Relative Contributions of the Slippage and Tunneling Mechanisms to Anion Net Efflux from Human Erythrocytes

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ABSTRACT The rates of anion net efflux from gramicidin-treated erythrocytes in the presence of a K gradient were measured at 25°C, pH 7.8, as rates of loss of K. The experiments served to estimate the relative contributions of two hypothetical mechanisms to Cl net efflux at low extracellular Cl concentrations. Cl, Br, and NO₃ net effluxes were measured into media of different Cl, Br, or NO₃ concentrations, respectively, to determine and compare the relative rates of the extracellular anion-inhibitable components. They were 48, 160, and 230 mmol/(kg Hb·min), respectively, at a membrane potential of about −90 mV. This indicates that the anion-inhibitable efflux is not due solely to the return translocation of the empty transport site (“slippage”) because slippage should be independent of the chemical nature of the anion. Cl net efflux was also measured as a function of the intracellular Cl concentration into media containing either 0 or 50 mM Cl. Under both conditions, net efflux was linearly dependent on Cl, between 30 and 300 mM Cl, and was 0 when back-extrapolated to 0 Cl. This observation is not compatible with the slippage process, which under these conditions would have been expected to be independent of Cl above 15 mM Cl. It was concluded that slippage contributes negligibly to Cl net efflux even at low extracellular anion concentrations and that the alternative process of “tunneling”—that is, movement of the anion through the anion transporter without a conformational change in a channel-type behavior—is the major, if not the sole, mechanism underlying Cl conductance.

INTRODUCTION

The physiological function of the anion transporter of the human red cell membrane is primarily a very tightly coupled one-for-one exchange transport of extracellular with intracellular anions (for recent reviews, see Cabantchik et al., 1978; Knauf, 1979; Lowe and Lambert, 1983). Most of the kinetic evidence favors a ping-pong kinetic mechanism of anion exchange in which a single transport site is alternately accessible to the two sides of the membrane (Gunn and Frohlich, 1979; Grinstein et al., 1979; Knauf et al., 1980; Passow et al., 1980; Jennings, 1982).

Recently, attention has shifted to another transport mode of the anion transporter, the anion net transport or conductance pathway, whose existence has
been known for a number of years (Hunter, 1971, 1977; Kaplan and Passow, 1974). The rate of Cl net transport is at least 1,000 times slower than the rate of Cl exchange, and it is mediated by the anion transport protein (Knauf et al., 1977). Only recently a more detailed look was taken to determine its possible mechanism (Kaplan et al., 1980, 1983; Knauf and Law, 1980; Knauf et al., 1983a; Fröhlich et al., 1983). It appears from these studies that Cl net transport cannot be completely described by the carrier-type kinetic scheme that served to characterize the Cl exchange mode. In the framework of this carrier scheme, Cl net transport was envisioned to be mediated by the "slippage" process (Vester-gaard-Bogind and Lassen, 1974; Kaplan and Passow, 1974; Knauf and Law, 1980), that is, the occasional translocation reaction of the unloaded anion transporter. The basic observation common to all these recent studies was that Cl net transport was not abolished at high Cl concentrations. Under these conditions, one would have expected full saturation of all anion transport sites and thus no empty sites available for the slippage reaction. In order to account for this phenomenon, it was postulated that Cl was also able to move through the anion transport protein without the need of a conformational change. I have named this process "tunneling" (Fröhlich, 1981; Fröhlich et al., 1983); it has been referred to as "barrier transit" or "ionic diffusion" by others (Knauf and Law, 1980; Kaplan et al., 1980, 1983; Knauf et al., 1983a).

The evidence against the existence of the slippage reaction at high anion concentrations is unambiguous and thus strongly favors the tunneling hypothesis under physiological conditions where only 2-3% of the transport sites are unoccupied and face the extracellular medium. However, it has not been resolved whether slippage occurs under conditions that are theoretically more favorable, such as in the absence of extracellular transportable anions. If, for example, Clo = 0, then virtually all transport sites are recruited into an outward-facing and unloaded state so that a maximal number of transport sites are available to return empty and thus to mediate net efflux by the slippage mechanism (Fröhlich et al., 1983). It has previously been shown that under these conditions of desaturating the outward-facing transport sites with decreasing Cl concentrations, net efflux is strongly increased, thus defining kinetically a Clo-dependent (Clo-inhibitable) component of Cl net efflux. This component was tentatively attributed to the slippage process and was named the "slippage component" (Fröhlich et al., 1983). According to this assignment, the slippage process was the main contributor to anion net efflux at low external anion concentrations, and tunneling was the predominant, if not the sole, component at high concentrations. An alternative explanation for the Clo dependence of Cl net efflux was put forward by Knauf et al. (1983a). They proposed that tunneling is possible through either the inward-facing or the outward-facing conformation of the transporter. Since increasing the extracellular Cl concentration causes the transport site to shift from an outward-facing to an inward-facing conformation (Fröhlich, 1982; Fröhlich et al., 1983; Knauf and Mann, 1984), one would expect that such a shift might also affect the observed net transport rates if the rates of tunneling through the different conformations are different. In other words, the slippage reaction might not contribute to anion net efflux at all, not even under slippage conditions.
The present paper reports on a study to distinguish between these two possible explanations of the Cl dependence of Cl net efflux at low extracellular Cl concentrations. I have used two specific predictions of the slippage scheme to test for the contribution of the slippage process to the overall Cl net transport rate. The experiments testing these predictions indicate that slippage does not appreciably contribute to the Cl conductance under any of the tested conditions, which contradicts previous assumptions. By elimination of the slippage process, the tunneling therefore becomes the most likely mechanism of net transport through the anion transporter of the red cell membrane. Knauf et al. (1983b) have reached this conclusion through a different argument, testing the action of a noncompetitive exchange inhibitor on Cl net efflux at low and high extracellular Cl concentrations. Preliminary reports on the data presented here have been given elsewhere (Fröhlich, 1983, 1984).

MATERIALS AND METHODS

The basic techniques for preparing cells and for measuring fluxes are the same as in a previous report (Fröhlich et al., 1983). The only significant difference is that in all experiments gramicidin was used as the voltage-clamping ionophore instead of valinomycin. Also, since gramicidin does not discriminate sufficiently among the alkali cations, K had to be replaced by the impermeant Tris instead of Na for establishing a K diffusion potential.

Preparation of Cells

All experiments were performed with cells from the author. For each experimental series, fresh blood was drawn into a heparinized container. White cells and plasma were removed by centrifugation and aspiration, and the red cells were washed three times in a medium containing 150 mM KCl (or KBr or KN0), 27 mM glycylglycine, pH 7.8 at 0°C, and bubbled extensively with nitrogen to remove CO2. The washed cells were then incubated in media of the same composition plus gramicidin at a hematocrit of 3–5%. The gramicidin was added from an ethanolic stock solution (0.4% wt/vol; 40 µl/ml of packed cells). The cells were incubated for 30 min on ice with occasional gentle agitation and were then collected by centrifugation and packed in nylon tubes (Gunn and Fröhlich, 1979; Fröhlich et al., 1983). These gramicidin-treated cells had a cation permeability comparable to the Cl exchange permeability as judged from K-Na exchange measurements, so that the cation conductance is at least 100 times higher than the Cl conductance (Fröhlich et al., 1983).

For changing the intracellular Cl concentration, the washed red cells were subjected to nystatin dialysis (Cass and Dalmek, 1975) prior to exposure to gramicidin. For this, media were prepared containing different concentrations of KCl, 20 mM glycylglycine, and 30 mM sucrose, pH 7.8 at 0°C, plus different concentrations of nystatin. Nystatin was added from a 50-mg/ml stock solution in ethanol/dimethylsulfoxide (1:1). The cells were incubated repeatedly in these media at a hematocrit of 5% to equilibrate the cell contents with the KCl concentrations of the media. The first incubation (5–10 min on ice) was at a nominal nystatin concentration of 0.1 mg/ml suspension, and the second incubation was at a concentration of 0.05 mg/ml. The cells to be equilibrated with <100 mM KCl were first equilibrated with 100 mM KCl and then with the intended KCl concentration, in order to minimize lysis. After equilibration of the cell contents with the dialysis media, the cells were washed at least five times (six to eight times for low KCl cells) at room
temperature in their respective dialysis media, but without nystatin (with a hematocrit of <5%). This removed the nystatin sufficiently well to restore a low background permeability for the electrolytes, as judged by the magnitude of the DNDS-insensitive net fluxes. These cells were then exposed to gramicidin in media containing the appropriate KCl concentrations plus 27 mM glycyglycine, pH 7.8 at 0°C, as outlined above, and packed in nylon tubes.

**Efflux Media and Reagents**

In the experiments with different anionic species, the efflux media were prepared from four N<sub>2</sub>-bubbled stock solutions: type A contained 150 mM KCl (or KBr or KNO<sub>3</sub>), 27 mM glycyglycine; type B, 120 mM Tris-Cl (or Tris-Br or Tris-NO<sub>3</sub>), 50 mM Tris base; type C, 25 mM K-citrate, 200 mM sucrose; and type D, 25 mM Tris-citrate, 200 mM sucrose, all with pH 7.8 at 25°C. These solutions were mixed to give media containing a K<sup>+</sup> concentration of 4 mM and different anion concentrations, with K<sup>+</sup> being substituted for by Tris<sup>+</sup> and Cl<sup>-</sup> (or Br<sup>-</sup> or NO<sub>3</sub><sup>-</sup>) being replaced by citrate/sucrose (Gunn and Fröhlich, 1979).

In the experiments with different intracellular Cl concentrations, the efflux media contained 50 mM KCl plus Tris-Cl, 27 mM glycyglycine, and sucrose to match the osmolality of the intracellular constituents. The K<sup>+</sup> concentration in the medium was designed to be 1/10 of the intracellular K<sup>+</sup> concentration, with Tris<sup>+</sup> added to allow for a Cl concentration of 50 mM. Intracellular K and Cl concentrations (in millimoles per kilogram cell water) were calculated from the cellular K and Cl and water contents (Gunn and Fröhlich, 1979).

The flux-stopping media consisted of 120 mM Tris-Cl, 50 mM Tris base, with 50 µM DNDS (4,4'-dinitrostilbene-2,2'-disulfonate) added as inhibitor of anion net flux (Fröhlich et al., 1983). For the experiments at high osmolalities, sucrose was added to achieve isotonicity.

DNDS was obtained from ICN K&K Rare Chemicals (Irvine, CA); gramicidin and nystatin were obtained from Sigma Chemical Co. (St. Louis, MO).

**Net Flux Experiments**

As previously, Cl net efflux was measured by determining the rate of K loss from the gramicidin-treated cells. All experiments were performed at 25°C. The packed cells (0.2-0.4 ml) were injected into the appropriate efflux medium (20 ml) in a thermostatted glass chamber and were rapidly dispersed by vigorous stirring. Duplicate samples of 2 ml were withdrawn at time intervals of 0.5–5 min and added to 8 ml of ice-cold stopping medium in polystyrene test tubes. Each tube also contained 0.5 ml dibutylphthalate on the bottom. The cells were passed through this oil layer by immediate centrifugation in a desk-top centrifuge. In the experiments calling for high sucrose concentrations in the stopping medium, the oil was omitted because its density was lower than the density of the aqueous phase, and the cell pellets were processed without the washing procedure below.

After centrifugation, the top aqueous layer was removed by aspiration, distilled water was layered on the oil for washing the tube walls, and water and oil were removed as much as possible by aspiration. The red cell pellet was then lysed in 2 ml of distilled water and the lysate was assayed. Hemoglobin (Hb) was determined spectrophotometrically with modified Drabkin's reagent (van Kampen and Zijlstra, 1961), and K was determined by atomic absorption flame spectrophotometry (model 460; Perkin-Elmer Corp., Norwalk, CT). The slope of the calculated K content per kilogram Hb vs. time yielded the net flux in units of millimoles of K<sup>+</sup> (or Cl<sup>-</sup>) (kg Hb·min). The time points were always chosen so as to lie in the linear phase of net efflux (usually <30% completion).
Control of Intracellular pH

During the anion net flux experiments, the red cells are exposed to K and Cl concentration gradients, both of which could contribute the driving force to potential proton movements. Protons could either move through the poorly cation-selective gramicidin channels, or they could move as a result of chloride-bicarbonate exchange via the anion transporter. In the first case, coupling to the K concentration gradient would cause proton movements into the cell, and in the second case, coupling to the Cl concentration gradients would drive protons (or proton equivalents) out of the cell (Jennings, 1976). In control experiments, I tested for possible pH shifts during the time course of the net flux experiments. Extracellular pH was monitored by a pH electrode in the flux chamber, and intracellular pH was determined in the hemolysates of the cell pellets obtained after centrifugation through the oil layer. Extracellular pH shifted by <0.03 pH unit within 4 min. The shift of intracellular pH was biphasic, consisting of an initial increase that was nearly complete by the time of the first time point (~20 s) and a slow upward drift. Both components were independent of the presence of gramicidin or valinomycin but depended on the initial Cl gradient. They were decreased in N₂-bubbled media and in the presence of DNDS, and were increased when small amounts of bicarbonate were added. This indicates that most of the observed pH changes were due to CO₂ contaminations and were not mediated by gramicidin. In N₂-bubbled media, the intracellular pH typically rose from 7.35 (before addition to the medium) to 7.6–7.7 (low Clₑ) or 7.4–7.5 (high Clₑ) within 30 s and then to 8.0–8.2 (low Clₑ) or 7.6 (high Clₑ) within 4 min. From these experiments, it cannot be ruled out that some of the observed proton outward movement was compensated for by gramicidin-mediated Kₐ-Hₑ exchange, which would simulate (DNDS-insensitive) Cl net efflux. However, these fluxes appear to be very low because one would have expected an intracellular acidification with high Clₑ media where only very little proton extrusion by chloride-bicarbonate exchange is expected because of the absence of a large Cl gradient.

Theoretical Considerations

It was previously proposed (Fröhlich et al., 1983) that anion net efflux is mediated by two different transport modes of the anion transporter, the slippage mechanism and the tunneling mechanism. The slippage mode is the mechanism expected for a transporter exhibiting classical carrier kinetics. In this mode, net transport is achieved by a reaction cycle in which (a) the substrate binds to the transport site on one side of the membrane; (b) the loaded transport site is translocated across the membrane by means of a (or a series of) conformational change(s); (c) the substrate is released on the other side of the membrane; and (d) the transporter undergoes the return reaction in the unloaded state.¹ The overall net transport cycle by the slippage mechanism therefore involves two conformational changes for each substrate molecule transported.

In the tunneling mode, on the other hand, an anion is assumed to move through the anion transporter without the need of a conformational change. This is tantamount to channel-like behavior.² I assumed in the modeling of anion net transport that the anion transporter has a specialized channel-like structure, which has both the carrier-type and the channel-type properties that are necessary

¹ It is the last step in this sequence that is referred to as the slippage reaction step and which gave the transport mode its name.
² The name "tunneling" was chosen to imply the movement of the anion through a channel-like structure. It does not imply a process analogous to quantum-mechanical tunneling.
to account for the anion exchange and net fluxes (Frohlich et al., 1983). For the sake of initial simplicity, I adopted the asymmetric, two-barrier, single-site channel of Läuger (1980), which can undergo conformational changes between two fundamental states. The two states of this channel differ from each other in that the relative heights of the two barriers are reversed. The two states are equivalent to states in our carrier-type scheme, which was initially developed to model anion exchange transport. For example, the conformation in which the outer barrier is lower is the same as the unloaded and outward-facing transporter conformation, \( C_o \), of the carrier scheme (Frohlich, 1984). In the same way, \( C_i \) symbolizes the channel conformation in which the inner barrier is lower, and \( C_Cl_o \) and \( C_Cl_i \) symbolize the corresponding states with the anion binding site occupied by Cl. Tunneling can occur in both directions and through both conformational states, \( C_i \) (and/or \( C_Cl_i \)) and \( C_o \) (and/or \( C_Cl_o \)), and the two basic conformational states may or may not have the same rate of tunneling (Knauf et al., 1983a). Also, because of the asymmetric potential energy profiles, the tunneling conductance for each conformation could be asymmetric, that is, different in the two directions of flow.

There are two alternative explanations for the dependence of Cl net efflux on Cl\(_o\). They are illustrated in Fig. 1. In the first explanation (Fig. 1a), which was the working hypothesis of our previous paper, net efflux is divided into two parts, a Cl\(_o\)-inhibitable component and a Cl\(_o\)-independent component. The Cl\(_o\)-inhibitable component is due to slippage ("slippage component") and the Cl\(_o\)-independent component is due to tunneling ("tunneling component"). Net efflux decreases with increasing Cl\(_o\), because with increasing Cl\(_o\), more extracellular Cl ions bind to the outward-facing transport sites and fewer empty transporter molecules are available for the slippage reaction. The tunneling component depends primarily on the intracellular Cl concentration, Cl\(_i\) (which is kept constant), and thus is essentially constant over the measured range of Cl\(_o\).

In the alternative explanation (Fig. 1b), originally proposed by P. A. Knauf (personal communication), the slippage reaction is not responsible for the Cl\(_o\) dependence of net efflux. Rather, the change of net efflux with Cl\(_o\) is attributed to a shift of the transporter among different conformations with changing Cl\(_o\). It has previously been shown (Frohlich et al., 1983) that at 0 Cl\(_o\), all transporter molecules are expected to be in the conformation \( C_o \), in which the anion binding/transport site is unloaded and faces the extracellular medium. With increasing

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5 This model is similar to the more descriptive model of Knauf et al. (1985a), with the potential advantage that it is directly translatable to a kinetic scheme for experimental testing.

4 Note that both states, although of extremely low conductance, are "open" states. The channel considered here has no "closed" state.

5 A third component that is insensitive to the anion transport inhibitor DNDS is neglected here because it is probably "nonspecific" and does not involve the band 3 protein.

6 For thermodynamic reasons, the tunneling component does depend on Cl\(_o\), as well, because at sufficiently high Cl\(_o\), the overall driving force would promote net influx. However, this dependence on Cl\(_o\) is only very slight and is not detectable in the studied range because net efflux would only be 0 if \([K]\times[Cl] = [K_o]\times[Cl_o]\), i.e., at Cl\(_o\) = 3.8 M in intact cells. The strong hyperbolic decrease of net efflux at low Cl cannot be explained by such a thermodynamic argument and has to be attributed to a kinetic mechanism.
Figure 1. Schematic representation of the two alternative explanations for the Cl\textsubscript{o} dependence of Cl\textsubscript{net} efflux. Shaded areas under the curve represent DNDS-inhibitable flux components; unshaded areas are the fluxes in the presence of DNDS and are considered a nonspecific and not band 3-mediated component. The inserts indicate the mechanism responsible for net efflux in the Cl\textsubscript{o} range below. They show the barrier profile of the simple asymmetric two-state channel with the high and the low energy barrier and the central binding site. The cytoplasmic end of the channel is on the left. The ion movement during tunneling is symbolized by the two curved arrows above the barriers. (a) At high Cl\textsubscript{o}, all of net efflux is due to outward tunneling (mainly through the inward-facing conformation), and at low Cl\textsubscript{o}, net efflux mediated by slippage is the major component. (b) All DNDS-inhibitable fluxes are due to tunneling, at Cl\textsubscript{o} = 0 through the outward-facing state, and at high Cl\textsubscript{o} mainly through the inward-facing state. In this scheme, the slippage reaction does not contribute at all to the net flux, and the inhibition by Cl\textsubscript{o} is brought about by the recruitment of the transporter from outward-facing to inward-facing.
Cl\textsubscript{o}, the sites are not only loaded but can also undergo inward translocation. As a consequence, the transporter molecules shift from an outward-facing to an inward-facing conformation until, at high Cl\textsubscript{o}, most of the transport sites face inward because of the intrinsic asymmetry of the transport mechanism (Knauf and Mann, 1984). If outward tunneling through the outward-facing conformation is assumed to be faster than outward tunneling through the inward-facing conformation, then the conformational shift with changing Cl\textsubscript{o} results in a decrease of the rate of net efflux with increasing Cl\textsubscript{o} (Knauf et al., 1983a; Fröhlich et al., 1983).

It is also possible that both alternatives apply and that the Cl\textsubscript{o}-inhibitable flux component is due to both slippage and tunneling. There are two criteria that one can use to estimate the relative contributions of slippage and tunneling. Both are based on specific predictions about the slippage mechanism and kinetic knowledge derived from previous anion exchange experiments. First, net efflux by the slippage mechanism should be the same for different anionic species even if their rates of tunneling differ. Second, net efflux by slippage should be maximally activated at low intracellular Cl concentrations (Cl\textsubscript{i} < 15 mM) and above that should be independent of Cl\textsubscript{i} (see below).

The first criterion is based on the expectation that the rate of anion net transport by the slippage mechanism should be the same for different anions, as long as they are exchanged much more rapidly across the membrane than they are transported in a net fashion. A relatively higher rate of exchange transport means that the rate of the translocation of the loaded transporter is faster than that of the unloaded transporter (the latter being the slippage step). The slippage step is therefore the rate-limiting step in the (slippage) net transport cycle. The slippage step does not involve an anion and is therefore independent of the nature of the transported anion. Judging from their rates of heteroexchange transport with Cl, both Br and NO\textsubscript{3} are exchanged rapidly (at least 10\% of the rate of Cl exchange; Gunn and Fröhlich, 1979; unpublished observations). For all three anions, Cl, Br, and NO\textsubscript{3}, net flux should be rate-limited by the anion-independent slippage step and should therefore have the same rate. If the amplitude of the trans-inhibitable component of net efflux (the slippage component) is not the same for all three anion species, this means that slippage is not the only contributing mechanism.

Knauf et al. (1983a) have provided arguments which at first might appear similar to this slippage criterion, but which are not applicable to the present experimental conditions. They argued that if the affinities of the transport site are different for different anions, then the extent of loading of the transport site by the different anions would be different at a given concentration. Consequently, the concentration of unloaded transport sites capable of the slippage reaction would be different, and the rates of net transport by slippage would occur in an order opposite to the order of the apparent transport affinities. This argument might be applicable for conditions under which not all transport sites are saturated; that is, at concentrations little above the half-saturation constant, $K_{\text{m}}$, $K_{\text{m}}$ is on the order of several millimolar if only the extracellular anion concentration is varied and it is $\leq 60$ mM if the anion concentration on both
sides of the membrane is varied (Gunn and Fröhlich, 1979). Knauf et al. (1983a) performed their experiments with halide concentrations of ~150 mM, which under their conditions should saturate nearly all of the outward-facing transport sites that are necessary for net efflux by slippage irrespective of the nature of the anion. A more stringent test is the test proposed here, namely to determine the amplitude of the Cl-dependant net efflux component that is obtained from the difference between net efflux at zero and at high extracellular anion concentration (150 mM).

The second criterion is also based on the rate-limiting nature of the slippage reaction step. Let us assume that there are no countertransportable anions available in the extracellular medium. In this case, it takes only very few loaded inward-facing transport sites (which are translocated rapidly) to match the rate of return by slippage. In other words, very low concentrations of Cl should be sufficient to recruit the transporter molecules predominantly into the outward-facing conformation, C0. Net efflux by slippage should therefore be maximally activated at very low values of Cl, far below the 60 mM that is the value of the apparent transport activation constant of Cl exchange by Cl (Gunn and Fröhlich, 1979). In an analogous argument, Jennings (1980) has explained the Cl dependence of influx of slowly transported extracellular sulfate in exchange for Cl. He estimated a half-saturation constant for Cl of <2 mM. One would therefore expect that Cl net efflux into 0 Cl would be essentially independent of Cl at Cl > 15 mM if it is mediated by the slippage mechanism.

RESULTS

The previous experiments on the concentration dependence of Cl net flux were performed with valinomycin-treated red cells to make the red cell cation-permeable for establishing cation (K⁺) diffusion potentials as the driving force of anion net flux (Fröhlich et al., 1983). It was demonstrated that under some conditions, the valinomycin-induced K⁺ conductance was not sufficient to clamp the membrane potential at the K⁺ diffusion potential. In the experiments presented here, the voltage-clamping ionophore was gramicidin, which is capable of introducing at least 100 times higher cation permeabilities (Fröhlich et al., 1983). In the data shown in Fig. 2, the membrane potential should therefore be nearly the same at all values of Cl (around ~90 mV). As previously, Cl net efflux decreased with increasing Cl at constant Cl. At high Cl, the DNDS-inhibitable flux decreased to slightly above 10% of its maximal value at Cl = 0. For comparison, net efflux by the slippage mechanism should have decreased to <3% of its maximum. As outlined previously (Fröhlich et al., 1983; Knauf et al., 1983a; Kaplan et al., 1983), this shows that slippage cannot be a significant contributor to net transport at high Cl.

Hunter (1977) and Knauf et al. (1983a) have shown that the Br net permeability at high Br is higher than the Cl net permeability at high Cl. These findings are confirmed in Fig. 3, where Br net efflux was measured from gramicidin-treated red cells into media containing different concentrations of Br. The shape of the curve is qualitatively similar to the data obtained for Cl (Fig. 2), but throughout, the Br flux rates are higher than those of Cl. Further-
more, the difference between the two curves is larger at low than at high extracellular anion concentrations. In other words, the amplitude of the slippage component is different for Cl and Br net efflux. This is the experimental test of the first criterion discussed in Theoretical Considerations. If the slippage com-

**Figure 2.** Cl net efflux from gramicidin-treated red cells in the absence (open circles) and the presence (closed circles) of 50 μM DNDS in the efflux medium. The membrane potential, calculated from the K concentration ratio (K_i = 140 mM, K_o = 4 mM), was about -90 mV.

**Figure 3.** Br net efflux under the same general experimental conditions as in Fig. 2, into media of varying Br concentrations (open circles). For comparison, the Cl data of Fig. 2 were included (+). Closed circles: Br net efflux in the presence of 50 μM DNDS.
Figure 4. NO₃ net efflux under the same general experimental conditions as in Figs. 2 and 3, into media of varying NO₃ concentrations. Fluxes were measured in the absence (○) and presence (×) of 50 μM DNDS.

Figure 5. Cl⁻ net efflux from nystatin-dialyzed cells containing different concentrations of KCl into isotonic media containing 50 mM Cl⁻ and a K concentration 1/10 as high as the intracellular concentration. Only the DNDS-inhibitable fluxes are shown. The DNDS-insensitive fluxes were in the range of 5 mmol/(kg Hb·min). The different symbols represent two different experimental series.
ponent had been due solely to slippage, then it should have had the same amplitude for Cl and Br. The data therefore indicate that the slippage mechanism is not the only mechanism contributing to the Cl$\text{in}$ (Br$\text{in}$)-inhibitable component of Cl (Br) net efflux. This notion is confirmed by the data in Fig. 4, which show that the NO$_3^-$-inhibitable component of nitrate net efflux is even larger than the corresponding component of Br or Cl net efflux.

If Cl net efflux at high Cl$\text{in}$ proceeds by a channel-type mechanism, one would expect it to depend on the intracellular Cl concentration. This hypothesis was

![Graph](image-url)

**Figure 6.** Cl net efflux from nystatin-dialyzed cells containing different concentrations of KCl into isotonic media containing 25 mM (K plus Tris), citrate plus sucrose, with the K concentrations adjusted to be $\frac{1}{10}$ as high as the intracellular concentration. The data are from two experimental series. Circles: DNDS-free efflux media; crosses, X's: efflux media containing 50 $\mu$M DNDS. Linear regression analysis of the data in the presence of DNDS (+, x) yields an extrapolated y-intercept (with standard deviation) of 8.1 ± 1.2 mmol/(kg Hb·min). The data in the absence of DNDS exhibit an intercept of 8.2 ± 0.5 (solid circles between Cl$\text{in}$ = 24 and 145 mM) or 6.6 ± 3.5 mmol/(kg Hb·min) (using all open and solid circles up to Cl$\text{in}$ = 300 mM, except open circles at Cl$\text{in}$ = 62 mM).

tested by measuring Cl net efflux from nystatin-dialyzed cells into media of high Cl concentrations. The different batches of cells in these experiments contained different concentrations of KCl, and the extracellular K$^+$ concentrations were chosen to give a 10-fold K$^+$ concentration gradient for constant membrane potential of about −60 mV throughout. Fig. 5 shows that Cl net efflux increases linearly with increasing Cl$\text{in}$ over a wide range of concentrations.

A similar dependence on Cl$\text{in}$ might also be expected for net efflux at 0 Cl$\text{in}$, if the Cl$\text{in}$-inhibitable component were mediated mainly by the tunneling mechanism. Alternatively, if the Cl$\text{in}$-inhibitable component were mediated by the
slippage mechanism, it would be independent of Cl above Cl\textsubscript{i} = 15 mM, as outlined in Theoretical Considerations above. This is the second criterion by which the relative contributions of slippage and tunneling to net efflux can be evaluated. Fig. 6 shows that also at Cl\textsubscript{o} = 0, Cl net efflux from nystatin-dialyzed cells depends linearly on Cl\textsubscript{i} over a wide range of Cl\textsubscript{i}. Furthermore, it is significant that extrapolation of the data points yields 0 Cl\textsubscript{o}-independent (and DNDS-inhibitable) flux at Cl\textsubscript{i} = 0, within the error limits of the experiment. This extrapolated flux rate would equal the contribution of the slippage process to net efflux if both mechanisms were operative in parallel. The fact that the extrapolation reveals only a Cl\textsubscript{o}-dependent component argues very strongly that the slippage mechanism plays only a very minor role in the Cl conduction process and that within the present error limits it is not measurable.

**DISCUSSION**

The aim of this study has been to test Cl net efflux with two different kinetic criteria and to estimate the relative contributions of two previously proposed mechanisms to the overall rate of net efflux at low anion concentrations. The two mechanisms are the slippage mechanism and the tunneling mechanism. The slippage mechanism is typical for a carrier-type transport mechanism, and the high rate of Cl exchange vs. net transport qualifies the anion transporter kinetically as a carrier-type transporter. The tunneling mechanism, on the other hand, is typical for a channel-type transport mechanism, which proceeds without a need for conformational changes of the transport protein. Läuger's (1980) simple two-conformation channel model provided the unifying scheme that could combine the carrier- and channel-type properties necessary to explain the experimental data, at least qualitatively. The purpose of this work is to provide a quantitative description of the relative importance of the two modes of anion net transport. My strategy was to test specific predictions possible for the slippage mechanism. The experimental data indicate that not only must there be another mechanism besides slippage contributing to the Cl\textsubscript{o}-inhibitable Cl net efflux, but also that this additional mechanism is the major, if not the sole, contributor to anion net flux. Since the data virtually exclude the slippage mechanism, the tunneling mechanism appears to be the best candidate to take its place. If tunneling is the mechanism responsible for anion net flux, then several conclusions can be drawn about the properties of the proposed channel-type transport pathway of Cl net transport.

(a) Despite the familiar channel-like appearance, the anion transporter should not be compared with other "true" channel-forming proteins. In what one might call the single-channel conductance of the anion transporter, the maximal Cl net efflux of 60 mmol/(kg Hb·min) at \( V_m = -90 \text{ mV} \) is equivalent to \( 3 \times 10^{-18} \) S per transport molecule—that is, about five to six orders of magnitude smaller than the cation channels of excitable membranes. Also, the maximal turnover number of about 20 Cl ions/s (at Cl\textsubscript{o} = 0) transported at 25°C by the tunneling mechanism needs to be compared with the turnover number of ~13,000 ions/s for Cl exchange at the same temperature (estimated from Brahm, 1977). This means that the rate of conformational change of the loaded transporter is much
higher than the rate by which Cl moves through the anion transporter without conformational changes.

(b) The rate of tunneling through the two basic conformations is different. Outward tunneling through the outward-facing conformation appears to be faster than through the inward-facing conformation. Whether this reflects a different height of the main barrier in the two conformations or different well depths of the binding site, or both, is not known. However, an answer to this question awaits further experimentation to test whether the simple potential energy profile assumed for the tunneling of Cl is adequate (see next paragraph).

(c) Läuger's two-barrier, single-site model, which has been used up to now for describing the basic concepts of slippage and tunneling, is probably an oversimplification. This is exemplified by a disagreement between the data presented in Fig. 5 and a prediction by the simple model for the Cl dependence of net efflux. One would expect that the rate of outward tunneling through Cl/CCl would be half-saturated at about the same concentrations of Cl as is Cl exchange. This is a consequence of the assumption that for both processes the inward-facing transport site needs to be loaded with Cl before translocation either by conformational change or by tunneling can take place. At high Cl, a large portion of the transport sites are in the inward-facing state (Knauf and Mann, 1984). One would therefore expect that net efflux would increase with increasing Cl with a half-saturation constant of ~60 mM. Fig. 5 shows that this is not the case. In fact, at least some of the nonlinearity at high Cl may be due to the fact that the Cl concentration, not activity, was used for the abscissa. At the present time, it is not possible, because of a lack of detailed experiments, to determine how the simple two-conformation channel model would have to be modified to describe the observations in a more quantitative manner. A possible modification of the simple model that could account for the discrepancy is to assume the existence of "pre"-binding sites on the transport pathway of Cl. Such sites would have to be in series with the central anion binding/transport site, located near the entry of the specialized channel with the function of guiding the anions to the translocation site. In terms of a formal kinetic scheme, this would correspond to a three-site, four-barrier scheme (or other, more complex schemes; see also the zipper model of Wieth et al. [1982] mentioned below). Another possible mechanism that might exhibit little saturation would be the one proposed by Knauf et al. (1983a), where an ion from the inside of the cell displaces the ion at the outward-facing transport site.

Finally, one could possibly construct a mechanism permitting conformational changes of the transporter that are controlled by low-affinity allosteric binding of Cl and thus lead to the linear dependence of Cl net efflux on Cl over a wide concentration range (P. A. Knauf, personal communication). Such a modified slippage scheme would in effect negate the arguments presented here, as well as all other arguments that have been put forward in favor of the tunneling (barrier transit/ionic diffusion) concept. It cannot be disproved by the available evidence, but it appears unlikely because of restraints imposed by some of this evidence. For example, the hypothetical allosteric binding site must be accessible to Cl on the inward-facing and on the outward-facing transporter, because the inhibition
of Cl net efflux by Cl, is the same over nearly the entire range of Cli (compare Figs. 5 and 6). Furthermore, since Cl net efflux is increased by increasing Cl, even at high Clo, where virtually all outward-facing transport sites ought to be occupied, this allosteric effect somehow has to affect not only the slippage but also the translocation of the loaded transporter, in a way that leads to Cl net transport. This complicates the modified slippage scheme to such an extent that the simpler tunneling concept becomes more attractive, irrespective of the appeal the tunneling concept has because of its implications about the structure of the biological carrier.

The conclusion of this study, that the slippage process contributes only negligibly to the Cl conductance in the presence of unloaded transporter (at low Clo), contrasts with our previous assumptions (Fröhlich et al., 1983). It was tentatively assumed that the Cl-inhibitable component was due to slippage, which would have meant that slippage was the major component of net efflux into 0-Cl media. I concluded from studies on the potential dependence of net efflux into Clo = 0 that the anion binding site bears a net positive charge that is translocated during the slippage reaction step. This line of evidence has lost its relevance with my new findings. Nevertheless, other evidence remains that speaks in favor of or is consistent with the notion of a net positive charge on the transport site that is neutralized when a Cl anion is bound: it has been shown that Cl exchange is not affected by changes in the membrane potential (Gunn and Fröhlich, 1979; Fröhlich et al., 1983), and Milanick and Gunn (1984) have shown that sulfate tracer influx into Cl-containing cells is enhanced by a positive (inside) membrane potential at pHo = 9.5, where it enters as a divalent anion, but not at pHo = 5.5, where it enters together with a proton. Finally, the large discrepancy between the translocation rate of the Cl-loaded site and the rate of slippage speaks in favor of a charge movement during slippage and of the absence of charge movement during Cl translocation.

The differences in the translocation rates of the empty and the loaded transport site is even larger now than previously estimated. At 25°C, the Cl exchange cycle, including both the inward and outward translocation of the loaded site, has an estimated turnover rate of 13,000 s⁻¹ (band 3 monomer)⁻¹ (Brahm, 1977). This is >10⁴ times faster than the rate of slippage under comparable conditions estimated from the present experiments: <1 s⁻¹. One explanation for this large difference is the proposed difference in the net charge on the empty and loaded transport sites (Milanick and Gunn, 1984), but the charge difference is probably not the only factor that influences the translocation rate.

Recently, Wieth and co-workers (1982) proposed a model of the anion transporter that is similar to the specialized channel model of this study. Based on their studies on the reactivity of several essential sites on the band 3 protein with protons and residue-specific inhibitory agents, they have constructed the zipper model of anion exchange. In this model, the anion transporter has a channel-like structure and possesses at least two sets of ion pairs made up of negatively and positively charged amino acid residues (arginine and possibly aspartate or glutamate). Cl exchange is mediated in this model by conformational changes resulting in the relative arrangement of the two residues in the ion pairs and
thus the energy barrier profile of the channel. This model could account for
slippage and tunneling in the same way as my specialized channel. The main
difference between the two models is that the zipper model is more specific
about the nature of the energy barriers.

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