should be maximal at $Cl_o = 0$ and be inhibited by increasing Cl_o, until at high Cl_o concentrations net efflux approaches zero because all outward-facing transport sites are occupied by chloride. The inhibitor constant of Cl_o for chloride net efflux K_I (Cl_o) according to Eqs. 1 and 3 is identical to $K_{1/2\text{-out}}$:

$$K_I (Cl_o) = a\left(\frac{b}{Cl_i} + e + f\right)^{-1} = K_{1/2\text{-out}}.$$

We have previously determined that $K_{1/2\text{-out}}$ is ~3 mM at 0°C with intact cells that have an intracellular chloride concentration of ~110 mM (Gunn and Fröhlich, 1979). Net efflux through slippage should therefore be half-inhibited at the same concentration at 0°C. Since we performed our net efflux experiments at 25°C we need an estimate of $K_{1/2\text{-out}}$ at this temperature. We obtained this from competition experiments between Cl_o and DNDS, a competitive inhibitor of chloride exchange transport (Fröhlich, 1982). In these studies we showed that the inhibition constant of Cl_o for DNDS binding to the transporter, which according to our kinetic scheme is identical to $K_{1/2\text{-out}}$, depended only very little on temperature. We therefore estimate that $K_I(Cl_o)$ should also be ~3 mM at 25°C.

As mentioned briefly above, the rate of slippage reaction may depend on the membrane potential, V_m. We can test this by measuring net chloride efflux as function of the membrane potential at fixed Cl_o. As long as Cl_i and Cl_o are fixed, C_o/C_{tot} should be clamped at a fixed value, and a change in M_{net} with membrane potential should primarily be due to a change of k_{-s}

with V_m under our conditions. This measurement is most sensitive at very low concentrations of Cl_o where C_o/C_{tot} and net efflux are nearly maximal.

RESULTS

The intrinsic cation permeability of the red cell membrane is lower than the chloride net permeability. To remove the electrostatic restrictions imposed on chloride net flux by the lack of co-permeating cations, valinomycin or gramicidin is added to the system to increase the cation conductance (Hunter 1971, 1977; Kaplan and Passow, 1974; Knauf et al., 1977; Cass and Dalmark, 1979). We performed two series of experiments to convert the membrane from chloride selective to potassium selective. In one series we measured net efflux of both potassium and tracer chloride from the same cells with several different nominal valinomycin concentrations and 0.7 mM KCl (plus Na-citrate/ sucrose) in the extracellular phase (Fig. 3A). At all valinomycin concentrations the net tracer chloride efflux exceeded the net potassium efflux by ~20 mmol (kg cell solids)$^{-1}$ min^{-1}. This parallel shift of Cl flux above K flux is possibly due to an additional exchange flux of intracellular tracer chloride with a very small contamination of bicarbonate in the extracellular fluid.[3] Above a concentration of 4 μM valinomycin, the fluxes were not increased further by increasing the valinomycin concentration. This could mean that then the fluxes are fully limited by the chloride net permeability of the membrane and that the membrane potential is dominated by a potassium diffusion potential, or it could mean that valinomycin did not partition further into the red cell membrane (Hunter, 1974). We tested these alternatives in another series of control experiments where we measured the rate of potassium efflux in exchange for extracellular rubidium (150 mM) over the same concentration range of valinomycin. The data of Fig. 3B show that the valinomycin-mediated K-Rb exchange did saturate at about the same nominal valinomycin concentration as K and Cl net efflux, which indicated that beyond 4 μM valinomycin, absorption into the red cell cell membrane is saturated (Hunter, 1974; Knauf et al., 1977). It is important to note, however, that the maximal rate of exchange, which is a measure for the K permeability, is about four times higher than the maximal rate of K net efflux. From this we conclude that despite the similar shapes of the experimental curves, the K permeability is several times larger than the maximal chloride permeability. Even though the membrane potential is not clamped tightly at the K diffusion potential, it is greatly influenced by the K gradient at saturating valinomycin concentrations. In the subsequent experiments where we used a nominal valinomycin concentration of 11 μM, we have consequently assumed that the K conductance was sufficiently high to change the membrane potential by changing the extracellular K concentration and thus analyze the data in a semi-quantitative manner (see also Discussion).

[3] Using a V_{max} of exchange transport at 25°C of 5 mol (kg cell solids)$^{-1}$ min^{-1}, estimated from the data of Brahm (1977), and a $K_{1/2-out} = 3$ mM, one can calculate that a concentration of ~10 μM of bicarbonate would be sufficient to cause the offset between the chloride and potassium fluxes.
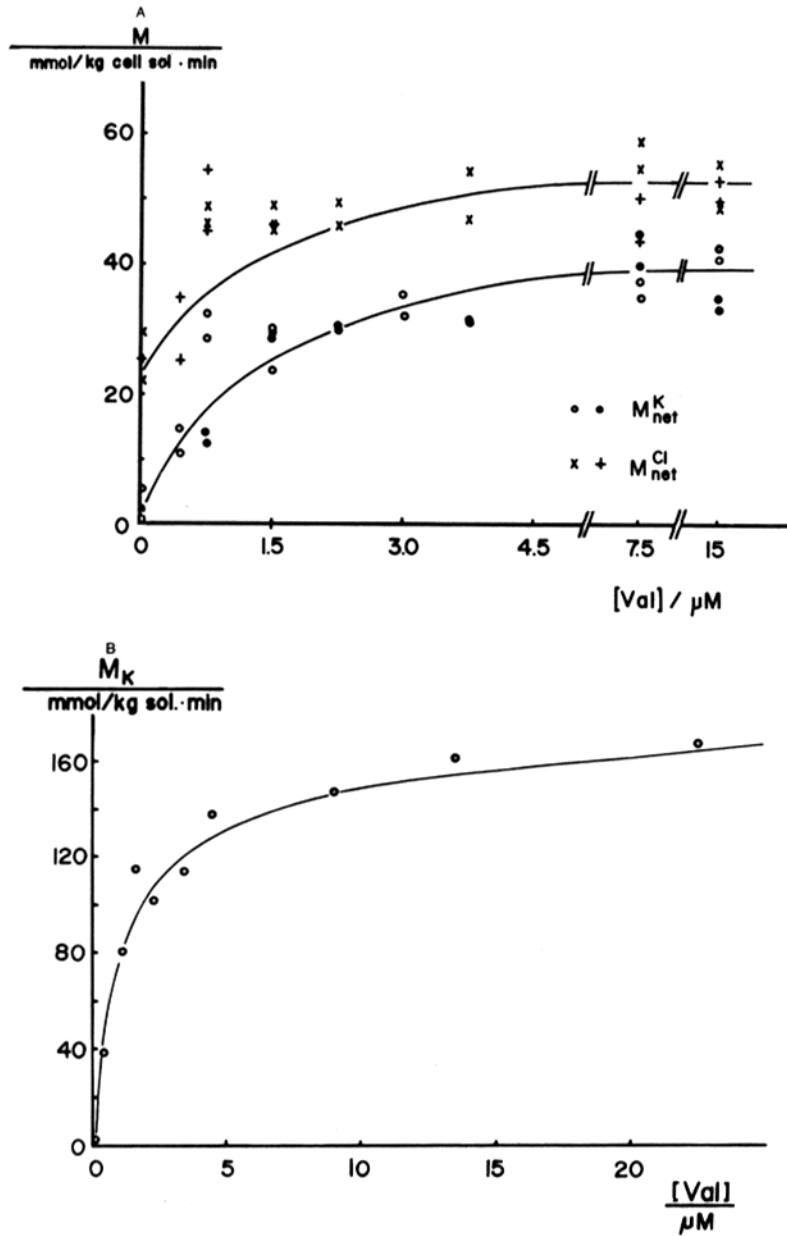
FIGURE 3.   (A) Potassium chloride net efflux from intact cells at 25°C, measured simultaneously in the same cells as potassium and tracer chloride net efflux, into media containing 0.7 mM KCl (plus Na_3-citrate/sucrose) and various valinomycin concentrations. The data are from two experimental series ($\bullet$, $\times$ and O, +). (B) Potassium efflux from intact cells in exchange for extracellular rubidium at 25°C. The efflux medium contained initially 150 mM RbCl and 27 mM glycylglycine, pH 7.8, and various (nominal) concentrations of valinomycin.

We next determined the effect of extracellular chloride on net efflux by measuring potassium net efflux from intact cells into media containing initially 0.3 mM potassium and different initial chloride concentrations. As expected from the considerations in our kinetic scheme (Fig. 2), net efflux was decreased when Cl_o was increased (Fig. 4A). Since the potassium gradient was the same in all experiments, we assume that changing the chloride gradient had only a relatively small effect on the membrane potential and that therefore a change in the efflux rate reflected mainly a change in the recruitment of the transporter into or away from the conformation C_o (see also Discussion). The chloride concentration at which half of the flux reduction was accomplished, ~ 3 mM (Fig. 4B), was in good agreement with the expected value, which was based entirely on chloride exchange measurements. At high concentrations of Cl_o, the fluxes approached a value of 14–16 mmol (kg cell solids)$^{-1}$ min^{-1} and were essentially constant between $Cl_o = 50 - 150$ mM (Fig. 5). This result was in disagreement with the theoretical expectation that net flux by slippage should decrease to zero. As a control we performed the same experiments with DNDS present in the efflux mixture at concentrations that would block at least 99% of chloride exchange transport. Figs. 4A and 5 show that the fluxes were not completely inhibited by DNDS. The DNDS-insensitive flux was ~5 mmol (kg cell solids)$^{-1}$ min^{-1} and did not vary with varying Cl_o. Our data at $Cl_o = 150$ mM agree with previous findings of Knauf et al. (1977) that under these conditions only 65% of the net flux could be inhibited by DIDS.

We have, therefore, divided anion net efflux into three components: a component that is not inhibitable by DNDS; a component that has the concentration dependence expected by slippage of the empty transporter between the conformations C_o and C_i; and a component that dominates at high extracellular anion concentrations where slippage should be negligible. We were interested in the characteristics of the net flux component caused by slippage which contributes most to the total anion net efflux at low Cl_o. Assuming that the DNDS-inhibitable component that was observed at $Cl_o = 150$ mM in Fig. 5 is independent of Cl_o, we can estimate that below $Cl_o = 1$ mM slippage accounts for >70% of the DNDS-sensitive net efflux. To measure the voltage dependence of the slippage component, we therefore measured tracer chloride net efflux into media containing 0.7 mM chloride of the same

FIGURE 4.   (*opposite*) (A) Potassium chloride efflux (measured as net K efflux) into media containing 0.3 mM K and various concentrations of Cl (upper curve). The lower curve shows the fluxes that were measured when 200 μM DNDS was added to the efflux media before the cells. The lines were drawn by eye. (B) Replot of the data of A. The flux values were corrected for the Cl_o-independent and the DNDS-insensitive flux components by subtracting 15 mmol (kg cell solids)$^{-1}$ min^{-1}. The straight line is a regression line ($r^2 = 0.82$) through the data points. Its intercept with the abscissa gives a half-saturation constant $K_{1/2}(Cl_o) = 3.3$ mM, and the intercept with the ordinate gives a value of the slippage component of 32 mmol (kg cell solids)$^{-1}$ min^{-1}. M_{sl} stands for the slippage component, which is defined as net efflux at the given Cl_o minus net efflux at $Cl_o = 150$ mM (i.e., as the Cl_o-inhibitable component).
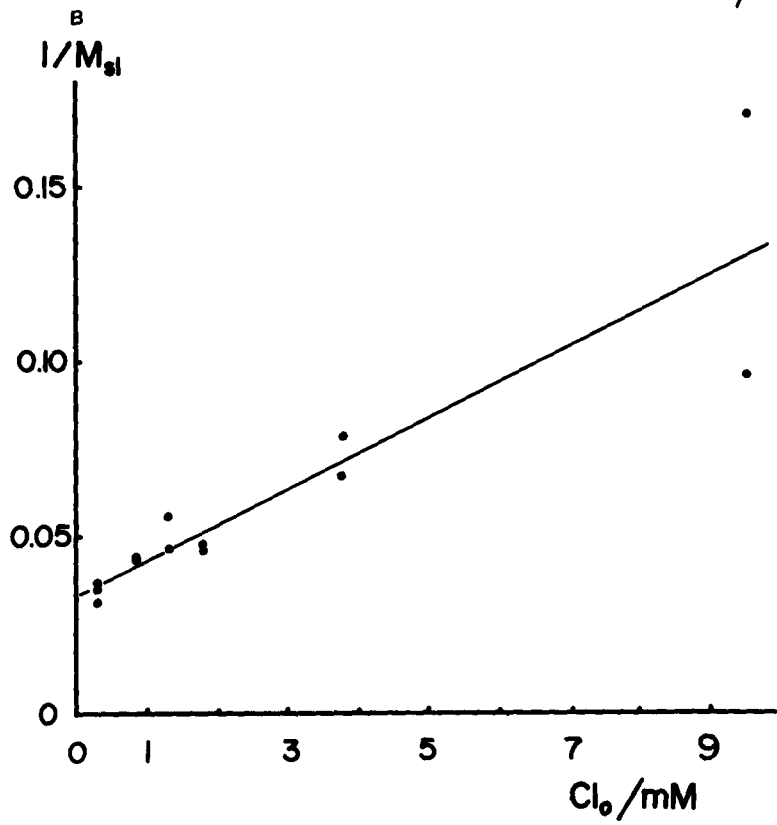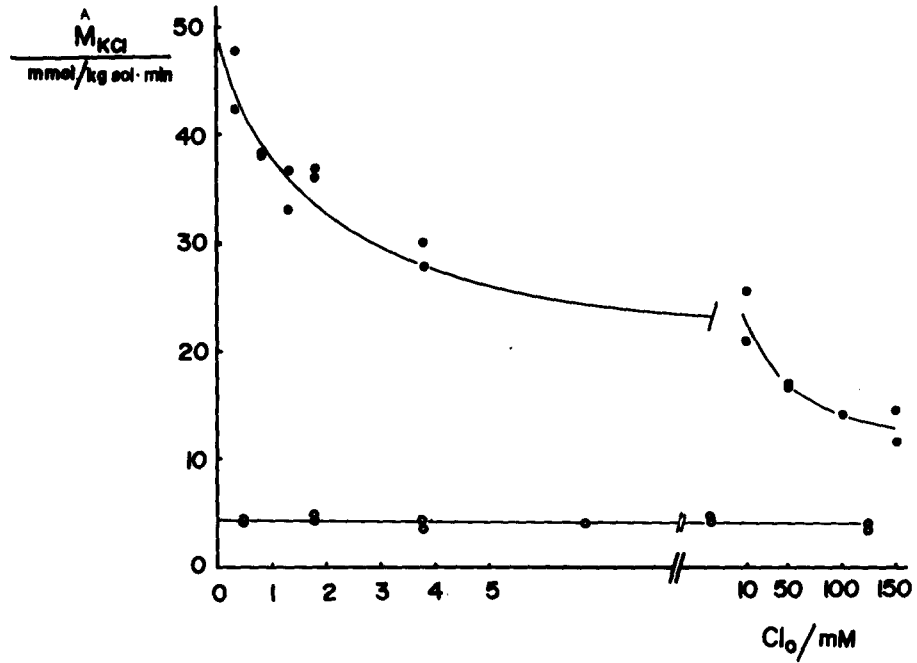
Figure 4

specific activity as the intracellular chloride, and different potassium concentrations, K_o. The results of these experiments are shown in Fig. 6. Net KCl efflux increased when K_o was decreased while the intracellular K concentration was the same in all cells.

From the data in Fig. 6 we conclude that anion net efflux at low Cl_o depends on the membrane potential. To conclude whether the translocational
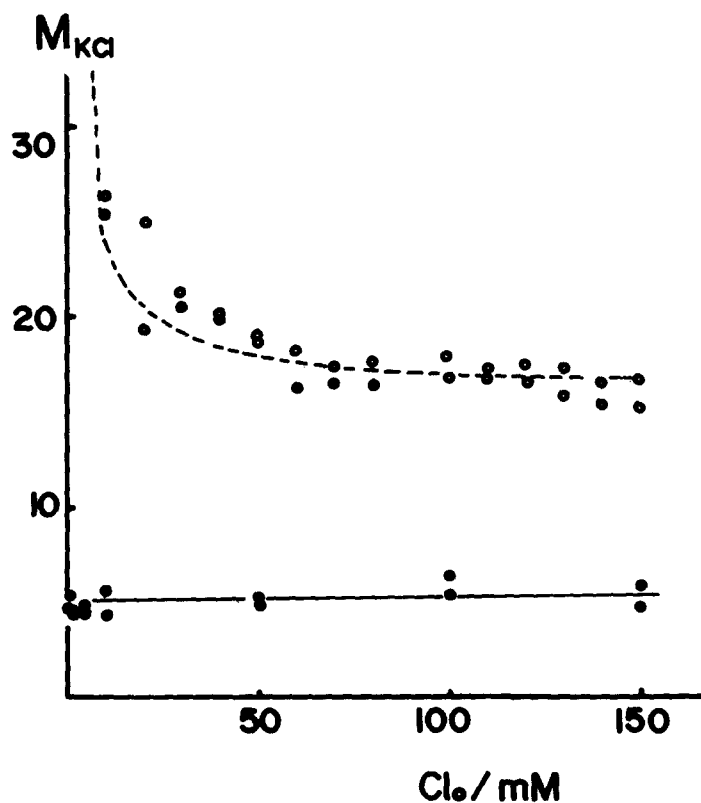

FIGURE 5. Repeat of the experiments of Fig. 4A at high extracellular chloride concentrations. The dashed line was calculated with Eqs. 6 and 10 by assuming $K_{1/2}(Cl_o)$ = 3 mM and a Cl_o-independent background flux of 16 mmol (kg cell solids)$^{-1}$ min^{-1}.

conformation change of the unloaded transporter or the translocation reaction of the loaded transporter or both were potential dependent, we measured chloride exchange transport at different membrane potentials. We previously reported (Gunn and Fröhlich, 1979) no significant change in the exchange flux characteristics at positive membrane potentials (chloride diffusion potential) compared with depolarized potentials in valinomycin-treated cells placed into media with low K_o. We repeated this type of experiment with a better time and concentration resolution using gramicidin as the voltage-clamping ionophore instead of valinomycin because at 0°C valinomycin is even less
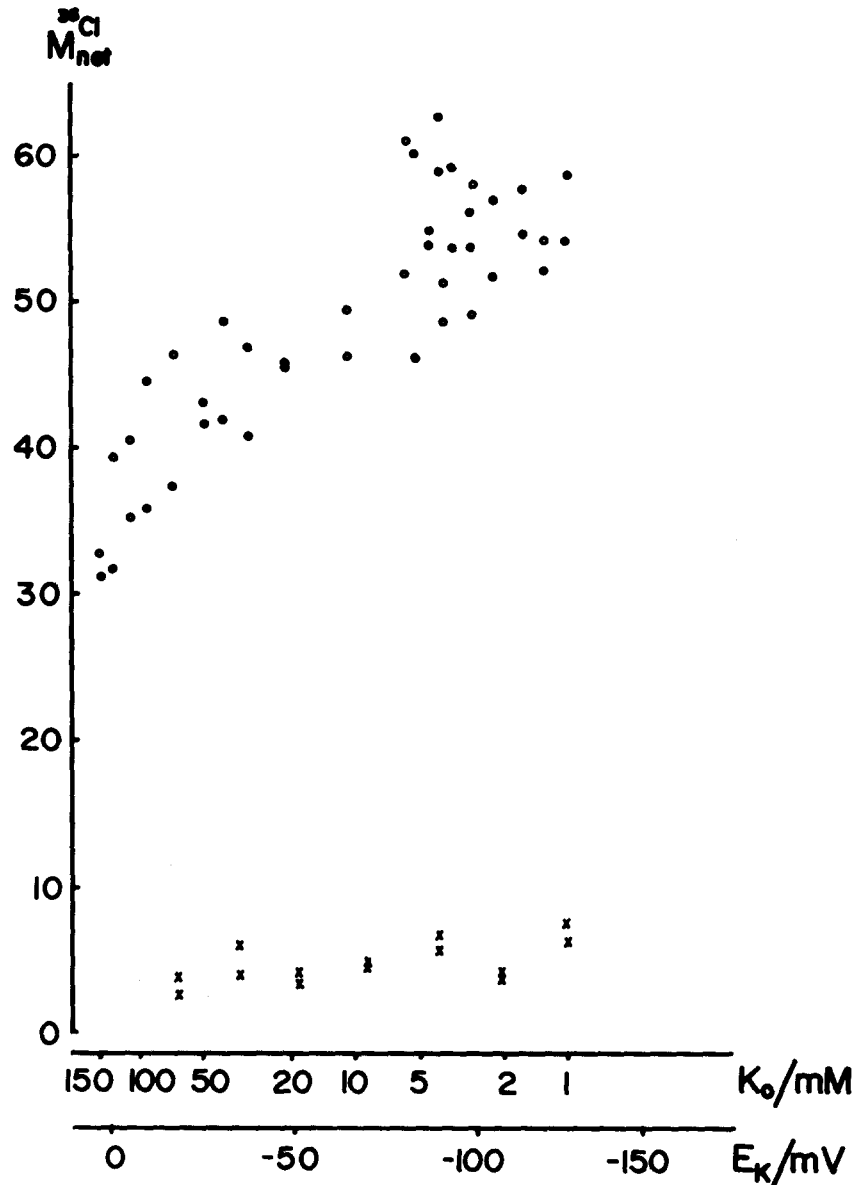
FIGURE 6.   Potassium chloride efflux (measured as tracer chloride net efflux) into media containing 0.7 mM ^{36}Cl of the same specific activity as the intracellular chloride and different concentrations of K. O: fluxes in the absence of DNDS with 75 mM (Na + K) citrate; ●: fluxes in the absence of DNDS with 25 (Na + K) citrate/sucrose; X: fluxes in the presence of 200 μM DNDS in the efflux media. The units of M_{Cl} are the same as the net fluxes of the previous figures: mmol (kg cell solids)$^{-1}$ min^{-1}.

effective at clamping the membrane potential at E_K than at 25°C (Hunter, 1974). Red cells were pre-equilibrated with gramicidin and used to measure the chloride tracer efflux at 0°C into media containing 75 mM K or Na and various concentrations of Cl (replacement with isotonic-isoionic citrate/sucrose). The rate of K efflux in exchange for extracellular Na was measured in several of the flux experiments by determining the K concentration in the same samples in which tracer chloride was determined. In these latter measurements the K flux was 1,000–1,500 mmol K (kg cell solids)$^{-1}$ min.$^{-1}$ This is >100 times the maximum chloride net flux at 0°C (obtained by extrapolation of the data of Fig. 8, see below). Therefore, in the experiments with gramicidin the membrane potential was close to the cationic diffusion potential (approximately −16 mV)[4] but it was between +50 and +100 mV in the absence of gramicidin. The results are shown in Fig. 7. The activation curve of Cl_o-stimulated chloride exchange was not significantly affected by a change in the membrane potential: in the absence of gramicidin $K_{1/2\text{-out}} = (2.2 \pm 0.2)$ mM and $V_{\text{max-out}} = (676 \pm 26)$ mmol (kg cell solids)$^{-1}$ min^{-1}, and in the presence of gramicidin $K_{1/2\text{-out}} = (1.8 \pm 0.2)$ mM and $V_{\text{max-out}} = (571 \pm 26)$ mmol (kg cell solids)$^{-1}$ min^{-1}.

We sought to dissect the three components of chloride net efflux by their temperature dependence. We measured the net efflux under three different conditions: at low Cl_o, at high Cl_o, and in the presence of DNDS (with either low or high Cl_o). The results are shown in Fig. 8. The relative proportions of the three components of net efflux were unaffected by temperature. Both the Cl_o-dependent and the Cl_o-independent component had the same activation energy. The activation energy of 17 kcal mol^{-1} (70 kJ mol^{-1}) agrees with the value Hunter obtained for net efflux at high chloride concentrations (Hunter, 1977), but is higher than the 42 kJ mol^{-1} reported by Cass and Dalmark (1979).

DISCUSSION

Before we can interpret the net flux experiments, the limited ability of valinomycin to clamp the membrane potential at a set value must be evaluated. Hunter (1974) demonstrated that the partitioning of valinomycin into the red cell membrane was a saturating function of the valinomycin concentration and depended on temperature. Since the native anion conductance of the red cell membrane is high, large amounts of valinomycin are necessary to render the membrane primarily cation permeable. Because of the limited partitioning of valinomycin into the membrane, high concentrations of valinomycin in the solution do not necessarily produce the K^+ permeability of the membrane required to clamp the membrane potential precisely at the K equilibrium potential. Hunter (1977), Kaplan and Passow (1974), and Knauf et al. (1977) employed the Goldman-Hodgkin-Katz equation and the

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[4] Actually, V_m is difficult to determine in this case because of the different specific conductances for K and Na, the K conductance being up to twofold higher (Hladky and Haydon, 1972). The value of $V_m = -16$ mV was calculated by assuming that Na behaved identically to K. Because of the higher K conductance the membrane potential may be somewhat more negative.

Goldman flux equation to model the activation of KCl net efflux by valino-
mycin and obtained a quantitative measure of the relative permeabilities for
K and Cl as well as the membrane potential. They found that at 37°C the
valinomycin-induced K permeability coefficient was up to 20 times greater
than the Cl permeability coefficient. Also, Knauf et al. calculated from
fluorescent probe data of Hoffman and Laris (1974) a K-to-Cl permeability
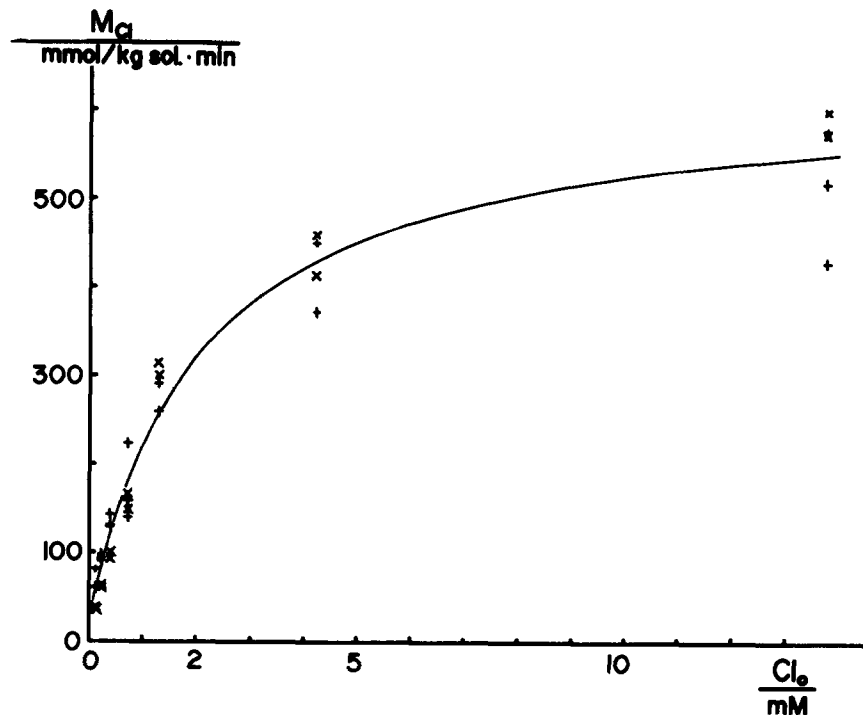
FIGURE 7. Tracer chloride efflux from gramicidin-treated cells (+) and control
cells (×) at 0°C into media containing different concentrations of chloride.
Chloride was varied by substituting with isoosmotic-isoionic citrate/sucrose (see
Methods for details). At Cl_o = 1.31, 4.25, and 13.8 mM Na_3-citrate was used in
the substitution medium in order to be able to determine the rate of K-Na
exchange as measure of the gramicidin-induced K permeability. The solid line
is a Michaelis-Menten curve with the parameters $K_{1/2\text{-out}}$ = 2.0 mM and
$V_{\text{max-out}}$ = 624 mmol (kg cell solids)$^{-1}$ min^{-1}.

ratio of up to 20 in valinomycin-treated red cells at room temperature (22–
23°C). All these previous experiments were performed at high concentrations
of Cl_o, where chloride net efflux (and with it the chloride conductance) was
minimal. In this study we found that at low Cl_o (Cl_o < 1 mM), net efflux was
several times larger than at higher physiological Cl_o (Cl_o = 150 mM). Our
experiments were designed to measure the fluxes and not to determine the
relative permeabilities of K and Cl. For purposes of comparison it is, however,
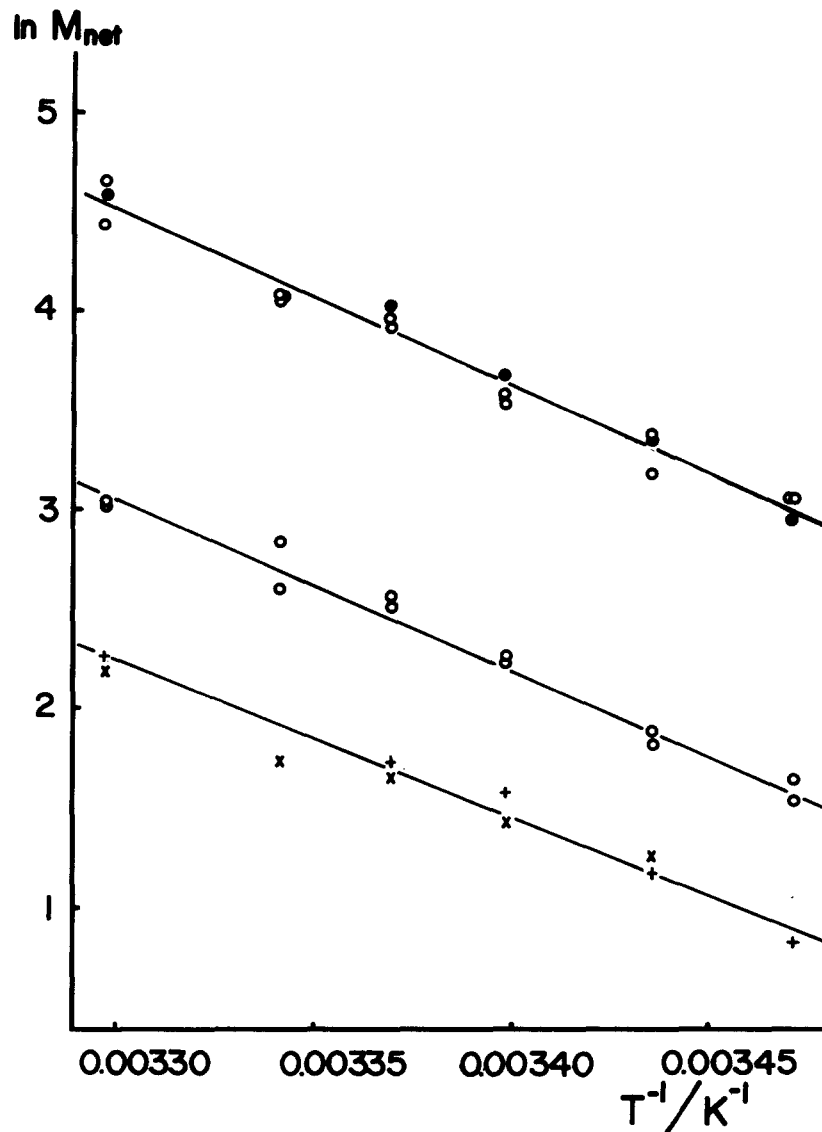useful to estimate the relative magnitude of the Cl and K conductance

FIGURE 8. Potassium chloride efflux (measured as K net efflux) into media containing 0.3 mM K at different temperatures between 15 and 30°C. The plots are Arrhenius plots of ln (flux) vs. 1/(abs. temperature). Upper line: efflux into media with Cl_o = 0.3 mM; middle line: efflux into media with Cl_o = 150 mM; bottom line: efflux into media with Cl_o = 0.3 mM (+) and Cl_o = 150 mM (×) plus 200 μM DNDS. The solid circles of the upper-line data are fluxes obtained with a nominal concentration of 22 μM instead of the usual 11 μM valinomycin, as control for saturating valinomycin effects at all temperatures. Regression analysis of the data gave activation energies of 17.4 kcal mol^{-1} (upper line), 17.1 kcal mol^{-1} (middle line), and 14.7 kcal mol^{-1} (bottom line). Units of flux are mmol (kg cell solids)$^{-1}$ min^{-1}.

pathways in the present study. At the usual nominal valinomycin concentration of 11 μM, K efflux into a Rb medium was 160 mmol (kg solids)$^{-1}$ min^{-1} (Fig. 3B), which corresponds to an exchange permeability of about $P_K = 0.7$ min^{-1} or 5×10^{-7} cm s^{-1} at 25°C. Provided that this permeability value can also be used to estimate net flux permeability[5] we calculate $P_{Cl} = 0.2$ min^{-1} or 1×10^{-7} cm s^{-1} at $Cl_o = 0.7$ mM and $P_{Cl} = 0.06$ min^{-1} or 5×10^{-8} cm s^{-1} at $Cl_o = 150$ mM from the net fluxes in Figs. 3B and 4A. Our value of $P_{Cl} = 5 \times 10^{-8}$ cm s^{-1} at high Cl_o and 25°C is higher than the value of 2.1×10^{-8} cm s^{-1} obtained by Cass and Dalmark (1979) at 25°C in gramicidin-treated cells, and higher than the values of 2.1×10^{-8} cm s^{-1} and 2.8×10^{-8} cm s^{-1} obtained at 37°C by Hunter (1977) and Knauf et al. (1977), respectively. Our value of $P_{Cl} = 1.5 \times 10^{-7}$ cm s^{-1} at low Cl_o is similar to the value of 1.2×10^{-7} cm s^{-1} reported by Wieth et al. (1973); however, the latter measurement was performed in the presence of phloretin, which may leave a stimulating effect on the chloride conductance, similar to phlorizin (Kaplan and Passow, 1974). These differences may be due to the approximate nature of our estimate and possibly also to variations among blood donors. Altogether, a reasonable estimate of the permeability ratios is $P_K/P_{Cl} = 4$–5 at $Cl_o < 1$ mM and $P_K/P_{Cl} = 12$–15 at $Cl_o = 150$ mM. Using the values of 4 and 12 and the Goldman-Hodgkin-Katz equation we calculate that the membrane potential was not very well "clamped" by valinomycin in the experiment shown in Fig. 4. It shifted from -37 to -54 and to -67 mV as Cl_o was increased from 0.7 to 10 and to 150 mM, respectively. Similarly, the membrane potential computed for the experimental conditions of Fig. 6 ranged from -41 mV at $K_o = 0.5$ mM to $+6$ mV at $K_o = 150$ mM (using a permeability ratio of $P_K/P_{Cl} = 4$).

We may still draw qualitative conclusions about the potential dependence of the slippage component of the flux although the estimated membrane potentials differ considerably from the K equilibrium potentials. We may exclude the possibility that changes in membrane potential cause the decline of net efflux with increasing Cl_o in Fig. 4. The increasing external chloride concentration along the abscissa would tend to hyperpolarize the membrane potential, which would accelerate the rate of efflux of anionic complexes or the influx of cationic complexes of the transporter. Since the flux declines rather than accelerates and since hyperpolarizing the membrane increases the flux at a fixed low external chloride (Fig. 6), the potential dependence cannot be the factor reducing the flux in Fig. 4. We therefore believe these data strongly support the role of inward slippage as the dominant mode of net chloride efflux at low Cl_o.

[5] This assumption has been made in all previous publications, but it probably represents only an approximation. K equilibrium exchange fluxes were usually measured at membrane potentials near zero, but under the conditions of a K gradient the value of the membrane potential can be fairly large. The valinomycin conductance can be a saturating, linear, or superlinear function of the potential, depending on the K concentration (Stark and Benz, 1971). In addition, due to the negative charges (from phosphatidyl serine) on the inner leaflet of the red cell membrane the conductance is probably strongly asymmetric. All these factors have not be considered in the value of the K permeability that was used in the calculations of the permeability ratios so that these ratios can be considered at best fair estimates.

The limited effectiveness of valinomycin particularly hampers the temperature experiments at low Cl_o shown in Fig. 8. At high Cl_o and in the presence of DNDS the permeability is still at least 10 times the Cl permeability so that the temperature dependence of the Cl_o-independent component and the DNDS-insensitive component are fairly estimated. But at low Cl_o the temperature dependence of the Cl_o-inhibitable fluxes may partially reflect the temperature dependence of the valinomycin-induced K permeability and thus may underestimate the temperature dependence of the slippage process. Even with these factors considered we do not believe that the activation energy of the slippage component is as high as the activation energy of the chloride exchange transport, ~110 kJ mol^{-1} (Dalmark and Wieth, 1972; Brahm, 1977).

We have demonstrated in this study that there are at least three phenomenologically different components that make up the chloride conductance of the human red blood cell membrane. One component is insensitive to DNDS, a stilbene disulfonate inhibitor of anion exchange transport (Barzilay et al., 1979; Fröhlich, 1982), and may therefore not utilize the anion transporter. Since net transport was measured as potassium net efflux this component may comprise not only stilbene-insensitive chloride conductance pathways but also net potassium transport pathways such as the Na-K pump, Na-K-Cl cotransport, Na-K exchange mediated by valinomycin, etc., as well as nonspecific leak pathways.[6] We have made no further effort to dissect this component.

The other two components are both DNDS inhibitable. They are distinguished phenomenologically by their dependence on Cl_o. The Cl_o-inhibitable component agrees well with the predictions of the slippage model in that it decreases hyperbolically with increasing Cl_o, and that it is half-inhibited by ~3 mM Cl_o (Figs. 4A and B). When slippage is maximal, at very low values of Cl_o, this component represents a flux of ~35 mmol (kg cell solids·min)$^{-1}$ and contributes ~70% to the overall net transport rate. Above Cl_o = 50 mM it is essentially abolished. Although the available data do not directly prove that this component is due to slippage, for the sake of simplicity we will refer to it as the slippage component. In the concentration range above 50 mM net chloride transport is accomplished only through the Cl_o-independent (DNDS-sensitive) and the DNDS-insensitive pathways. In agreement with previous reports (Knauf et al., 1977), we found that at Cl_o = 150 mM, ~65% of the net efflux was DNDS sensitive and 35% was insensitive (Fig. 5). In addition, when extrapolated to Cl_o = 0 mM, the Cl_o-independent component contributes ~20% to net efflux and the nonspecific component contributes ~10%.

Physically one could envision the anion transporter as a specialized channel that contains at least one energy barrier on each side of the main anion binding/transport site, as in the theory of the two-conformation channel described by Läuger (1980). Depending on the conformation of the transporter, C_i or C_o, one barrier is high and the other barrier is low so that in effect

[6] For comparison, the Cl net movement through the unmodified lipid bilayer is much lower. Assuming a bilayer conductance of 10^{-8} S cm^{-2}, one can estimate that the unmodified bilayer gives rise to Cl net fluxes that are at least orders of magnitude smaller than the net fluxes reported here.

the anions have ready access to the site only from one side of the membrane at a time. Net ion flow in the sense of ions passing through the channel, one after the other, is strongly impeded (see below). Instead, exchange transport is facilitated if the loaded transporter can undergo conformational changes such that the relative heights of the two barriers are easily reversed. As a result, the anion binding site is alternately accessible to both sides of the membrane and it acts kinetically like the single-site, carrier-type transporter we have experimentally described and used in our kinetic modeling of anion exchange transport (Gunn and Fröhlich, 1979; see review by Knauf, 1979). With respect to exchange, this scheme is analogous to the gated channel or lock-type carrier concepts developed earlier (Patlak, 1957; Rothstein et al., 1976; Gunn, 1979; Passow et al., 1980).

A conformational change may also occur occasionally among the two empty conformations of the transporter, C_i and C_o. This is the slippage process that we believe dominates the measured net flux at low extracellular chloride concentrations. At the higher concentrations where virtually all outward-facing transport sites are occupied, slippage is not possible and a distinctly different mode of anion movement has to be postulated. In the preceding paper, Knauf et al. (1983) propose that the anions are able to move through the entire length of the channel without the need of a conformational change of the transporter (Knauf et al., 1983; see also Kaplan et al., 1980; Knauf and Law, 1980). In the framework of the simple two-barrier channel model this would mean that the anions can occasionally also cross the second, main barrier in the channel. We have previously referred to this process as "tunneling," in loose reference to the assumption that the protein does not undergo a conformational change during this mode of translocation (Fröhlich, 1981). Tunneling may occur in at least four different ways: ion influx or efflux through either the inward- or the outward-facing conformation (see also Knauf et al., 1983). In terms of the conformations of the ping-pong kinetic scheme in Fig. 1 the four modes can be expressed as $C_i + Cl_o \rightarrow CCl_i \rightarrow C_i + Cl_i$; $C_o + Cl_i \rightarrow CCl_o \rightarrow C_o + Cl_o$; $C_i + Cl_i \rightarrow CCl_i \rightarrow C_i + Cl_o$; and $C_o + Cl_o \rightarrow CCl_o \rightarrow C_o + Cl_i$. In more complicated channel models other modes could be constructed as well.

Alternatives exist to the slippage concept to explain Cl_o-dependent net efflux at low extracellular chloride concentrations that we cannot exclude at the present time. For example, the rates of tunneling through different conformations of the transporter channel may be different. With respect to our experiments net outward tunneling through C_o ($C_o + Cl_i \rightarrow CCl_o \rightarrow C_o + Cl_o$) could be faster than through C_i ($C_i + Cl_i \rightarrow CCl_i \rightarrow C_i + Cl_o$). According to our kinetic scheme, as Cl_o increases, C_o decreases hyperbolically to zero (Fig. 2), and C_i and CCl_i increase hyperbolically from zero to a value determined by Cl_o and Cl_i, with the same half-saturation constant as the decrease of C_o (~ 3 mM). As a result one could expect a similar hyperbolic curve as in Fig. 4A by assuming just tunneling through C_o and C_i without need for slippage. The net flux values at the two extremes of the curve then correspond to the outward tunneling through C_o and C_i.

One might hope to distinguish between tunneling and slippage by their activation energies. Unfortunately, the temperature dependences of all three net flux components are very similar (Fig. 8).

In principle, the low turnover rate calculated from the net flux data at $25°C$ would be physically reasonable for either tunneling or slippage. For example, the rate of the slippage component in Fig. 4 corresponds to a turnover rate of 13 ions per second for each individual transport unit. Considering that the loaded transporter can undergo the conformational change between CCl_o and CCl_i at a rate of $400 \ s^{-1}$ at $0°C$, $13,000 \ s^{-1}$ at $25°C$, and nearly $10^5 \ s^{-1}$ at $37°C$ (Brahm, 1977), this calculated slow slippage rate is very reasonable. At present our working hypothesis is that the Cl_o-dependent component in Fig. 4A is mostly due to slippage and that contributions to net efflux by tunneling are only minor at low Cl_o.

In Figs. 6 and 7, we have attempted to provide evidence about the net charge on the translocated empty site of the transporter. If the transport site contains a net charge then the conformational change between C_o and C_i ought to depend on the membrane potential, V_m, because the position of this charge relative to the main diffusion barrier is changed upon translocation. This is equivalent to movement of the charge across the potential drop. On the other hand, if the anion transport site is neutral, net transport ought not to be affected by V_m under our experimental conditions.[7] Fig. 6 shows that chloride net efflux under slippage conditions was accelerated by a negative (inside) potential. That is, given a slippage mechanism, the movement of the unloaded transporter behaves as if a net positive charge were moved across the membrane potential barrier. The notion that the unloaded transporter is positively charged (C_o^+) is consistent with the notion that the loaded transporter $(CCl, CHSO_4)$ is neutral (Gunn, 1972) and its movement has negligible potential dependence.[8] We previously demonstrated the potential independence of the exchange of chloride using valinomycin-treated red cells (Gunn and Fröhlich, 1979). Because of the difficulties inherent in the use of valinomycin, we repeated those experiments using gramicidin and achieved a permeability ratio of $P_K/P_{Cl} > 100$, even at low Cl_o. Fig. 7 shows that also under these better-controlled conditions exchange is affected very little, if at all, by a change in the membrane potential.

We do not conclude from Fig. 6 that the slippage mechanism is the only net transport pathway that exhibits potential-sensitive fluxes. There is no reason

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[7] It should be borne in mind that V_m is a driving force for net chloride transport regardless of the mechanism. Nevertheless, if the anion transport site is neutral and Cl_o is very low, the net efflux will not significantly depend on V_m. In this case, V_m can only alter the rate of net transport through redistribution of the steady state concentrations of the different transporter conformers, but the transporters are almost exclusively in the conformation C_o due to the very low Cl_o.

[8] In this argument it is implied that the translocation step of the loaded transporter is the rate-limiting step in the transport. This is a widely shared but not proven assumption. However, it is relatively safe to assume that at least at $0°C$, and probably also at higher temperatures, the rate of release of chloride from its binding site (the other reaction step that could limit the V_{max} of exchange) could occur more rapidly than the overall rate of $400 \ s^{-1}$ observed for chloride exchange (Gunn and Fröhlich, 1979).

to assume that the tunneling component should be independent of V_m, since tunneling is a movement of charge in the electric field. Fig. 6 demonstrates that the DNDS-insensitive net flux depends on V_m. Our proposal that there is a net positive charge on the anion transport site is based on the assumption that most of the Cl_o^--inhibitable component is actually due to slippage and that, therefore, most of the potential-dependent flux is due to the potential dependence of slippage; and based on the observations in Fig. 7 that chloride exchange is not affected by the membrane potential. This single net positive charge on the transport site would be translocated across the membrane barrier during slippage. The charge would be neutralized by chloride binding, and only one chloride ion would be bound to each transport site (Gunn and Fröhlich, 1979). This proposal does not exclude the suggestion of Passow et al. (1980) that there are other positively charged groups near the chloride transport site.

In conclusion, the net chloride pathway in human erythrocytes has three operational components. One component dominates at low *trans* chloride concentrations and behaves like slippage or return of empty transporters. The potential dependence of this component flux is consistent with a cationic empty transport site. Another component dominates at high *trans* chloride concentrations and may be the tunneling of chloride through the transporter without any change of conformation or access of sites. The third and smallest component is both chloride and DNDS insensitive and may not be mediated by band 3 protein. Surprisingly, all three components have about the same apparent activation energies of 70 kJ mol^{-1}.

APPENDIX

With $X_i = {}^{36}Cl_i$, $Y_o = Cl_o$, and $X_o = Y_i = 0$, the flux equation of anion exchange flux from the scheme in Fig. 1 is (neglecting the influence of the slippage reaction):

$$v = \frac{Cl_i Cl_o}{aCl_i + bCl_o + (e + f)Cl_i Cl_o}$$

or

$$1/v = a/Cl_o + b/Cl_i + (e + f),$$

where v is the rate of chloride exchange per transporter unit, a, b, e, and f are constants, and Cl_i and Cl_o are the intracellular and extracellular chloride concentrations. The constants a–f can be expressed in terms of the rate constants of Fig. 1:

$$a = (k_{-1}k_{-2} + k_{-1}k_{-3} + k_2k_{-3})/k_{-1}k_{-2}k_3$$

$$b = (k_{-1}k_{-2} + k_{-1}k_{-3} + k_2k_{-3})/k_1k_2k_{-3}$$

$$e = (k_{-1} + k_2 + k_{-2})/k_{-1}k_{-2}$$

$$f = (k_{-3} + k_2 + k_{-2})/k_2k_{-3}.$$

By varying only Cl_o and keeping Cl_i constant in chloride exchange experiments one can obtain $K_{1/2\text{-out}}$, the half-saturation constant, and $V_{max\text{-out}}$, the maximal flux, of Cl_o-stimulated chloride exchange:

$$K_{1/2\text{-out}} = a(b/Cl_i + e + f)^{-1};$$

$$V_{max\text{-out}} = (b/Cl_i + e + f)^{-1}.$$

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