Relationship of Net Chloride Flow across the Human Erythrocyte Membrane to the Anion Exchange Mechanism

PHILIP A. KNAUF, FOON-YEE LAW, and PAMELA J. MARCHANT

From the Research Institute, Hospital for Sick Children, Toronto Ontario, Canada M5G 1X8, and the Department of Radiation Biology and Biophysics, University of Rochester School of Medicine, Rochester, New York 14642

ABSTRACT The parallel effects of the anion transport inhibitor DIDS (4,4'-diisothiocyanostilbene-2,2'-disulfonate) on net chloride flow and on chloride exchange suggest that a major portion of net chloride flow takes place through the anion exchange system. The "slippage" model postulates that the rate of net anion flow is determined by the movement of the unloaded anion transport site across the membrane. Both the halide selectivity of net anion flow and the dependence of net chloride flux on chloride concentration over the range of 75 to 300 mM are inconsistent with the slippage model. Models in which the divalent form of the anion exchange carrier or water pores mediate net anion flow are also inconsistent with the data. The observations that net chloride flux increases with chloride concentration and that the DIDS-sensitive component tends to saturate suggest a model in which net anion flow involves "transit" of anions through the diffusion barriers in series with the transport site, without any change in transport site conformation such as normally occurs during the anion exchange process. This model is successful in predicting that the anion exchange inhibitor NAP-taurine, which binds to the modifer site and inhibits the conformational change, has less effect on net chloride flow than on chloride exchange.

INTRODUCTION

Although small anions such as Cl^- and HCO_3^- are exchanged very rapidly across the red cell membrane (Brahm, 1977; Klocke, 1976; Wieth and Brahm, 1980), the net flow of anions (accompanied by cations) occurs much more slowly. For example, the apparent permeability of the membrane to Cl^- as measured by isotope exchange is $\sim 4 \times 10^{-4}$ cm/s, whereas the permeability for net chloride flow is only $\sim 2.5 \times 10^{-8}$ cm/s (Hunter, 1971, 1977; Knauf et al., 1977; Schubert and Sarkadi, 1977), $\sim 10,000$ times less than the exchange permeability. This finding has two principal implications. The first is that the anion exchange mechanism is very tightly coupled, a fact which strongly suggests that anions do not diffuse freely across the membrane, but rather cross the membrane by combining with some membrane component, a

"carrier" in the most general sense (Gunn, 1972, 1979; Cabantchik et al., 1978; Knauf, 1979). The second is that because of the relatively low permeability of the membrane to net anion flow, comparatively small changes in cation permeability (Glynn and Warner, 1972; Hoffman and Knauf, 1973) or cation pump rate (Hoffman et al., 1979) can have a large effect on the membrane potential.

The mechanism of the anion exchange process has been extensively investigated (Dalmark, 1975a, b; 1976; Gunn et al., 1973; Gunn, 1978, 1979; Gunn and Fröhlich, 1979; Schnell et al., 1978), and has led to the formulation of a ping-pong carrier model for the transport mechanism shown in Fig. 1.
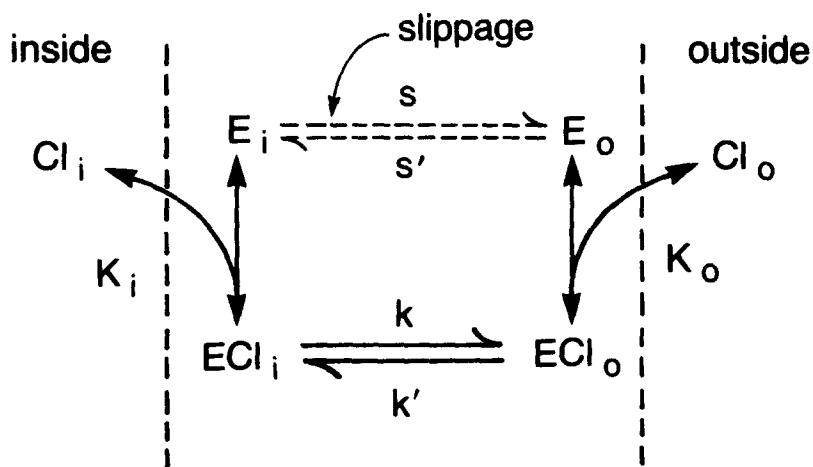

FIGURE 1. Schematic model for the anion exchange system. The anion exchange protein can exist in either of two conformations, one in which the transport site faces the inside of the cell (E_i) and another in which the transport site faces outward (E_o). K_i and K_o are the dissociation constants for chloride at the inside and outside of the membrane, respectively; k is the rate constant for the conformational change from inside-facing to outside-facing when the transport site is loaded with chloride; k' is the rate constant for the reverse conformational change. The constants s and s' are the corresponding rate constants for the conformational change when the transport site is empty. If s and s' are zero, the conformational change can only occur when an anion is bound, so an obligatory one-for-one exchange of anions takes place. If s and s' are non-zero, the system can change conformation without transporting an anion. This "slippage" of the unloaded carrier results in net transport of one anion when the transport site binds an anion and goes back across the membrane.

According to this model, anions cross the membrane by combining with the substrate site, designated E, after which they cross the membrane as an E-Cl complex. The tightly coupled exchange is accounted for by assuming that the unloaded forms of the carrier, E_o and E_i, cannot cross the membrane. Studies with chemical probes (Cabantchik and Rothstein, 1974; Passow et al., 1975) have shown that anion exchange is mediated by an integral 95,000-mol wt

transmembrane protein known as band 3 (Fairbanks et al., 1971), which is asymmetrically oriented in the membrane and which comprises ~25-30% of the total membrane protein (Fairbanks et al., 1971; Steck, 1974). The transport process almost certainly does not involve diffusion of an actual carrier across the membrane, but rather a conformational change in the band 3 protein, such that the transport site changes from inside-facing to outside-facing or vice versa (Gunn, 1978; Knauf, 1979), a process which is kinetically equivalent to the carrier model (Patlak, 1957). Dalmark (1976) has obtained evidence for a second anion binding site of lower affinity, known as the modifier site. When anions are bound to this site, translocation of the substrate site-anion complex is inhibited.

The net anion transport process has been much less thoroughly characterized, despite its importance in determining the membrane potential. In particular, the relationship of net anion flow to the anion exchange system is controversial. On the one hand, net anion flow exhibits a different temperature dependence (Hunter, 1977; Cass and Dalmark, 1973), pH dependence (Scarpa et al., 1970; Knauf et al., 1977), and anion selectivity (Passow et al., 1975; Knauf et al., 1977; Hunter, 1977) from that of anion exchange. Certain inhibitors, notably phlorizin, have different effects on net and exchange chloride permeability (Kaplan and Passow, 1974). On the other hand, dipyridamole has similar effects on net and exchange chloride permeability (Hoffman and Knauf, 1973; Cotterrell, 1975), and Kaplan et al. (1976) have found a parallelism between the side dependence of the effects of certain inhibitors on net and exchange chloride transport. Perhaps the strongest evidence for a common mechanism is the finding that the highly selective inhibitor of anion exchange DIDS (4,4'-diisothiocyano-2,2'-stilbene disulfonate) inhibits net and exchange fluxes of chloride and sulfate in parallel (Knauf et al., 1977). Under the conditions of these experiments, DIDS binds very selectively to band 3 (Ship et al., 1977), which suggests that the band 3 protein is involved in both net and exchange anion flow. Further evidence for this is provided by the finding that vesicles from which almost all of the major membrane proteins other than band 3 have been extracted still manifest DIDS-sensitive net and exchange sulfate fluxes (Wolosin et al., 1977).

In terms of the carrier mechanism shown in Fig. 1, the parallel effects of inhibitors on net and exchange fluxes could be most simply explained by supposing that the net flow represents a small component of "slippage" of the unloaded carrier (E_i or E_o) across the membrane (Vestergaard-Bogind and Lassen, 1974; Sachs et al., 1975; Kaplan et al., 1976; Knauf et al., 1977; Cabantchik et al., 1978; Gunn, 1978). Binding or covalent reaction with inhibitors such as DIDS would reduce the total number of active carriers, and thereby would inhibit both net and exchange flows in parallel. Since the rate-limiting step for net flow would be different from that for exchange (translocation of the unloaded and loaded carriers, respectively), differences in the pH dependence, temperature dependence, and response to certain noncompetitive inhibitors would be expected. This model provides a single explanation for both net and exchange anion flow, and it further suggests that studies of

net anion flow may provide insights into certain steps of the anion exchange mechanism.

This model has the further advantage that it makes certain striking predictions which can be tested experimentally. In the first place, since it is the flow of unloaded carrier that is rate-limiting for net anion transport, the net flow rate for different anions would be expected to be more nearly similar than the exchange rates for the same anions, which are determined by the translocation rate for the carrier-anion complex. The experimental results are in qualitative agreement with this prediction (Knauf et al., 1977; Hunter, 1977; Gunn, 1978). Furthermore, it would be predicted that those anions that bind more strongly to the substrate site should have lower net permeabilities, since net flow depends on the amount of unloaded carrier, and this decreases if the carrier is complexed with anions. A test of this prediction is presented in the first section of the Results.

The slippage model also predicts that as the concentration of any anion is increased, the net flux of that anion should decrease. This is so because as the anion concentration is raised, more carrier-anion complexes are formed, and the amount of uncomplexed carrier decreases. Since the net flow depends on the amount of unloaded carrier, it must also decrease (see Appendix). Stimulated by Passow's (1977) report that net sulfate fluxes increase with increasing sulfate concentration, contrary to expectation, we examined the dependence of net chloride flux on the chloride concentration. This requires that the cells be loaded with different KCl concentrations by using the antibiotic nystatin (Passow, 1977; Dalmark, 1975b). To guard against the possibility of nonspecific leak pathways induced by nystatin treatment, we compared the fluxes in cells with and without nystatin treatment, and in addition used sensitivity to DIDS as a marker for the portion of net anion flux that is apparently associated with the exchange pathway. Preliminary reports of such experiments (Knauf and Law, 1980; Kaplan et al., 1980) suggest that the net chloride flux increases with increasing chloride concentration, contrary to the slippage model.

In earlier experiments with DIDS (Knauf et al., 1977), we found that ~20–30% of the net chloride flux was not inhibited by DIDS, even when 99% of the exchange flux was inhibited. This DIDS-insensitive flux might represent a separate pathway for net chloride flow, or it might simply reflect a partial inhibition by DIDS of the net flow through the exchange mechanism. To investigate this question, we have examined the effects of different inhibitors and of changes in the chloride concentration, pH, and temperature on these two components of the net chloride flux.

METHODS

Nystatin Treatment

Blood was obtained from apparently healthy adults with heparin as an anticoagulant. The cells were washed three times in 160 mM NaCl and 5 mM HEPES at room temperature and the white cells were removed by aspiration.

All solutions were titrated to pH 7.2 at room temperature, unless otherwise stated. The cells were divided into three portions and treated with nystatin according to a modification of Dalmark's method (1975*b*). The first portion (A) was washed in 75 mM KCl, 120 mM sucrose, and 5 mM HEPES, and then resuspended in the same solution (ice cold) at 10% hematocrit with 75 μg/ml nystatin (E. R. Squibb & Sons, Inc., Princeton, NJ) (added as a 5-mg/ml solution in methanol) for 10 min. The cells were then centrifuged and resuspended in ice-cold 75 mM KCl, 27 mM sucrose, and 5 mM HEPES with 75 μg/ml nystatin. The cells were incubated for 10 min on ice and were then centrifuged and washed five times at room temperature in 75 mM KCl, 27 mM sucrose, and 5 mM HEPES without nystatin and were stored overnight in this buffer. On the next day, the cells were washed two more times in the same buffer, brought to 50% hematocrit, and used for the flux measurements. The second portion of cells (B) was treated in a similar manner, except that the cells were treated with nystatin in 160 mM KCl, 27 mM sucrose, and 5 mM HEPES, and washed in the same buffer. The third portion of cells (C) was treated and washed with 300 mM KCl, 27 mM sucrose, and 5 mM HEPES.

It was found necessary to keep the ice-cold cells in these high-potassium media since otherwise the cells lose potassium before the flux measurement. This presumably occurs because nystatin is much more effective at low temperatures (Cass and Dalmark, 1973), so a tiny amount of residual nystatin can cause a substantial K^+ loss over a period of hours in ice-cold cells, even though no effect of nystatin pretreatment was seen at 37°C. In an earlier series of experiments (Knauf and Law, 1980), cells were stored on ice in low-K^+ medium, and this caused a loss of potassium from the cells before some of the flux determinations. This in turn (because of the experimental design, in which fluxes for some conditions were measured before others) led to a systematic underestimation of the fluxes at high chloride concentrations in some experiments.

Net KCl Flux

To begin net KCl efflux, cells were suspended at 0.1% hematocrit in a flux medium at 37°C, pH 7.04, which contained 1.33 μM valinomycin (Calbiochem-Behring Corp., La Jolla, CA) and 1.33% ethanol. For measurements of the DIDS-sensitive component of net Cl flux, 10 μM DIDS was added to the flux medium. For the A cells the flux medium was 70 mM NaCl, 5 mM KCl, 5 mM HEPES, and 27 mM sucrose; for the B cells it was 150 mM NaCl, 10 mM KCl, 5 mM HEPES, and 27 mM sucrose; and for the C cells the medium was 280 mM NaCl, 20 mM KCl, 5 mM HEPES, and 27 mM sucrose.

Samples were taken as soon as possible after the start of the flux and usually at 5-min intervals thereafter. For each sample, ~10 ml of the suspension was poured into a 50-ml centrifuge tube, and 20 ml of ice-cold washing buffer was added. Since lowering the temperature virtually stops the valinomycin-mediated flux, the time of the sample was taken as the time when the ice-cold buffer was added. For the A cells the washing buffer was 75 mM NaCl and 5 mM HEPES; for the B cells it was 160 mM NaCl and 5 mM HEPES; and for the C cells it was 300 mM NaCl and 5 mM HEPES. The cells were washed twice in the washing buffer and then lysed in 2.2 ml distilled de-ionized water. 1 ml of the lysate was added to 2 ml Drabkin's solution for determination of hemoglobin as cyanmethemoglobin (Crosby et al., 1954) by measuring optical density at 540 nm against a cyanmethemoglobin standard (Hycel Inc., Houston, TX). A second aliquot of the lysate was appropriately diluted for K^+ determination by flame photometry (National Instrumentation Laboratories), using lithium as an internal standard.

Since the cells were stored in media containing KCl, some K^+ was added to the flux medium with the cells. To determine this, samples of the medium were taken for K^+ determination by flame photometry. For the experiments reported here, the external K^+ for the A cells was 5.76 mM ± 0.15 mM (SD); for the B cells it was 11.3 ± 0.3 mM; and for the C cells it was 23.6 ± 1.1 mM.

Determination of Cell K^+, Cl^-, and Water

For determination of the intracellular chloride, cells were suspended at 15% hematocrit in the buffer used for nystatin treatment (without nystatin), containing 0.1 μCi/ml ^{36}Cl (Amersham Corp., Arlington Heights, IL, or ICN Chemical and Radioisotope Div., Irvine, CA) and 1 μCi/ml [^3H]-methoxyinulin (New England Nuclear, Boston, MA). The suspension was incubated at 37°C for 10 min and then centrifuged at 13,000 rpm in an HB-4 rotor in a Sorvall RC-2B centrifuge (E. I. DuPont de Nemours and Co., Newtown, CT). Aliquots of 25 μl of the cell pellet or the supernatant were added (Aliquanter; Hamilton Co., Reno, NE) to 1 ml of 5% trichloroacetic acid (TCA), and 0.8 ml of the supernatant was counted in 8 ml Aquasol (New England Nuclear) in a Packard liquid scintillation counter (Packard Instruments, Inc., Downers Grove, IL). Additional 25-μl aliquots were lysed and diluted in Drabkin's solution for hemoglobin measurement, and the remainder of the cell pellet was weighed, dried for at least 24 h at 80°C, and then reweighed for determination of the wet and dry weight. From these measurements and an assumed cell density of 1.097, we calculated the ratio of intracellular to extracellular chloride concentration and the amount of hemoglobin per liter cell water. The intracellular chloride concentration was then determined as the product of the chloride ratio and the extracellular chloride concentration.

For measurement of intracellular potassium concentration, 100-μl aliquots of the 50% cell suspensions used for the flux experiments were washed twice in the appropriate wash buffer to remove external potassium. The pellets were then resuspended in 2.2 ml distilled deionized water and samples were prepared for determination of hemoglobin and potassium as described above for the timed flux samples. From the measurement of potassium per kilogram of hemoglobin and the value of kilograms of hemoglobin per liter cell water determined above, the potassium in millimoles per liter cell water was calculated. For the pH experiments in Fig. 6, the potassium was determined directly by flame photometry of a dilution of the radioactive cell pellet used for measuring the chloride ratio.

Treatment of Cells with DIDS or NAP-Taurine

Fresh red cells were washed three times with 150 mM NaCl, 10 mM KCl, and 5 mM HEPES (HNK), pH 7.2, at room temperature. They were then treated with various concentrations (0–10 μM) of DIDS at 25% hematocrit for 30 min at 37°C in the same buffer. In the case of NAP-taurine, cells were suspended at 9% hematocrit with an NAP-taurine concentration of either 136 or 500 μM and were irradiated with white light from a tungsten source for 10 min at 0°C as described previously (Knauf et al., 1978b). After treatment, all cells were washed twice in HNK plus 0.5% bovine serum albumin (Sigma Chemical Co.), then once in HNK. The cells were stored overnight in 27 mM sucrose, 10 mM glucose, 112 mM KCl, and 38 mM NaCl, to minimize changes in the intracellular ion concentrations. On the next day, a portion of each cell sample was washed twice in HNK, brought to 25% hematocrit, and 1 μCi of ^{36}Cl was added to 0.2 ml of this suspension, which was then incubated at room temperature for 15 min and then on ice for 15 min. Chloride exchange was measured by the rapid

filtration technique of Dalmark and Wieth (1972) as previously described (Knauf et al., 1978a). The following day, another portion of each cell sample was washed twice in buffer and net KCl flux was measured as described above for B cells (with nystatin treatment omitted).

Theory and Calculations

When high-K^+ red cells are suspended in low-K^+ media in the presence of valinomycin, there is a net KCl loss from the cells, accompanied by an isotonic flow of water. As a result, the volume of the cell decreases in parallel with its K^+ content, so the K^+ concentration remains relatively constant. For this reason, the driving force for the net KCl efflux does not change very much, so the flux is quite constant during the first 30–50% of intracellular K^+ loss (see Fig. 4). The net K^+ efflux can therefore be determined from the initial slope of a plot of the cell K^+ content per kilogram of hemoglobin vs. time (see Fig. 4). Since net flows of H^+, OH^-, and HCO_3^- are small in comparison with the net K^+ flow under these conditions (Knauf et al., 1977), the net K^+ flow is nearly equal to the net Cl^- flow.

If one assumes that the flows of ions are independent of each other and that there is a constant electric field through the membrane, the membrane potential (inside with respect to outside) is given by the Goldman-Hodgkin-Katz equation (Goldman, 1943; Hodgkin and Katz, 1949):

$$E_m = \frac{RT}{F} \ln \frac{P_K K_o + P_{Cl} Cl_i}{P_K K_i + P_{Cl} Cl_o}. \tag{1}$$

Only terms in K^+ and Cl^- are included, since in the presence of valinomycin these are by far the most permeable ions. Under the conditions of these and our previous (Knauf et al., 1977) experiments, the external potassium concentration is appreciable. Thus, at high valinomycin concentrations (high P_K) the $P_K K_o$ and $P_K K_i$ terms dominate the numerator and denominator of Eq. 1, so the membrane potential becomes nearly equal to the Nernst potential for potassium, that is, $E_m = (RT/F)\ln(K_o/K_i)$.

The net flow of chloride may be described by the Goldman flux equation as:

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

where $B = \exp(-FE_m/RT)$. Since under our conditions P_{Cl} has very little effect on E_m (and therefore on B), the net chloride flux is almost a linear function of the net chloride permeability, P_{Cl}. In particular, increases in the K permeability have very little effect on the net KCl efflux (Knauf et al., 1977). It is therefore comparatively easy and accurate to calculate P_{Cl} from the measured KCl flux and the internal and external K^+ and Cl^- concentrations. For our calculations, we used a value of P_K at 1.33 μM valinomycin of 3.56 mmol/kg hemoglobin·min·mM, determined from ^{42}K exchange experiments (Knauf et al., 1977), but inaccuracies in this value would have little effect on the results. This is in sharp contrast to other methods (Hunter, 1971, 1977; Kaplan et al., 1980 and unpublished data), in which the precision of the P_{Cl} value depends on the accuracy of both the net flux determination and the P_K value.

One additional advantage of our method is that the ratio of internal and external K^+ concentrations can be held constant, thereby keeping the membrane potential nearly constant. This condition is difficult to achieve with techniques in which external K^+ is low, but may be necessary for precise results, since in a separate study

we found that the value of P_{Cl} is strongly dependent on the membrane potential (Knauf and Marchant, 1977).[1]

Fluxes are most easily expressed in millimoles per kilogram hemoglobin per minute, and the driving force (the terms other than P_{Cl} on the right-hand side of Eq. 2) in millimolar, so the permeability is given in units of millimoles per kilogram of hemoglobin per minute per millimolar. To convert this to the commonly used unit of min^{-1}, the permeabilities should be multiplied by kilograms of hemoglobin per liter cell water, which for the experiments reported here, except for the pH experiments in Fig. 6, was ~0.412 ± 0.024 (SD).

An advantage of expressing the permeabilities in units of millimoles per kilogram of hemoglobin per minute per millimolar is that the permeability is not affected by the initial cell volume, as are permeabilities expressed in min^{-1}. Since the amount of hemoglobin is linearly proportional to the number of cells, permeabilities in these units provide an easy means of calculating the flux per cell if the driving force from the Goldman equation (Eq. 2) is known. We have chosen not to express fluxes or permeabilities per unit of surface area, both because of the variations in literature values for surface area and because at least for the DIDS-sensitve fluxes, the anions flow through discrete sites in the membrane, rather than through the entire membrane surface. Thus, the total flux is related to the number of transport sites per cell, and the surface area of the cells is irrelevant. If it is desired for comparative purposes to convert to units of centimeters per second, this can be done by multiplying the permeability in millimoles per kilogram of hemoglobin per minute per millimolar by the factor 4.3×10^{-7}, calculated from the values of surface area and volume given in Knauf et al., 1977.

Treatment of Red Cells with Inhibitors of Water Permeability

Fresh red cells were washed and treated with 10 μM DIDS as described above. After three washes with PBS (1 part 310 mosmol phosphate buffer, pH 7.4 [Dodge et al., 1963], 9 parts 165 mM NaCl), portions of the cells were treated with either 1 mM PCMBS (parachloromercuribenzenesulfonic acid) or 1 mM DTNB (5,5'-dithio-bis-2-nitrobenzoic acid) (concentration in the medium at time zero) at 25% hematocrit for 60 min at 21-22°C in phosphate-buffered saline (PBS). The cells were then washed three times in 20 mM Tris, 130 mM NaCl, and 10 mM KCl, pH 7.4, and the net K^+ efflux at 37°C in the presence of 1.33 μM valinomycin was measured as described above. To check the effectiveness of PCMBS, net K^+ efflux was measured with 0.1 mM ouabain and without valinomycin. In all experiments reported, PCMBS pretreatment caused at least a fivefold increase in the net K^+ efflux.

Halide Substitution Experiments

Fresh red cells were washed three times in solutions consisting of 136 mM NaX, 10 mM KX, 0.5 mM Na metabisulfite, and 20 mM HEPES, pH 7.85, at room temperature, where X was Cl, Br, F, or I. Portions of the cells were then treated with 10 μM DIDS as described above. Efflux of K^+ was measured with 1.33 μM valinomycin as described above, except that X^- replaced Cl^- in the flux and washing buffers. In the case of fluoride, cells were washed in medium containing 146 mM NaF, and the flux was measured in HEPES-buffered medium containing 136 mM NaF and 10 mM

[1] Knauf, P. A., P. J. Marchant, and F.-Y. Law. Effects of potassium concentration and membrane potential on the apparent net chloride and sulfate permeabilities of the human erythrocyte membrane. Manuscript in preparation.

KCl. Free anion distribution ratios were estimated by measuring the chloride ratio with 1 mM NaCl added to the buffer, using the technique described above. Chloride ratios were similar for the various halide media, and varied from 0.635 to 0.794 in the different experiments.

pH Experiments

Fresh blood was washed three times in 160 mM KCl, 27 mM sucrose, and 5 mM either HEPES or PIPES buffer, pH 7.2, at room temperature, and the buffy coat was removed. Cells were resuspended at 10% hematocrit and washed three times in the same buffer, except at different pH values. Before each centrifugation, the cells were allowed to stand 5 min at 37°C to permit pH equilibration. Since even this treatment did not result in complete pH equilibration of the cells in low pH buffer, for the experiments with PIPES at pH 6.1 at 37°C, the cells were titrated to the desired pH by bubbling CO_2 through the suspension, after which the cells were washed three times in PIPES buffer to remove CO_2 and bicarbonate. In all cases, the stated pH is that of the cell suspension in the last wash buffer at 37°C. The cells were made up to 20% hematocrit and fluxes were measured at 0.33% hematocrit with 1.33% ethanol and 1.33 μM valinomycin at 37°C as described for the nystatin-treated cells, using media with 150 mM NaCl, 9 mM KCl, 27 mM sucrose, and 5 mM HEPES or PIPES, to give a final external K^+ concentration of ~10 mM. Chloride ratio, intracellular K^+ concentration, and cell water were measured as described above.

RESULTS AND DISCUSSION

Halide Selectivity

Since the affinities of the various halide anions for the anion exchange system are known (Dalmark, 1976), the rate of net flow for each halide can be calculated according to the "slippage" model. Those halides, such as iodide, which bind tightly to the substrate site, should reduce the number of unloaded transport sites and therefore should have a low net flow rate. To test this experimentally, red cells were equilibrated with solutions containing 10 mM KX, 136 mM NaX (where X is Cl$^-$, Br$^-$, F$^-$, or I$^-$), 20 mM HEPES (pH 7.85 at room temperature), and 0.5 mM sodium metabisulfite, to keep the halides in the ionized form (Parker et al., 1977). Net efflux of potassium in the presence of valinomycin was measured at 37°C.

Fig. 2 shows the net K^+ flux for cells equilibrated with either Cl$^-$ or I$^-$ medium and exposed to various valinomycin concentrations. For Cl$^-$ the flux tends to reach a plateau with increasing valinomycin concentration. This is expected since as the K^+ permeability is increased, net KCl efflux is limited by the rate of net Cl$^-$ flow (Knauf et al., 1977). In the case of iodide, the flux is larger and the plateau is reached at higher valinomycin concentrations. This would be expected if the membrane permeability for I$^-$ net flow is greater than for Cl$^-$, since the I$^-$ permeability should not become rate-limiting until the potassium permeability (that is, the valinomycin concentration) is raised to higher values.

The results in Fig. 2, which suggest that the net I$^-$ permeability is much greater than the net Cl$^-$ permeability, are in agreement with Hunter's (1977) values obtained by a different technique, and would seem to provide strong

evidence against the "slippage" hypothesis. This is only true, however, if the large net I^- flow is actually related to the anion exchange system and does not take place via a parallel pathway through the membrane. This latter possibility exists even though metabisulfite was present to prevent formation of I_2 and I_3^-. To test this, net K^+ fluxes with 1.33 μM valinomycin for I^- and the other halides were compared in DIDS-treated and control cells.
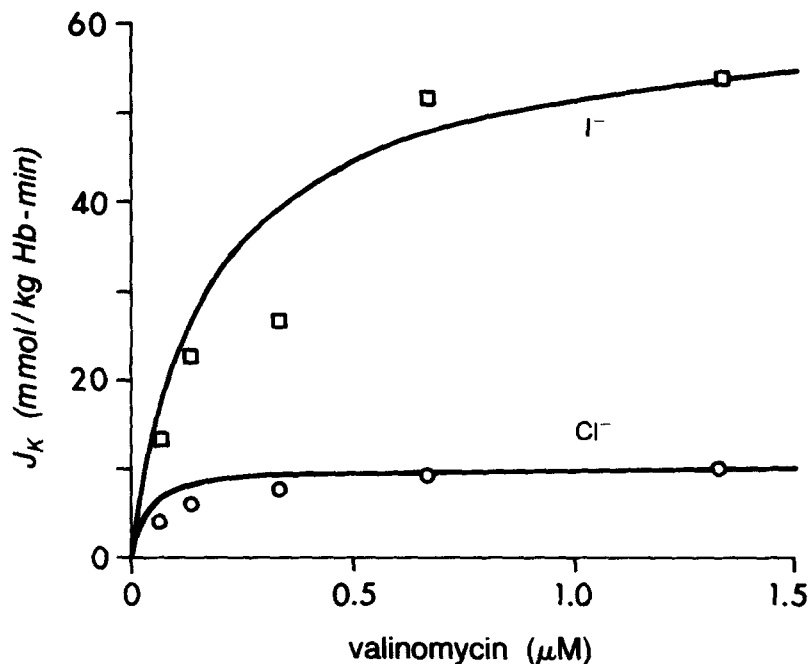

FIGURE 2.   Effects of valinomycin on net K^+ efflux in chloride or iodide media. Cells were washed in chloride or iodide media as described in Methods and the net KCl efflux into medium with 10 mM K^+ was measured after addition of various amounts of valinomycin. Solid lines are theoretical calculations of the K^+ flux, using P_K values for the different valinomycin concentrations determined previously (Knauf et al., 1977). For the upper line, the P_I value (determined from the rightmost point) was 0.2904 mmol/kg Hb·min·mM, whereas for the lower line, the P_{Cl} value was 0.0436 mmol/kg Hb·min·mM. The somewhat lower fluxes at low valinomycin concentrations, relative to the theoretical predictions, may be partly due to a decrease in net anion permeability which occurs at the lower membrane potential values under these conditions.[1]

After washing with the various halide solutions, the cells were divided into two portions, one of which was treated with 6.7 μmol DIDS per liter cell suspension (at 33% hematocrit) for 30 min at 37°C, then washed three times with buffer containing 0.5% albumin and three times with buffer. The results, shown in Table I, demonstrate that for I^- there is no statistically significant DIDS-sensitive net flux. A DIDS-sensitive component may exist, but it is

small and is completely masked by the scatter in the much larger DIDS-insensitive net I^- flux. Thus, the large net flux of iodide must take place by a parallel pathway through the membrane and is not relevant to the question of the halide selectivity of the net anion flux that takes place through the anion exchange mechanism (defined as the DIDS-sensitive component).

The absence of DIDS-sensitive iodide net flux might be explained on a trivial basis if there were some difference in the response of iodide exchange to DIDS, or if DIDS were a less potent inhibitor in iodide than in chloride media. That this is not the case is shown in Fig. 3, where the iodide exchange flux at 20°C as a percent of control is plotted against the number of DIDS molecules per cell to which the cells were exposed. For the low DIDS concentrations, where there is little nonspecific binding (Ship et al., 1977), there is a very good correlation ($r^2 = 0.994$) between the inhibition of iodide

TABLE I

NET K^+ EFFLUX AT 37°C FROM CELLS LOADED WITH DIFFERENT HALIDE ANIONS

Halide	Control		P	DIDS treated		P	DIDS sensitive		P
F^-	19.8±5.8	(3)	<0.02	4.3±1.2	(3)	<0.05	15.4±4.7	(3)	<0.05
Cl^-	9.5±1.4	(4)	—	2.1±0.4	(3)	—	7.0±1.4	(3)	—
Br^-	12.8±1.4	(3)	<0.05	3.3±0.8	(3)	<0.1	9.7±0.8	(3)	<0.05
I^-	52.1±18.5	(15)	<0.02	48.4±14.0	(11)	<0.001	10.1±16.1	(5)	>0.5

Cells were loaded with different halides as described in Methods. Net K^+ fluxes into media containing 10 mM K^+ in the presence of 1.33 μM valinomycin are expressed in millimoles K^+ per kilogram of hemoglobin per minute. Values are given ± standard error of the mean. The numbers in parentheses indicate the number of experiments. P values indicate the significance of the difference between the flux with a particular halide present and that with Cl^- present, as determined by an unpaired Student's t test. For the control and DIDS-treated cells, individual fluxes were compared statistically. The DIDS-sensitive component was determined by subtracting the average flux in DIDS-treated cells taken from a given donor from the average flux in control cells taken from the same donor. For this reason, the number of determinations of the DIDS-sensitive flux is smaller and the weighting of the observations is somewhat different.

exchange and the number of DIDS molecules per cell. The extrapolated value for the maximum number of molecules per cell required for 100% inhibition, assuming that every DIDS molecule added is bound to the transport sites, is $\sim 1 \times 10^6$. This is in very good agreement with estimates of the number of transport sites derived from measurements of the effects of DIDS (Ship et al., 1977) or other inhibitors (Zaki et al., 1975; Lepke et al., 1976; see summary in Knauf, 1979) on chloride or sulfate exchange fluxes and suggests strongly that iodide and chloride share a common exchange pathway, as would be expected since iodide is a competitive inhibitor of chloride exchange (Dalmark, 1976).

For the other halides (Table I), the difference between the net flux in the presence and absence of DIDS is highly significant ($P < 0.02$). For the DIDS-sensitive component, the flux with F^- is greater than that with Cl^-, which indicates a higher net permeability for F^- than for Cl^-. The increased flux

cannot be due to an increased K^+ permeability in the presence of F^-, since at 1.33 μM valinomycin the K^+ permeability is so large that a further increase in K^+ permeability causes no significant increase in net K flux (Knauf et al., 1977). The high net F^- permeability fits with the "slippage" model, since F^- has a lower affinity for the transport site than does Cl^-. On the other hand, the fact that the DIDS-sensitive net Br^- permeability is significantly ($P <$ 0.05) larger than that for Cl^- is not predicted by this model, since Br^- has a higher affinity for the transport site than does Cl^-, and hence should have a
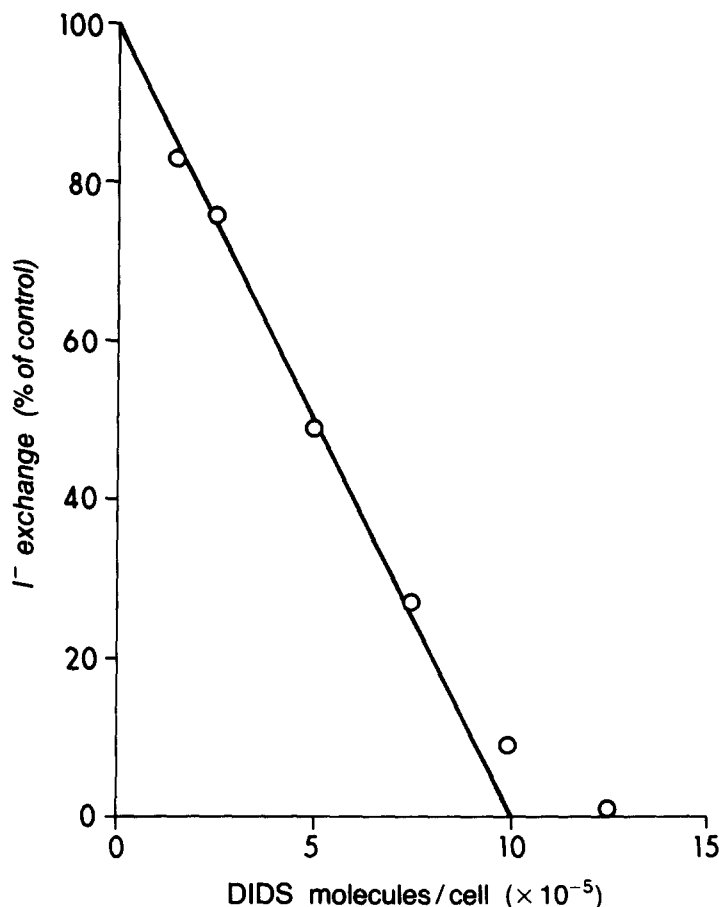

FIGURE 3. Effect of DIDS on iodide exchange. Aliquots of cells were treated with various amounts of DIDS as described in Methods. The iodide exchange rate constant (expressed as percent of control), measured at 20°C, is plotted against the number of molecules of DIDS added per cell. The straight line was fitted to the first four points by the method of least squares. Points for higher DIDS concentrations were not used since nonspecific binding becomes more prominent at higher concentrations (Lepke et al., 1976). The slope was −9.97 ± 0.31 (SD), which corresponds to 1.0 × 10⁶ molecules per cell required for complete inhibition.

lower net flow. Because of the comparatively small difference between Cl^- and Br^- affinities and the small difference in net fluxes, however, these results do not provide conclusive evidence against the model.

Effects of Chloride Concentration on Net Chloride Flux

To test the slippage model more stringently, cells were loaded with different KCl concentrations by use of nystatin, and the efflux of KCl into a low-K^+ medium was measured in the presence of 1.33 μM valinomycin. Under these circumstances, the K^+ permeability is high enough that the membrane potential is essentially equal to the potassium equilibrium potential (E_K), and the membrane potential was kept nearly constant by varying external K^+ together with internal K^+. Fig. 4 shows a plot of the potassium content
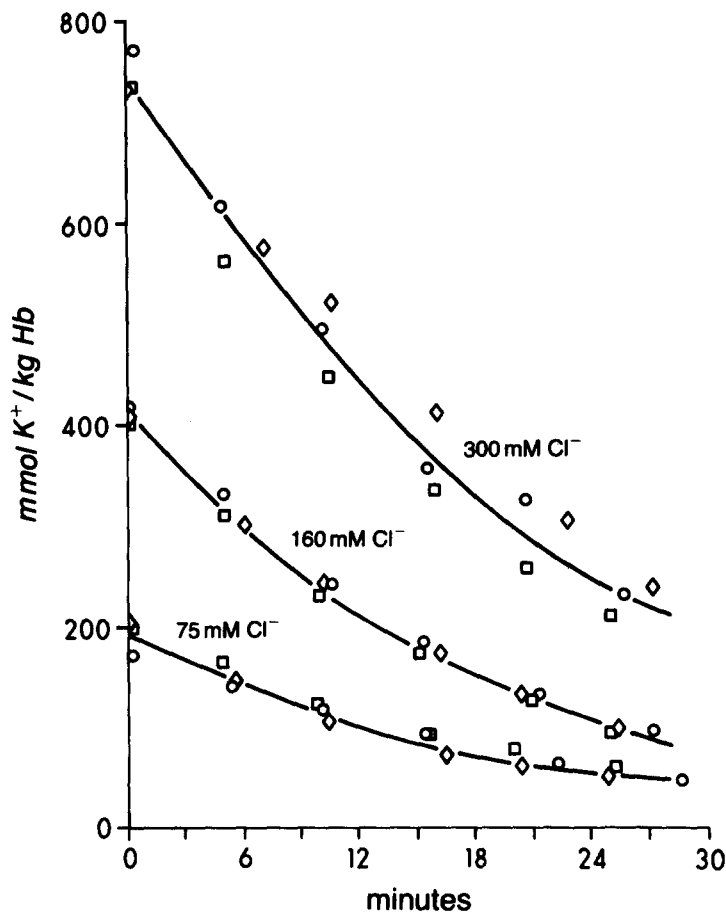

FIGURE 4. Loss of K^+ from cells treated with valinomycin in media with various Cl^- concentrations. Cell K^+ content was determined as described in Methods at various times after exposure of cells to 1.33 μM valinomycin at 37°C. O, □, and ◇ points refer to values from different experiments. Lines were drawn by eye.

(expressed as millimoles K^+ per kilogram of hemoglobin) of cells treated with nystatin in either 75, 160, or 300 mM Cl^- as a function of time after addition of 1.33 μM valinomycin. The initial time course of the efflux is linear, which permits extrapolation of the lines to zero time to obtain the initial rate of net K^+ loss. From Fig. 4 it is clear that the net KCl efflux increases as the chloride concentration increases, contrary to the predictions of the slippage hypothesis.

As in the case of the halide fluxes, however, this finding is only significant if the flux takes place via the DIDS-sensitive anion exchange system, and not by some parallel pathway through the membrane, perhaps resulting from nystatin treatment. This latter possibility would seem unlikely, since at 160 mM chloride, net KCl fluxes measured with and without nystatin treatment were not significantly different (data not shown). Nevertheless, to evaluate the effects of chloride concentration on the DIDS-sensitive and DIDS-insensitive components of net chloride flux, fluxes were measured in the presence and absence of 10 μM DIDS in the flux medium. The results for several such experiments are shown in Fig. 5. All the components of net chloride flux show an increase with increasing chloride concentration. For the DIDS-insensitive flux, the increase is nearly linear, whereas there is some tendency for the total and DIDS-sensitive components to saturate with increasing chloride concentration. There is also a tendency for the DIDS inhibition to decrease as the chloride concentration increases. This effect is not due to a decrease in the inhibitory effect of DIDS at high chloride concentrations (Shami et al., 1978), since the chloride exchange measured at 0°C was >99% inhibited by 10 μM DIDS, even at 600 mM chloride (data not shown).

The slippage model would predict that the DIDS-sensitive flux should decrease as chloride concentration increases, since there are fewer unloaded carriers to slip back across the membrane. The results are so dramatically different from this prediction that they make it very unlikely that the slippage mechanism accounts for any appreciable portion of net chloride flow under these conditions.

Effect of Inhibitors of Water Flow

The linearity of the response of net chloride flux to increases in chloride concentration, particularly in the case of the DIDS-insensitive net chloride flux, suggests that at least part of the net chloride flow may occur by free diffusion of the chloride across the membrane. Hunter (1977) has suggested that net anion flow might take place through the postulated "pores" that are thought to mediate most of the water flow across the membrane. If anions and water use the same channels, agents that block the flow of the very small water molecule would be expected to cause an equal or greater inhibition of net anion flow. To test this, we treated intact red cells with two sulfhydryl (SH)-reactive inhibitors, PCMBS and DTNB, at concentrations that have been reported to inhibit osmotic water permeability. Cells were pretreated with 1 mM PCMBS or DTNB for 60 min at 21–22°C, which should inhibit osmotic water transport by ~80 and 50%, respectively (Macey and Farmer, 1970; Naccache and Sha'afi, 1974). As can be seen from Table II, neither
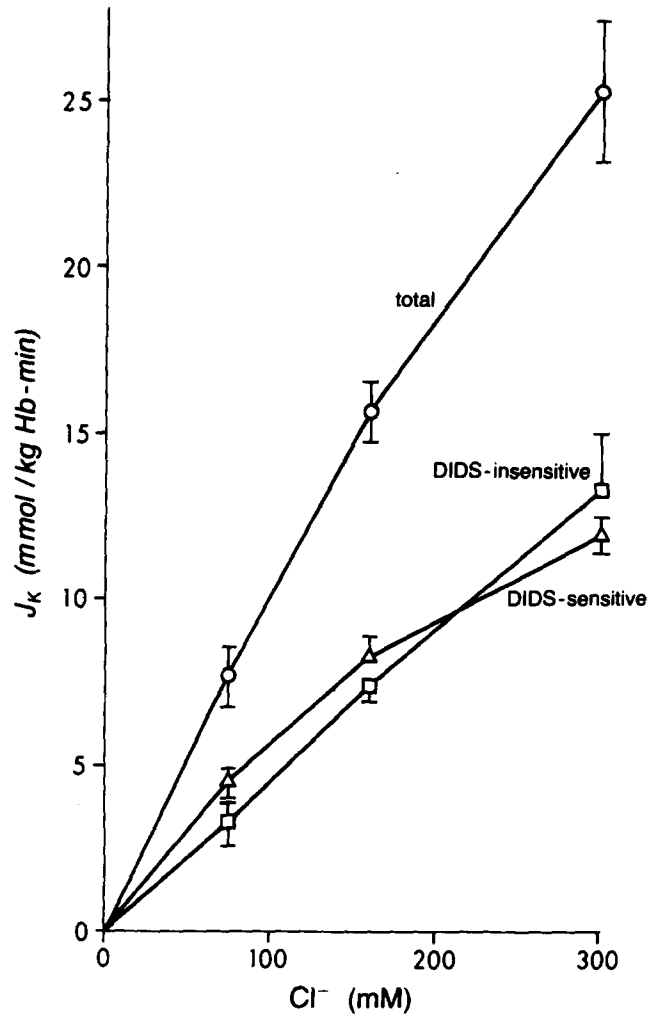
Figure 5. K^+ efflux from valinomcyin-treated cells in media with different Cl^- concentrations. Cells were loaded with different KCl concentrations by use of nystatin as described in Methods, and efflux of K^+ into low-K^+ media at 37°C in the presence of 1.33 μM valinomycin was measured. For the cells in 75 mM Cl^- medium, the external K^+ concentration was 5.76 ± 0.15 mM (SD), the chloride ratio was 0.935 ± 0.057, and the internal K^+ concentration was 86.1 ± 11.8 mM. For the cells in 160 mM Cl^-, K_o was 11.3 ± 0.3 mM, Cl_i/Cl_o was 0.954 ± 0.039, and K_i was 170.3 ± 15.4 mM. In 300 mM Cl^-, K_o was 23.6 ± 1.1 mM, Cl_i/Cl_o was 0.977 ± 0.053, and K_i was 297.1 ± 32.9 mM. For all experiments, the calculated membrane potential was between −63.1 and −74.4 mV. Symbols represent the mean of four experiments ± SEM. In each experiment, fluxes were determined in duplicate or triplicate.

PCMBS nor DTNB caused any significant reduction in either the total net chloride flux or its DIDS-sensitive or DIDS-insensitive components. Although there was considerable scatter in the data, the net chloride fluxes after treatment with PCMBS or DTNB tended to be, if anything, slightly larger than in the control cells. This result is the opposite of that predicted if anions flow through water channels, which suggests that no significant component of net anion flow takes place through this route.

Effects of pH

Gunn (1972) has postulated that the anion exchange carrier can exist in two forms. Titration with one hydrogen ion converts the monovalent anion carrier to a form that carries the divalent anion sulfate. It has been suggested (Gutknecht and Walter, 1982) that the divalent form of the carrier might

TABLE II

EFFECTS OF WATER PERMEABILITY INHIBITORS ON P_{Cl}

Experiment	Inhibitor		$P_{Cl}/P_{Cl}^{control}$[*]	DIDS insensitive[‡]	DIDS sensitive[§]
A	None		1.00±0.08 (2)[∥]	0.28±0.02 (2)	0.72±0.08
	PCMBS	(1 mM)	1.12±0.15 (3)	0.48±0.09 (3)	0.64±0.17
	DTNB	(1 mM)	1.88±0.60 (3)	0.25±0.09 (3)	1.63±0.61
B	None		1.00±0.04 (4)	0.14±0.04 (3)	0.86±0.06
	DTNB	(1 mM)	1.18±0.05 (4)	0.23±0.02 (3)	0.95±0.05

[*] P_{Cl} was measured as described in Methods and was expressed as a fraction of the value of P_{Cl} for untreated cells in the same experiment.

[‡] The DIDS-insensitive component of P_{Cl} was measured in cells that had been treated with 10 μM DIDS as described in Methods and was expressed as a fraction of the control P_{Cl} value.

[§] The DIDS-sensitive component of P_{Cl} was determined by subtracting P_{Cl} in the DIDS-treated cells from the total P_{Cl}.

[∥] Values are expressed ± standard deviation. The numbers in parentheses are the number of P_{Cl} determinations. None of the effects of DTNB or PCMBS on P_{Cl} were significant ($P > 0.05$), except for the slight increase in the control P_{Cl} by DTNB in experiment B, which was marginally significant ($0.02 < P < 0.05$). Two other experiments with 1 mM extracellular potassium instead of 10 mM gave similar results.

participate in net chloride flow by transporting two chloride ions in one direction and then returning with only a single chloride ion. Such a model would be capable of explaining the observed increase in net flux with increasing chloride concentration if the affinity of the divalent carrier for chloride were so low that it is not saturated at the chloride concentrations used in these experiments. This model would, however, imply that the flux should increase with decreasing pH, as more of the monovalent carrier is converted into the divalent form. Earlier investigations by ourselves (Knauf et al., 1977) and others (Scarpa et al., 1968, 1970), using light-scattering techniques, had suggested that chloride permeability does increase with decreasing pH. To further investigate this point, we measured the net chloride permeability over the range from pH 6.1 to 7.6, using the net K^+ efflux technique.

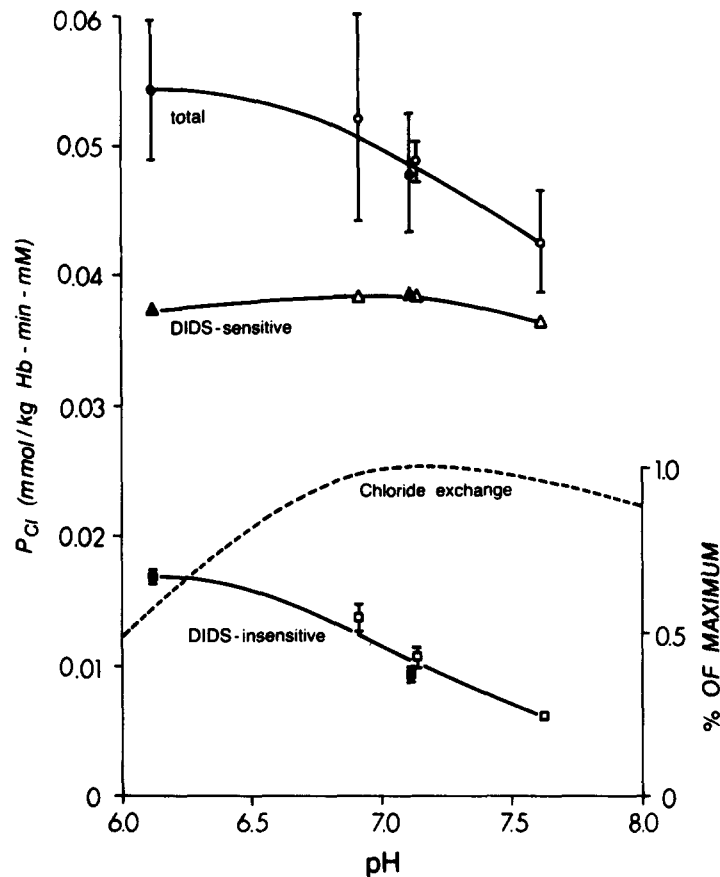As can be seen from Fig. 6, the total net chloride permeability increased

FIGURE 6. pH dependence of net chloride permeability. Solid symbols represent experiments with PIPES buffer, open symbols with HEPES. ●, ○:total P_{Cl}; ■, □:P_{Cl} in the presence of 10 μM DIDS; ▲, △:difference between P_{Cl} in the absence and presence of DIDS. Bars indicate SEM of at least 5 and up to 11 separate determinations of permeability in a series of 7 experiments. In all but one experiment, blood from a single donor was used, to minimize donor-to-donor variations in P_{Cl}. Calculated membrane potentials ranged from −49 to −74 mV. Standard errors are not shown for the DIDS-sensitive permeability; they are similar to those shown for the corresponding total P_{Cl}. For the DIDS-insensitive permeability, all of the values in the same buffer at different pH were significantly different ($P < 0.05$). For the total and DIDS-sensitive P_{Cl}, none of the values were significantly different ($P > 0.05$). pH values given are those of the suspension of cells in the last wash before the flux; the corresponding pH values of the flux media at 37°C are 6.12 and 7.12 for PIPES and 6.36, 7.04, and 7.80 for HEPES. Lines were drawn by eye. For comparison, the pH dependence of chloride exchange in red cell ghosts at 38°C (Brahm, 1977) is indicated by the broken line and the right-hand ordinate.

slightly with decreasing pH, but the increase was not statistically significant.[2] This was apparently entirely due to a very significant increase in the DIDS-insensitive component, with the DIDS-sensitive component showing no significant change. The increase in the DIDS-insensitive component was not due to a decrease in the reactivity of DIDS at low pH (Ship et al., 1977), since in these experiments DIDS was present in the flux media so the inhibition was independent of the covalent reaction of DIDS.

These results suggest that the DIDS-sensitive and DIDS-insensitive components of net chloride permeability have different pH dependencies and therefore are probably independent processes with different rate-determining steps. Both net flux components also exhibit a pH dependence that is significantly different from that of chloride exchange. Over a pH range where the chloride exchange decreases by >40%, the DIDS-sensitive net chloride permeability is unaffected, whereas the DIDS-insensitive component actually increases more than twofold. Although the increase in this latter component is highly significant, it is far less than the more than 10-fold increase that would be expected if net chloride flux were mediated by the divalent form of the anion exchange system. The same is true for the DIDS-sensitive component, which shows no change. Thus, the pH data argue strongly against involvement of the divalent form of the anion exchange carrier in net anion flow and suggest that different processes determine the rates of net and exchange anion flow.

Is the Net Chloride Flux Saturable?

The values for total, DIDS-sensitive, and DIDS-insensitive net chloride permeabilities, calculated from the flux experiments presented in Fig. 5, are shown in Fig. 7. For the DIDS-insensitive flux, there is no significant difference between the permeabilities measured at different chloride concentrations, which is consistent with the concept that this flux represents simple diffusion across the membrane, perhaps through the lipid bilayer. For the DIDS-sensitive component, however, there is a small but significant ($P < 0.02$) decrease in permeability as the chloride concentration is raised from 75 to 300 mM. Evidence for a decrease in P_{Cl} as the Cl^- concentration is increased from 50 to 100 mM has also been obtained by Kaplan et al. (1980 and unpublished data), using a different technique for measuring net Cl^- flux. At present, we cannot rule out the possibility that this apparent saturation is a side effect of the change in ionic strength, rather than representing true saturation behavior. Such saturation would, however, be expected if net anion flow involves binding to the anion exchange site, as suggested by the parallel effects of DIDS on net and exchange chloride fluxes (Knauf et al., 1977).

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[2] The difference in these results from those obtained earlier may possibly be attributed to the smaller pH range studied in the earlier experiments (Knauf et al., 1977), to the different method used for measuring net chloride fluxes, or to the higher membrane potential in the previous experiments.
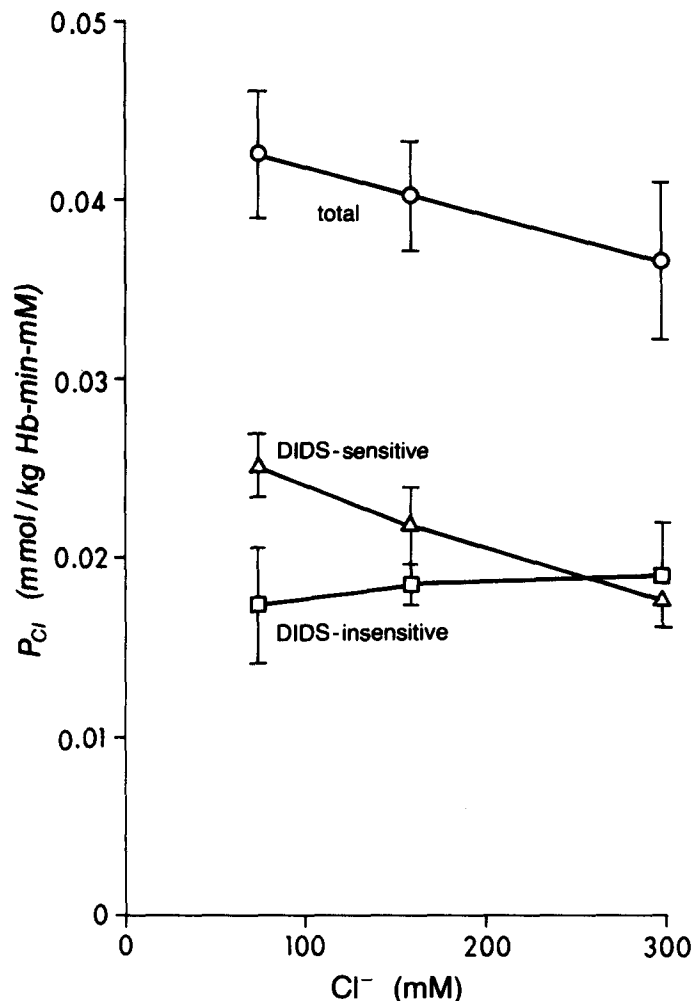
FIGURE 7. Variation of P_{Cl} with external Cl^- concentration. P_{Cl} values were calculated from the flux data shown in Fig. 5. P_{Cl} was calculated from each individual flux measurement, and the results for each experiment were averaged. Each symbol represents the mean of four such experiments ± SEM.

Model for Net Anion Flow through the Anion Exchange System

Recent evidence from the structure of the band 3 protein and from the kinetics of anion exchange suggests that the anion exchange system may function as a lock carrier (Gunn, 1978; Knauf, 1979). According to this model (Fig. 8), the transport protein can exist in two conformations, one in which the transport site faces inward (E_i) and the other in which it faces outward (E_o). This conformational change may be brought about not by movement of the transport site itself, but rather by a change in position of the diffusion barriers
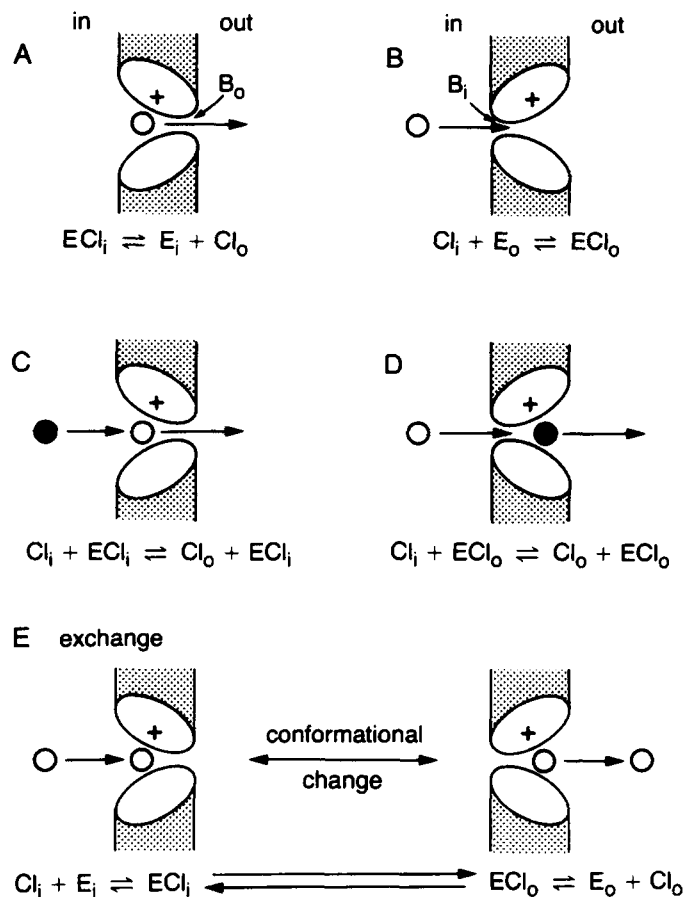
FIGURE 8. Model for net anion flow through the anion exchange system. Only processes resulting in Cl^- efflux are shown. Cl^- influx would involve the same processes, operating in the opposite direction. A. With the system in the inward-facing, loaded form (ECl_i), Cl^- can cross the barrier (B_o) at the outside of the transport site (+). B. Chloride might also cross the barrier toward the inside of the transport site (B_i), so that internal Cl^- could combine with the outward-facing, unloaded form of the transport system (E_o). Process A should display saturation because the ion must be bound to the site before crossing B_o. Process B may decrease with increasing Cl^-, because an ion bound at the site might inhibit the Cl^- ion from crossing B_i. C and D represent similar processes in which there is simultaneous displacement of an ion at the transport site as the ion crosses the permeability barrier. These processes would not be expected to exhibit saturation behavior. In all of these net transport events, a chloride ion crosses the diffusion barriers adjacent to the transport site, with no change in the protein conformation. This contrasts with the situation during anion exchange (panel E), where the ion crosses the membrane by virtue of a protein conformational change in which the transport site changes from inside-facing to

(B_i and B_o) in series with the transport site. To explain the tight coupling of anion influx and efflux, it is assumed that the conformational change occurs only when an anion is bound to the transport site (Fig. 8E).

In terms of such a model, it is relatively easy to see how DIDS-sensitive net anion flow might take place, and why it might tend to saturate with increasing chloride concentration. If the diffusion barriers are not completely impermeable to anions, then it is possible for an anion to cross the membrane without the usual change in protein conformation. For example, as in Fig. 8A, if the transport system is in the inward-facing conformation, an ion might diffuse from the site across the barrier B_o to the external solution. Net anion efflux might also occur with the system in the outward-facing conformation if an anion crosses the barrier B_i from the inside solution to the binding site (Fig. 8B). Since the probability of crossing the barrier should be related to the binding of anions to the transport site, some saturation should be observed. If, however, the transport can occur by processes such as depicted in Figs. 8C and D, the system may not exhibit the same saturation characteristics as anion exchange. Also, binding of chloride to the modifier site may affect the properties of the barriers B_o and B_i.

Without further information regarding the rates of the individual steps, it is impossible to calculate precisely the expected net chloride flow as a function of chloride concentration from this model. Nevertheless, it is easy to see how this model could account for the DIDS sensitivity of net anion flow, since binding of DIDS should block the transport site and prevent net flow through the barriers.

Effects of NAP-Taurine on Net Chloride Flow

Despite the difficulties in making quantitative predictions from this model, certain qualitative predictions may be made. Various lines of evidence suggest that external NAP-taurine binds to the modifier site of the anion exchange system and inhibits transport by locking the system in the outward-facing form (Knauf et al., 1978a, 1980; Knauf, 1979).[3] Since the protein conformational change is required for anion exchange, but is not necessary for net chloride flow, NAP-taurine should have a smaller inhibitory effect on net chloride flux than on chloride exchange. Some effect of NAP-taurine on the net flow might be expected, of course, because NAP-taurine will increase the proportion of transport sites in the E_o form and the permeabilities of the internal and external diffusion barriers (B_i and B_o) may not be equal.

[3] Knauf, P. A., F.-Y. Law, T. Tarshis, and W. Furuya. Use of NAP-taurine to detect effects of the transport site conformation on the modifier site of the human erythrocyte anion exchange protein. Manuscript in preparation.

outside-facing, or vice versa. The protein conformation "remembers" the direction of the last ion transport event, so that the next event is always in the opposite direction, giving rise to a one-for-one coupling of anion flows in opposite directions.

As a basis for comparison, the effects of DIDS on net and exchange chloride
fluxes were measured. Portions of cells were treated with various amounts of
DIDS, excess DIDS was washed away with albumin, and net and exchange
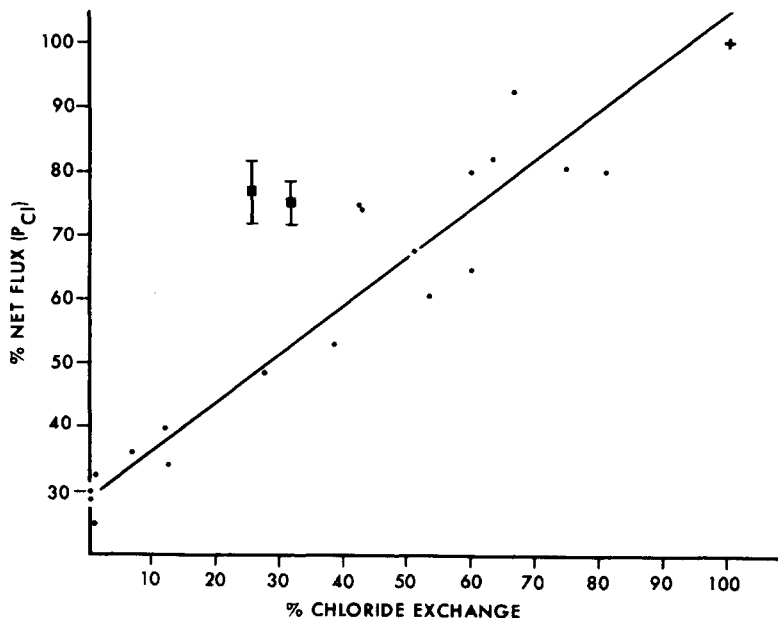fluxes of chloride were measured. The results, shown in Fig. 9, are very similar

FIGURE 9. Effects of DIDS and NAP-taurine on chloride exchange and net
chloride flux. Aliquots of cells were treated with various amounts of either DIDS
or NAP-taurine as described in Methods, and the chloride exchange rate
constant was determined at 0°C as well as the value of P_{Cl} (the permeability for
net chloride flow) at 37°C. As in previous experiments using a different
technique for measurement of P_{Cl} (Knauf et al., 1977), there was a strong
correlation (r^2 = 0.895) between the effects of DIDS (circles) on chloride
exchange and net flow, but 29.4 ± 2.9% (SD) of the net flux was insensitive to
DIDS. The square points show the results for 500 (left) and 136 μM (right)
NAP-taurine ± SEM for 2 and 10 experiments, respectively. For NAP-taurine
the inhibition of net chloride permeability was significantly less (P < 0.001)
than that caused by DIDS.

to those obtained earlier (Knauf et al., 1977) using a light-scattering technique
to measure net chloride fluxes. The relationship between net and exchange
flux is linear, with a correlation coefficient (r^2) of 0.895. About 30% of the net
flux was insensitive to DIDS, whereas <1% of the chloride exchange was
DIDS insensitive. For the cells treated with NAP-taurine and exposed to light,
the inhibition of net chloride flow was significantly (P < 0.001) less than the

inhibition caused by a DIDS treatment that had an identical effect on anion exchange.[4]

These data further reinforce the concept that the DIDS-sensitive component of net chloride flux takes place through the anion exchange mechanism. They argue strongly against any model in which the conformational change, inhibited by NAP-taurine, is necessary for net anion flow. For example, Hunter (1977) has suggested that net chloride flow could occur if two chloride ions are loaded on a single transport site. Translocation of these chloride ions would be expected to require the same conformational shift as that which is involved for normal chloride translocation, and therefore should be inhibited by NAP-taurine as well as chloride exchange is inhibited. Since NAP-taurine inhibits sulfate exchange as well as chloride exchange, the data provide further evidence against the possibility (Gutknecht and Walter, 1982) that net chloride flow is mediated by the divalent (sulfate-transporting) form of the carrier. The data argue in favor of models such as that presented in Fig. 8, in which net anion flow occurs without a change in transport site orientation.

CONCLUSIONS

The data presented here are inconsistent with several postulated mechanisms for net anion flow. Water transport inhibitors have little or no effect on net chloride flux (Table II), which suggests that net chloride flow does not take place through water channels. Both the halide selectivity (Table I) and the concentration dependence of net anion flow (Fig. 5) strongly suggest that net flow does not involve backflow ("slippage") of the unloaded chloride carrier (or a conformational change of the unloaded carrier in the model shown in Fig. 8). In addition, it does not appear that the sulfate-transporting form of the carrier is involved, since under the conditions of the experiments reported here, lowering the pH does not cause a dramatic increase in net anion flow (Fig. 6), as would be expected from this model. Furthermore, transport does not seem to involve a doubly loaded form of the anion exchange carrier (Fig. 9).

The DIDS-insensitive portion of net chloride flow may involve a pathway separate from the anion exchange system, since this component of the flux increases linearly with concentration and is not sensitive to inhibitors of anion exchange. In the case of nystatin-treated cells, it is possible that part of this flux goes through nystatin channels, since in some nystatin experiments the DIDS-insensitive component amounted to 50% of the total flux, compared with the more usual value in untreated cells of 20–30% (see, e.g., Figs. 6 and 9). The majority of the DIDS-insensitive flux, however, cannot be attributed

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[4] Since NAP-taurine locks the system in the E_o form and inhibits net chloride flux somewhat, it might seem that net chloride efflux occurs more readily when the system is in the ECl_i form than when it is in the ECl_o form (that is, the processes shown in Figs. 8A and C are more probable than that shown in D). This speculation is likely to be in error, however, if NAP-taurine causes a change in the structure of the ECl_o form which decreases its net anion permeability.

to nystatin channels. In the case of iodide, it seems probable that diffusion through the lipid accounts for the very large DIDS-insensitive flux. Data indicating that another lipid-soluble anion, nitrate, has a net permeability >15 times that of chloride (Reichstein and Rothstein, 1981) would support this concept. The high activation energy for the DIDS-insensitive component (22.0 kcal/mol as compared with 14.6 kcal/mol for the total P_{Cl}, based on net flux measurements at 25 and 37°C; data not shown) would also be compatible with this model. Since in these experiments the membrane contains valino-mycin, it is also possible that the formation of complexes between the more polarizable anions such as I^- and valinomycin-K^+ may contribute to their observed high conductance.

On the other hand, it still seems possible that for ions such as chloride, bromide, and fluoride, much of the DIDS-insensitive flux may take place via the anion exchange mechanism. The failure to observe saturation for this component might be due to superposition of flux through nystatin channels, which would add a linear component to the flux and thereby make saturation more difficult to observe. Also, it is possible that DIDS might alter the system in such a way as to block the transport site but still to permit some net flow of chloride through the system. The similar selectivities of the DIDS-sensitive and DIDS-insensitive components for F^-, Br^-, and Cl^- would favor this concept. The differences in temperature dependence and pH sensitivity suggest that the characteristics of the rate-determining processes for DIDS-sensitive and -insensitive net chloride flow differ significantly, but even this could be reconciled to the concept of a common band 3 pathway if DIDS substantially affects the band 3 structure and hence alters the nature of the net flow process. Further data are required to resolve this question.

It is highly probable that the major (50-80%) DIDS-sensitive component of net chloride flux is mediated by the anion exchange system. The parallel effects of DIDS on chloride exchange and on this component of net chloride flow support this concept, as does the evidence for saturation of this component at high chloride concentrations.

The proposed model for net anion flow (Fig. 8) explains net chloride flow on the basis of diffusion of chloride through barriers in series with the transport site, a process that does not require the protein conformational change that normally accompanies anion exchange. This process can be called "transit"[5] in the sense that the ion goes through (permeates) the diffusion barrier, with no protein conformational change, as opposed to "slippage," where the transport mechanism itself slips from one conformation to another with no ion present. It is interesting to note that this barrier transit model grows directly out of the lock-carrier model for anion exchange. According to a

[5] Fröhlich et al. (1983) have referred to this process as "tunneling." The term "transit" is chosen because of its Latin root meaning "he (it) goes across or through," in contrast to the term "transport," whose root meaning is "it is carried across." Thus, "transit" implies movement of the ion itself across fixed diffusion barriers, in contrast to the "transport" process, in which the ion is carried across by virtue of a movement of the barriers (Fig. 8E).

classic diffusible carrier model, the process shown in Fig. 8A, where the inward-facing carrier-anion complex releases a chloride ion to the outside, could never occur. According to the lock-carrier model, such a process arises naturally from the likely possibility that the barrier B_o has a finite permeability for anions. Similarly, the other processes shown in Figs. 8B–D are recognized as possibilities only in terms of the lock-carrier model. Thus, even though the lock-carrier and diffusible carrier models are kinetically equivalent for explaining anion exchange (Patlak, 1957), the different molecular models corresponding to the lock-carrier concept suggest a new mechanism for net anion flow through the exchange system.

In terms of saturation, the model proposes processes such as shown in Figs. 8A and B, which should exhibit saturation or which should decrease with increasing Cl^- concentration, along with processes that should increase (Figs. 8C and D). This fits rather well with the weak saturation behavior that has been observed (Fig. 7). As noted in Methods, the stronger saturation seen in a preliminary study (Knauf and Law, 1980) was due partly to an experimental artifact.

Fröhlich et al. (1983) have presented evidence that net chloride flux increases dramatically at very low extracellular chloride concentrations and have suggested that this probably represents slippage of the unloaded form of the transport site under these conditions, where many of the transport sites are in the E_o form. An alternative possibility, also noted by Fröhlich et al. (1983), is that the permeability of the barrier B_i may be large, so that a large net flux occurs by the process depicted in Fig. 8B under these conditions. Increasing the external Cl^- concentration would decrease the number of outward-facing, unloaded (E_o) sites, so that a larger fraction of the transport sites would be in the E_i and ECl_i forms. If the permeability of the barrier B_o is less than that of the barrier B_i, then this shift in conformation would give rise to the observed decrease in net chloride flux. This model would explain the dependence of the net flux on membrane potential just as well as would the slippage model, except that in this case the increase in net chloride efflux with membrane hyperpolarization would be ascribed to the effect of potential in driving the chloride ion outward, rather than in driving the unloaded, positively charged transport site inward. Further experiments are required to resolve the mechanism of net anion transport at low chloride concentrations, but it seems that all of the available data can be fitted just as well to the barrier transit model as to the slippage model. It seems clear, however, that under physiological circumstances slippage does not account for any significant fraction of net chloride flow.

The barrier transit model is successful in predicting that NAP-taurine, which inhibits the conformational change, should have a smaller effect on net anion transport than does DIDS, which blocks the transport site. Much further work is required to test various aspects of this model. In the first place, the kinetic constants for the processes depicted in Fig. 8 should be determined. This might be facilitated by use of NAP-taurine or other probes to hold the system in the E_o conformation so that the number of variables is reduced.

Because of the intrinsic asymmetry of the transport system (Gunn and Fröhlich, 1979; Furuya, 1980; Knauf et al., 1980),[3] it seems that the native system is primarily in the E_i form (Jennings, 1980; Knauf, 1982). Thus, it should be possible to determine the rate constants for each form of the system separately and to improve the precision of the kinetic model. This in turn may permit more accurate predictions, which could be used to test the model.

The new model has the ability in principle to explain several aspects of net anion transport as well as or better than previous models. For example, the similarity between the rates of net sulfate and chloride transport (Knauf et al., 1977), despite the 10,000-fold difference in their rates of exchange, can be explained in terms of the different rate-limiting processes involved. The exchange rate depends on the rate of the protein conformational change, which in turn can be affected by the nature of the ion bound to the transport site. Net flow, on the other hand, depends on the ability of the ion to diffuse across the permeability barriers. Ions that are poorly transported by the exchange mechanism may actually disturb the protein conformation and this may increase the permeability of the barriers. In this connection, it is interesting to note that both F^- and Br^-, which are less readily exchanged than Cl^- (Tosteson, 1959; Wieth et al., 1973), have higher rates of DIDS-sensitive net transport (Table I). In part, this observation may be rationalized by considering that when an anion is bound to the inward-facing form of the transport system to form the ECl_i complex, it can either diffuse across the barrier B_o, as in Fig. 8A, or can undergo a conformational change to the ECl_o form, as in Fig. 8E. Inasmuch as this represents an either/or choice, the anion exchange process may be said to compete with net anion flow. Thus, a high anion exchange rate would be expected to correlate with a low net flux.

Similar reasoning may explain the different effects of temperature, pH, and inhibitors on net and exchange anion fluxes. A particular case of interest is external phlorizin, which strongly inhibits chloride exchange (Schnell et al., 1973), but causes a 10-fold increase in P_{Cl} (Kaplan and Passow, 1974). The same disruption of the anion transport protein that inhibits the delicate conformational change involved in anion exchange might be expected to disorganize the protein so as to increase the permeabilities of the diffusion barriers adjacent to the transport site.

It is a bit more difficult to rationalize the failure of acid pH to inhibit net chloride flow, even though chloride exchange is substantially inhibited. Gunn and Milanick (1982) have presented evidence that internal Cl^- does not bind to the protonated form of the transport system when the internal pH is above 5.7, which would seem to preclude the processes in Figs. 8A and C for the protonated carrier. On the other hand, there is evidence for binding of external Cl^- to the protonated carrier (Milanick and Gunn, 1982), so processes such as those in Figs. 8B and D could occur. Protonation might affect the barrier permeabilities in such a way that the total transport rate is unaffected. Much further work is needed to determine the mechanism of net anion flow under acidic conditions, but at present it seems that in principle the model presented can account for the observations.

Finally, the model may explain the dependence of the net chloride and sulfate permeabilities on membrane potential. This phenomenon seems difficult to rationalize on the basis that net flow occurs through the anion exchange system, since an ideal anion exchange system with no slippage is by definition unresponsive to potential. In terms of the new model, however, it would seem quite plausible that the permeability of the barriers could be affected by changes in potential. These changes in barrier permeability would affect net flow, but would have no effect on the conformational change involved in anion exchange.

If the new model for net chloride flow is indeed correct in general outline, it has some interesting implications. First, measurements of net anion flow under certain conditions may provide insight into the permeability of the diffusion barriers adjacent to the transport site and into the effects of various agents such as enzymes and chemical probes on these structures. Second, because of effects of chloride gradients on the conformation of the transport system (Furuya, 1980; Knauf et al., 1980),[3, 6] it is possible that the net chloride flow may exhibit rectification under some circumstances.

Finally, although the new model for net anion flow is quite successful in fitting the data, it is by no means unique. In particular, Passow and co-workers (Passow et al., 1980; Kaplan et al., 1980; Passow and Fasold, 1981) have proposed a nonconducting gate model for the anion exchange system, in which anion binding causes a reorientation of positively charged groups such that a gate is alternately open to one side or the other of the membrane. They propose that occasionally an anion may pass through the gate without causing the site reorientation, thereby giving rise to net anion flow. Although we feel that the detailed mechanism of this model is somewhat less plausible than the model presented in this paper, the calculations of Passow and co-workers indicate that this model could explain many of the characteristics of net anion flow just as well as the model presented here.

APPENDIX

The slippage model shown in Fig. 1 assumes that the rate-limiting step in the net efflux of chloride is the net inward "flow" of the unloaded form of the carrier. Thus, it seems logical that the net chloride efflux should decrease at high chloride concentrations, where the amount of carrier in the unloaded form decreases. Here this is demonstrated for two different forms of the carrier model.

Case I: Symmetric Carrier

If we assume that the chloride dissociation constant at the inside surface of the membrane, K_i, is equal to the chloride dissociation constant at the outside, K_o, then $K_i = K_o = K$, where K is the concentration of chloride that half-saturates the

transport system with equal chloride at the two sides of the membrane. (In all of these calculations, interactions at the modifier site are ignored, so the model system will exhibit simple saturation. Since any interactions with chloride at the modifier site are likely to be inhibitory, inclusion of such effects in the model should only emphasize the decrease in net flux with increasing chloride concentration.) For a symmetric system, we further assume that the rate constant for transport of the carrier-chloride complex from inside to outside, k, is equal to the rate constant for transport in the opposite direction, k'. Since the net chloride efflux is determined by inflow of unloaded carrier, the equation for the net efflux, J, is:

$$J = s'E_o - sE_i \tag{1A}$$

where s and s' are the rate constants for outflow of the inward-facing unloaded carrier, E_i, and inflow of the outward-facing unloaded carrier, E_o, respectively (see Fig. 1). The rate constants s and s' are assumed to be functions of the membrane potential. For a symmetric carrier, the amounts of E_o and E_i, expressed as a fraction of the total carrier, E_t, are given by:

$$E_o/E_t = E_i/E_t = 0.5/(1 + Cl/K) \tag{2A}$$

where $Cl = Cl_i = Cl_o$. Substituting this into Eq. 1A:

$$J = E_t \frac{s' - s}{2(1 + Cl/K)}. \tag{3A}$$

The existence of the term Cl/K in the denominator means that as the chloride concentration is increased, the flux will decrease. At very low chloride concentrations, the decrease in flux should be small, but under no circumstances will the flux increase with increasing chloride concentration. For the specific case when the unloaded carriers are assumed to flow like monovalent ions according to the Goldman (1943) equation, the flux is given by:

$$J = -PE_t \frac{FE_m}{2(1 + Cl/K)} \tag{4A}$$

where F is the faraday, E_m is the membrane potential (inside with respect to outside), and P is the permeability of the unloaded carrier.

Case II: Asymmetric Carrier

If $K_i \neq K_o$ and $k \neq k'$, and if we define L as k/k', then the expression for E_o/E_t is:

$$E_o/E_t = \frac{LK_o/Cl_o}{\dfrac{K_i}{Cl_i} + \dfrac{LK_o}{Cl_o} + 1 + L}; \tag{5A}$$

whereas the corresponding expression for E_i/E_t is:

$$E_i/E_t = \frac{K_i/Cl_i}{\dfrac{K_i}{Cl_i} + \dfrac{LK_o}{Cl_o} + 1 + L}. \tag{6A}$$

The net chloride flux with $Cl_i = Cl_o = Cl$ is given by substituting Eqs. 5A and 6A into 1A:

$$J = E_t \frac{s'K_oL - sK_i}{K_i + LK_o + Cl(1 + L)}.$$ (7A)

Since L, s, s', E_t, K_o, and K_i are all independent of Cl, if the membrane potential is kept constant, the net chloride flux will decrease as Cl increases.

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