

Stoichiometry of a Half-Turnover of Band 3, the Chloride Transport Protein of Human Erythrocytes

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ABSTRACT The kinetics of human red blood cell Cl transport have been studied under nonequilibrium conditions to determine whether or not an outward Cl gradient can recruit the transport protein from an inward-facing to an outward-facing configuration. Three kinds of evidence are consistent with this outward recruitment. First, the initial net Cl efflux into a Cl-free phosphate medium is independent of the intracellular Cl concentration in the range 20–170 mM. Second, an outward Cl gradient strongly enhances the inhibitory potency of DNDS (4,4'-dinitro-2,2'-stilbene disulfonate), which suggests that DNDS binds primarily to outward-facing states. Finally, we have estimated the number of Cl ions transported during the putative outward recruitment. Re-sealed red cell ghosts containing only 70 μM ^{36}Cl were resuspended at 0°C in a Cl-free, HCO_3 -free Na_2SO_4 medium. In the first 10 s, $\sim 10^6$ Cl ions leave each ghost, followed by a much slower further loss of Cl. The rapid loss of 10^6 Cl ions per ghost, which is abolished by pretreatment with DIDS (4,4'-diisothiocyanato-2,2'-stilbene disulfonate), appears to represent the Cl that is transported during the first half-turnover of the transport cycle. These data are strong evidence that the influx and efflux events in the catalytic cycle for anion transport do not take place simultaneously, and that the stoichiometry of the transport cycle is close to one pair of anions exchanged per band 3 monomer.

INTRODUCTION

Inorganic anion transport across the human erythrocyte membrane is catalyzed by band 3, the major transmembrane protein (Cabantchik and Rothstein, 1974; Passow et al., 1975; Ho and Guidotti, 1975). The physiologic mode of this anion transport is Cl- HCO_3 exchange, which is one of the steps in CO_2 elimination. Band 3, though sometimes called an anion "channel," does not function in a manner similar to the Na and K channels of nerve and muscle, because the anion transport is an obligatory one-for-one exchange (Hunter, 1971; Knauf et al., 1977). As discussed recently by Gunn and Fröhlich (1979) and Knauf (1979), there are two general ways in which this exchange may take place. One possible mechanism is a sequential¹ reaction,

¹ The terms "ping-pong" and "sequential" were introduced by Cleland (1963) to describe the kinetics of multi-substrate enzymes.

in which a ternary complex is formed among the protein, an intracellular anion, and an extracellular anion. The anion exchange would come about when the two bound anions switch places with each other. The other possibility is a ping-pong mechanism: only one transportable anion is bound at a time, and the intra- and extracellular anions take turns crossing the membrane.

A major difference between the ping-pong and sequential mechanisms is that the ping-pong model postulates that there are two stable, structurally distinct forms of the transport protein: an inward-facing state and an outward-facing state. The inward-facing state can bind intracellular anions and transport them outward; the outward-facing state does the reverse. To account for the electrically silent nature of the transport, a ping-pong model must require that the transition between inward- and outward-facing states be 10^4 -fold faster when Cl or HCO_3 is bound to a transport site than when the site is empty. A consequence of this requirement is an important prediction of the ping-pong model (see Knauf, 1979): changes in the Cl gradient should cause large changes in the proportions of inward- and outward-facing transport proteins. This hypothetical recruitment of transport systems by the Cl gradient predicts several phenomena that have been observed experimentally. First, the apparent affinity for extracellular Cl depends on the intracellular Cl concentration (Gunn and Fröhlich, 1979). Second, an outward Cl gradient increases the inhibitory potency of NAP-aurine [*N*-(4-azido-2-nitrophenyl)-2-amino ethylsulfonate] and H_2DIDS (4,4'-diisothiocyano-1,2-diphenylethane-2,2'-disulfonate) (Knauf, 1979; Knauf et al., 1980). Finally, an outward Cl gradient dramatically reduces the unidirectional SO_4 efflux, as expected if the Cl gradient recruits all of the transport systems into an outward-facing configuration (Jennings, 1980).

The data presented here include further steady-state anion flux data that support the ping-pong model, but the major result is a measurement of the amount of chloride transported outward under conditions where only one half-turnover of the catalytic cycle for transport is permitted to take place. These data provide direct evidence that, as predicted by the ping-pong model, the outward and inward translocation events take place at different times. We also have been able to estimate that the stoichiometry of a single catalytic cycle is close to one pair of anions exchanged per band 3 monomer.

A report of some preliminary experiments for this paper has been presented (Jennings, 1981).

MATERIALS AND METHODS

Materials

Human red blood cells (EDTA anticoagulant) were obtained from the Lipid Research Laboratory of the University of Iowa and were used after at most 3 d of storage at 4°C as packed cells in plasma. MOPS [3-(*N*-morpholino) propanesulfonic acid] was purchased from Sigma Chemical Co., St. Louis, Mo., and purified by passage down a Rexyn 201 (Fisher Scientific Co., Pittsburgh, Pa.) anion exchange column that had been washed extensively with 1 N H_2SO_4 and then distilled water. The zwitterionic MOPS was only slightly retarded on the column, whereas >99% of any possible

chloride contaminant was adsorbed (judging from the adsorption of ^{36}Cl added with the MOPS on a test run). DNDS (4,4'-dinitro-2,2'-stilbene disulfonate) was purchased as a technical grade powder from Aldrich Chemical Co., Milwaukee, Wis. For the half-turnover experiments, NaCl was removed from the DNDS by passing a DNDS solution in 100 mM Na_2SO_4 down a Sephadex G-10 column. The high ionic strength promotes the adsorption of the aromatic DNDS to the Sephadex (Janson, 1967), but the Cl contaminant was not adsorbed. The DNDS was recovered by washing the column with distilled water. The concentration of the purified DNDS was determined by measuring the absorbance at 353 nm and assuming a molar extinction coefficient of $30,000\text{ cm}^{-1}$ (Barzilay and Cabantchik, 1979). DIDS (4,4'-diisothiocyano-2,2'-stilbene disulfonate) was purchased from Pierce Chemical Co., Rockford, Ill. The DNDS and DIDS were stored in the dark, and solutions made with either inhibitor were protected from unnecessary exposure to light (Fröhlich and Gunn, 1981). All other salts were obtained from Fisher Scientific Co.

Cl-P_i Exchange

Red cells were washed four times at a >4:1 dilution in 20 mM Na-citrate, pH 6.4, plus combinations of 150 mM NaCl and 140 mM Na-phosphate, such that the Cl concentration varied from 20 to 150 mM, and the P_i concentration varied from 121 to 0 mM. The suspensions were incubated for 10 min at 37°C before each spin to allow equilibration of the anions and pH between cells and medium. Packed cells were loaded with ^{36}Cl (ICN Chemical and Radioisotope Division, Irvine, Calif.) in a 50% suspension containing 1 $\mu\text{Ci } ^{36}\text{Cl}/\text{ml}$. The ratio of the intracellular to extracellular Cl concentration, r_{Cl} , was calculated from the wet and dry weights of the cells present, the hematocrit, and the extracellular counts per minute per milliliter before and after addition of the cells. In the Cl-P_i exchange experiments, r_{Cl} was 0.90–1.15. Cells loaded with ^{36}Cl were packed by centrifugation for 3 min at 13,000 rpm and resuspended at 27°C in a Cl-free, HCO_3 -free (30 min N_2 bubbled), 140 mM Na-phosphate, 20 mM Na-citrate medium, pH 6.40, and the time-course of Cl loss was measured using the inhibitor stop method described previously for Cl-Cl exchange (Ku et al., 1979). We established that the majority of the Cl loss was indeed as exchange for P_i (presumably H_2PO_4 ; see Gunn et al., 1980) by measuring the Cl loss from the same cells into 220 mM sucrose, 20 mM Na-citrate, pH 6.40. The initial Cl efflux into sucrose/citrate was between 5 and 15% of that into the phosphate medium for all intracellular Cl concentrations used. The initial Cl efflux was calculated from the initial fractional rate of loss (min^{-1}) times the initial intracellular Cl contents (millimoles per kilogram solids).

Cl-SO₄ and SO₄-SO₄ Exchange

Cells were titrated at room temperature with 1 N HCl to an equilibrium extracellular pH of 6.0 in 150 mM NaCl, 20 mM Na-citrate, and then washed four times in either 150 mM NaCl or 100 mM Na_2SO_4 , each buffered with 20 mM Na-citrate at pH 6.0. For SO_4 loading, the cells were incubated for 10 min at 37°C before each spin. For $^{35}\text{SO}_4$ influx measurements, 0.20 ml packed cells was added to 10 ml 100 mM Na_2SO_4 , 20 mM Na-citrate, pH 6.0, 27°C, containing 10 $\mu\text{Ci } ^{35}\text{SO}_4$ (New England Nuclear, Boston, Mass.). Aliquots of 1.0 ml were removed at timed intervals, plunged into 10 ml ice-cold buffer to stop the $^{35}\text{SO}_4$ influx, and centrifuged for 3 min at 5,000 rpm. The pellet was washed once in 10 ml cold buffer and the intracellular radioactivity was determined after lysis of the pellet in water and precipitation of the hemoglobin with 8% trichloroacetic acid (TCA). It was not necessary to include

inhibitor in the stopping or washing buffer because SO_4 transport is so slow at 0°C . The unidirectional $^{35}\text{SO}_4$ influx (mmol/kg cell solids·min), which is equal to the net SO_4 influx in the case of Cl- SO_4 exchange, was calculated from the extracellular specific activity, the number of kilograms of cell solids per milliliter suspension, and the initial rate of increase of intracellular radioactivity.

Resealed Ghosts

Resealed red cell ghosts were prepared by a method similar to those developed by Passow and co-workers (e.g., Schwach and Passow, 1973), modified to produce ghosts that contain very low chloride concentrations. Red cells were first washed four times at a 5:1 dilution in unbuffered 100 mM Na_2SO_4 , with a 10-min incubation at 37°C before each spin to allow the Cl to leave the cells. Then 8 ml of chilled, packed cells was lysed in 150 ml ice-cold 4 mM MgSO_4 , 1 mM citric acid. After 3 min, the ionic strength of the lysate was increased by the dropwise addition of 3 ml 0.9 M $\text{Na}_3\text{-citrate}$, 0.1 M $\text{H}_3\text{-citrate}$; the final citrate concentration was 20 mM, the osmolality was 68 mosmol/kg H_2O , and the pH was 6.15. This pH value and all others in this paper refer to 22°C , the temperature at which the pH was measured. After another 3 min at 0°C , the suspension was warmed and incubated 45 min at 37°C for resealing.

The resealed ghosts were washed twice at a 20:1 dilution in "ghost wash," which consisted of 50 mM Na-MOPS, pH 6.8, 2 mM Na_2SO_4 (73 mosmol/kg), and twice more at a 5:1 dilution. For the last two washes, the "ghost wash" was made with purified MOPS (see *Materials*) and was bubbled with N_2 for 30 min to remove atmospheric CO_2 . Before the first three centrifugations, the suspensions were incubated for 15 min at 37°C to allow the intracellular SO_4 (initially 6 mM) to exit and the intracellular pH (initially 6.15) to rise, via $\text{SO}_4\text{-HCO}_3$ or $\text{SO}_4\text{-OH}$ exchange. The ghosts were loaded with ^{36}Cl by mixing equal volumes of ghosts and ghost wash, to which 150–200 μM ^{36}Cl (0.46 mCi/mmol) had been added. After 10 min at 20°C , the ghosts were chilled for 30 min and centrifuged for 5 min at 35,000 g . Despite the very low Cl concentration, the ratio of intra- to extracellular Cl concentration, r_{Cl} , was very reproducible in these ghosts. In 24 ghost preparations, the measured r_{Cl} averaged 0.72 (range, 0.65–0.84). The r_{Cl} in ghosts was measured as it was for red cells (see above), except that it was assumed that in the packed ghosts, 17% of the water is extracellular, and that the ghosts themselves are 96% (wt/vol) water. The 17% extracellular water is consistent with the measured fraction of the ^{36}Cl that is initially outside the ghosts (see Results).

DIDS Pretreatment

In some experiments, 55–75% of the band 3 monomers were inhibited irreversibly with DIDS by adding 40 nmol of DIDS to 2.5 ml packed ghosts plus 2 ml ghost wash and incubating for 20 min at 37°C . Flame photometric analysis of the DIDS stock solution indicated a sizeable NaCl contamination (~ 1.5 mol/mol DIDS); this is not unexpected since the final recrystallization of the DIDS is in concentrated NaCl. Most of this added Cl was removed from the ghost suspension by an extra wash (10-fold dilution) before ^{36}Cl loading. It is estimated that the Cl added with the DIDS, and not removed by the subsequent wash, causes at most a 2% systematic error in the determination of the Cl contents of the DIDS-treated ghosts.

^{36}Cl Efflux

The chilled, ^{36}Cl -loaded, resealed ghosts were resuspended under N_2 in ice-cold 30 mM Na_2SO_4 , 1 mM Na-MOPS, pH 6.8, at $t = 0$, and at subsequent times the Cl

efflux was interrupted by adding 0.7 ml of the flux suspension to 0.5 ml of 30 mM Na_2SO_4 medium, which contained 30 μM freshly dissolved DIDS. The 30 mM Na_2SO_4 was made from H_2SO_4 (Fisher Scientific Co.; 0.2 ppm Cl; 0.5 ppm NO_3) and ultrapure NaOH (Alfa Div., Ventron Corp., Danvers, Mass.; 1 ppm Cl). The titrations of the flux solution to pH 6.8 were made by adding NaOH to 100 ml of solution and sampling the pH of a 5-ml aliquot, which was then discarded. This precludes the possibility of Cl entry into the flux solution from the combination pH/reference electrode. The flux solution had been purged of CO_2 by 30 min of vigorous N_2 bubbling at pH <6.0 before the final titration to pH 6.8, which was followed by a further 20-min N_2 treatment. This N_2 treatment is believed to lower the HCO_3^- concentration to $\ll 1 \mu\text{M}$ (Jennings, 1976), but even a trace of HCO_3^- could cause a comparatively rapid loss of all of the intracellular Cl via the Jacobs-Stewart (1942) cycle. Therefore, 2.5 μM ethoxzolamide was included in the flux medium and the packed ghosts in order to inhibit carbonic anhydrase. This concentration of ethoxzolamide has no effect on red cell anion exchange (Wieth, 1979).

The above procedure was also used to measure the rate of Cl-Cl exchange, except that the ghosts were loaded with a much higher ^{36}Cl concentration (2.2–2.4 mM final extracellular Cl; $r_{\text{Cl}} = 0.60\text{--}0.71$; $n = 6$). To allow the larger amount of Cl to enter the ghosts, the suspensions were incubated at 37°C for at least 20 min before being chilled and centrifuged. The extracellular medium for the Cl-Cl exchange experiments was identical to that for the half-turnover experiments except that 2 mM NaCl was included.

Stoichiometry of Half-Turnovers

The amount of rapid Cl loss into the Cl-free, HCO_3^- -free medium was calculated from the intracellular ^{36}Cl concentration (μM), the number of ghosts per milliliter packed ghosts, and the fractional loss of cellular ^{36}Cl in the rapid phase of the efflux (see Results). The intracellular ^{36}Cl concentration was calculated from the final extracellular counts per minute per milliliter, r_{Cl} , and the ^{36}Cl specific activity (counts per minute per micromole), obtained from titrating the H^{36}Cl stock against 1.00 N NaOH. It is assumed that all of the Cl initially inside the red cells has been washed away. This is a reasonable assumption, because if the Cl reaches equilibrium for all of the washes during ghost preparation, the original intracellular Cl should be diluted by a factor of $>10^7$. The number of ghosts per milliliter packed ghosts was determined by total phosphorus (Bartlett, 1959). Of the total, ~10% is intracellular organic phosphorus, measured in the first ghost supernatant, and virtually all of the remainder is membrane lipid phosphorus. The conversion factor we used was 4.7 μmol total ghost phosphorus per 10^{10} cells, obtained from two preparations in which a known number of cells (1 g cell solids = 3.1×10^{10} cells; Funder and Wieth, 1976) was used, and the loss of ghosts during the preparation was minimal. This amount of phosphorus per ghost compares well with the known amount of lipid phosphorus per ghost (4.0×10^{-10} μmol ; Guidotti, 1972).

RESULTS

Chloride-Phosphate Exchange

The catalytic cycle for the ping-pong mechanism of anion exchange is represented in Fig. 1. It consists of binding, translocation, and release—first in the outward direction and then in the inward direction. The rate-limiting steps in the cycle are assumed to be translocation rather than binding or

release. The number of transport systems facing inward depends on the intrinsic properties of the transport protein as well as the anion concentrations on each side of the membrane. For example, if chloride is symmetrically distributed and if the transport system is itself symmetric with regard to chloride binding and translocation, then half the transporters will face in each direction. If, on the other hand, chloride is present only inside the cells and no rapidly penetrating anion is outside, then nearly all of the transport systems should be recruited into an outward-facing configuration (Knauf, 1979; Jennings, 1980). Suppose specifically that chloride-containing cells are sus-

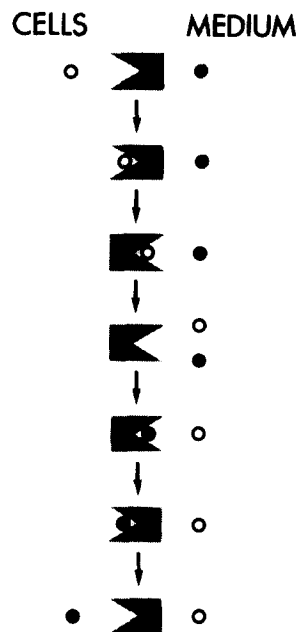


FIGURE 1. Catalytic cycle for anion exchange by way of a ping-pong mechanism. An intracellular anion (○) binds to an inward-facing transport system, is transported outward by way of a conformational change in the protein-anion complex, and is released into the extracellular medium. Then another extracellular anion (●) binds and is transported inward to complete the cycle.

pending in a CO_2 -free, Cl-free, 140 mM Na-phosphate medium. The ping-pong model predicts that the outward Cl gradient should recruit all of the transport systems into an outward-facing state; this is a consequence of the fact that Cl-Cl exchange rates are $\sim 10^4$ larger than those of inorganic phosphate (Brahm, 1977; Gruber and Deuticke, 1973). After this initial outward recruitment of transport systems, the net Cl- P_i exchange should proceed in a steady state, in which nearly all of the transport systems remain facing outward until the Cl gradient is dissipated. Another way to state this is that the catalytic cycle for the Cl- P_i exchange should be limited by the P_i influx rather than the Cl efflux. Therefore, the ping-pong model implies that

the Cl-P_i exchange flux should be *independent of the intracellular Cl*, as long as Cl_i ≫ Cl_o.

Fig. 2 shows the time-course of the net Cl loss at 27°C from red cells loaded with varying concentrations of chloride (P_i is the replacement anion) and resuspended in an all-P_i medium. The time-course is not an exponential—it is linear for well over half of the efflux, as is true for net Cl-SO₄ exchange (Jennings, 1980). The fractional Cl loss per minute depends strongly on the Cl concentration, but the flux (mmol/kg cell solids·min) is independent of the

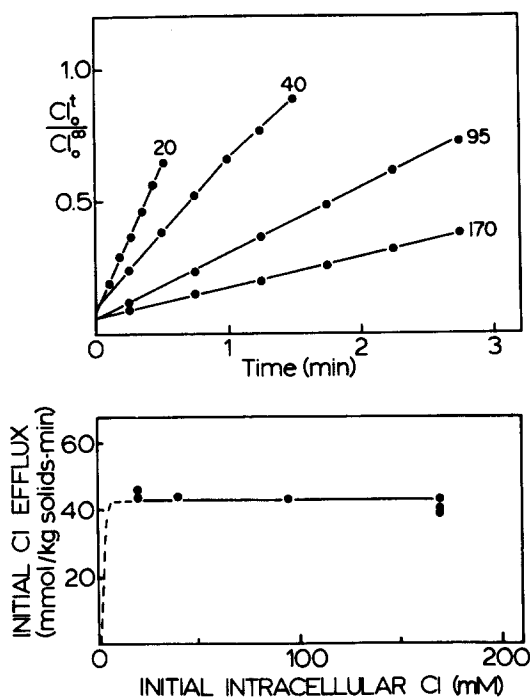


FIGURE 2. Top: time-course of net Cl efflux into initially Cl-free, HCO₃-free, 140 mM Na-phosphate, 20 mM Na-citrate medium, pH 6.4, 27°C. Cells were loaded at pH 6.4 with 20, 40, 95, or 170 mM intracellular Cl, as indicated, with phosphate as the replacement anion. Bottom: the initial Cl-P_i exchange flux, obtained from the data in the top portion of the figure plus at least one further experiment at each initial Cl concentration.

intracellular Cl (Fig. 2); that is, the transport protein *appears* to be completely saturated with Cl at an intracellular Cl concentration of 20 mM. The intracellular Cl concentration necessary for half-maximal Cl-P_i exchange cannot be determined from these data, but it is certainly less than 2 mM. In contrast, the true dissociation constant (K_c^i) for intracellular Cl is believed to be >50 mM (Brahm, 1977; Gunn and Fröhlich, 1979). The much lower apparent K_c^i for Cl-P_i exchange is not expected for a sequential mechanism, but is completely in accordance with the ping-pong mechanism.

Outward Cl Gradient Enhances DNDS Potency

The reversibly acting disulfonic stilbene DNDS and the stilbene derivative H₂DIDS both compete with Cl for binding sites on the extracellular surface of band 3 (Fröhlich and Gunn, 1981; Shami et al., 1978). Moreover, chemical labeling experiments indicate that disulfonic stilbenes bind more strongly to the putative outward-facing form of band 3 than to the inward-facing form (Passow and Zaki, 1978; Grinstein et al., 1979; Passow et al., 1980). If this is so, and if the ping-pong model is correct, then the Cl gradient should strongly influence the inhibitory potency of DNDS.

Specifically, suppose the unidirectional SO₄ influx is measured for red cells at Donnan equilibrium in a Cl-free 100 mM Na₂SO₄ medium. Only an unknown fraction (f^{eq}) of the transport systems face outward, and the unidirectional SO₄ influx is proportional to this fraction, according to the ping-pong model. If Cl, rather than SO₄, is the intracellular anion, then virtually all of the transporters should face outward because of the outward Cl gradient. Therefore, the initial SO₄ influx into Cl-loaded cells should be larger than the SO₄-SO₄ equilibrium exchange flux in a given medium by a factor of $1/f^{eq}$ (Jennings, 1980). Moreover, an inhibitor that binds only to the outward state should be a more potent inhibitor of the Cl-SO₄ than of the SO₄-SO₄ exchange by the same factor of $1/f^{eq}$. This is a direct consequence of the ping-pong model (see Appendix).

Fig. 3 shows that the initial Cl-SO₄ exchange flux at an extracellular pH of 6.0 is eightfold larger than the equilibrium SO₄ exchange flux in the same medium, assayed in the same manner. If the ping-pong model is correct, this eightfold acceleration of the SO₄ influx by the intracellular Cl implies that, at equilibrium in the SO₄ medium at pH 6.0, one-eighth of the transport systems face outward. Therefore, an inhibitor that binds only to the outward-facing state should be eightfold more potent in inhibiting Cl-SO₄ exchange than SO₄-SO₄ exchange in the same medium. The data in Fig. 3 show in fact that this is very nearly true for DNDS. Dixon plots of the data show that the K_I for DNDS inhibition of Cl-SO₄ exchange is 0.3 μ M. The K_I for SO₄-SO₄ exchange in the same medium is 1.8 μ M, or sixfold higher. These data therefore indicate that DNDS binds preferentially, if not exclusively, to the outward-facing state.

Half-Turnovers of Band 3

The data in Figs. 2 and 3 are consistent with the idea that when Cl-containing red cells are suspended in a Cl-free medium (e.g., P_i or SO₄), virtually all of the transport systems are recruited into the outward-facing configuration. If this recruitment actually takes place, then it must involve the net loss of a small amount of Cl from the cells, because the transition from inward- to outward-facing state is most rapid when Cl is bound to the transport system. The following experiments were designed to measure the amount of Cl lost during this putative outward recruitment. The recruitment, according to the ping-pong model, is the result of an arrested transport cycle, in which only the outward steps take place because the extracellular anions are so slowly penetrating. The problem in trying to detect the number of Cl ions transported

outward during this half-turnover of the transport system is to arrange conditions such that the number of Cl ions initially inside the cells is not much larger than the number of inward-facing transport proteins. Fortunately, band 3 is a very abundant membrane protein; there are $\sim 10^6$ monomers/cell (Fairbanks et al., 1971), or 20 $\mu\text{mol/liter}$ cells. We have developed a preparation of resealed red cell ghosts that contain only slightly more Cl than band 3. The intra- and extracellular ionic compositions of this preparation are given in Table I.

The low Cl ghosts were resuspended in a Cl-free, HCO_3 -free, 30 mM Na_2SO_4 medium; the time-course of the appearance of extracellular ^{36}Cl is shown in Fig. 4. About 23% (range 19–26%, $n = 10$) of the radioactivity is

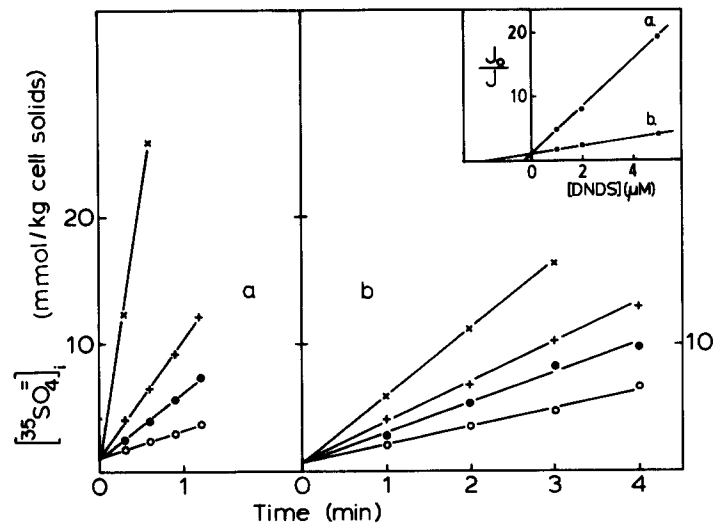


FIGURE 3. Time-course of $^{35}\text{SO}_4$ influx from a Cl-free, 100 mM Na_2SO_4 , 20 mM Na-citrate medium, pH 6.0, 27°C. The medium contained zero (\times), 1 (+), 2 (\bullet), or 5 (\circ) μM DNDS. In *a*, the cells had been loaded with 180 mM Cl. In *b*, the cells were at Donnan equilibrium with the Cl-free Na_2SO_4 medium. The inset (upper right) is a Dixon plot of the data in *a* and *b*, represented as the control flux, J_0 , divided by the inhibited flux, J , vs. the DNDS concentration.

initially extracellular, as shown by pretreating the ghosts with an excess of DIDS after ^{36}Cl loading. With no DIDS pretreatment, the time-course of Cl efflux has two distinct phases. About one-third of the Cl leaves the ghosts in <10 s; this rapid Cl loss is followed by a much slower further loss. We believe that the rapid Cl loss represents the Cl that is transported outward during the first half-turnover of initially inward-facing transport systems. After the release of the Cl outside the cells, further loss of Cl is much slower because SO_4 is so slowly penetrating at 0°C and because the extracellular medium contains 4 μM DNDS, which binds preferentially to outward-facing states (see above). A similar rapid loss is observed if the DNDS is omitted, but the subsequent slower efflux is not nearly so slow as in Fig. 4. Quantitation of the rapid efflux is therefore very difficult without the extracellular DNDS.

Partial DIDS Pretreatment

The rapid Cl efflux is prevented by pretreatment with enough DIDS to completely (>98%) inhibit Cl-Cl exchange (Fig. 4), as would be expected if the Cl efflux represents a half-turnover of the transporter. It is possible, however, that the extracellular medium contains a rapidly penetrating anionic impurity. For example, if there were 1 μM Cl originally in the 30 mM Na_2SO_4 medium, this Cl could exchange for the intracellular ^{36}Cl until the intra- and

TABLE I
INTRA- AND EXTRACELLULAR ANIONIC CONSTITUENTS OF
THE LOW Cl RESEALED GHOSTS

Intracellular	Extracellular
70 μM ^{36}Cl	100 μM ^{36}Cl
~ 1 mM SO_4	2 mM SO_4
20 mM citrate	50 mM MOPS
pH ~ 6.6	pH 6.8

The sole extracellular and major intracellular cation is Na. Ethoxzolamide (2.5 μM in 0.05% ethanol) is also present both inside and outside the ghosts. The intracellular pH and SO_4 concentration are estimates based on r_{Cl} .

The average ghost size is 70×10^{-15} liter, based on total phosphorus per milliliter packed ghosts. The ghosts are therefore $\sim 20\%$ smaller than native red cells.

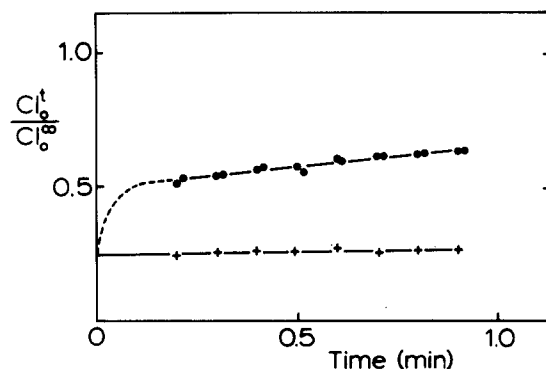


FIGURE 4. Time-course of ^{36}Cl efflux from low-Cl ghosts (see Table I), suspended at a 3% hematocrit in ice-cold Cl-free, HCO_3 -free, 30 mM Na_2SO_4 , 1 mM Na-MOPS, pH 6.8, 4 μM DNDS, N_2 atmosphere. The closed circles represent two separate experiments using the same ghost preparation. The lower points (+) refer to ghosts that were pretreated with an excess of DIDS (40 nmol/ml packed ghosts) after ^{36}Cl loading.

extracellular specific activities were equal. The result would be the observed rapid loss of about one-third of the cellular ^{36}Cl , which could be prevented by pretreatment with an excess of DIDS. The nominal extracellular Cl concentration is only about 0.1 μM , so exchange should not represent a large fraction of the rapid tracer efflux. It is still necessary to address the possibility that some or all of the rapid loss of ^{36}Cl represents exchange with an extracellular contaminant (Cl, NO_3 , or HCO_3).

The control we have done is to pretreat the ghosts with enough DIDS not to completely inhibit Cl transport, but to inhibit 50–75% of the band 3 monomers. Such a DIDS pretreatment should reduce the amount of ^{36}Cl lost in the rapid phase, if this efflux represents a half-turnover of the transport cycle. If, on the other hand, the rapid efflux represents exchange for an impurity, then the DIDS pretreatment should not reduce the amount lost, because the same impurity should be present independent of the DIDS pretreatment. Fig. 5 shows that a DIDS pretreatment of 16 nmol/ml packed ghosts² reduces the amount of ^{36}Cl , which leaves the ghosts in the rapid phase by ~50%. Fig. 6 summarizes the results of several such experiments. The same DIDS pretreatment inhibits the rate of ^{36}Cl -Cl exchange (2 mM Cl_o , 1.5 mM Cl_i) by $65 \pm 10\%$ (right) and reduces the “half-turnover” ^{36}Cl efflux from 0.9

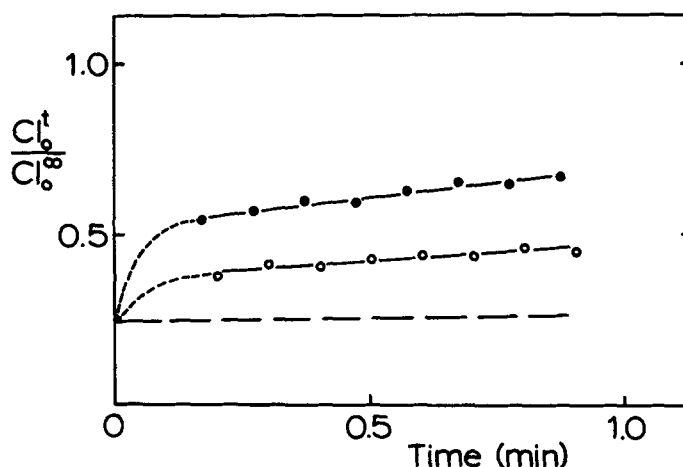


FIGURE 5. Time-course of ^{36}Cl efflux from low Cl ghosts, as in Fig. 4. The closed circles refer to untreated ghosts (a separate preparation from those in Fig. 4). The open circles refer to ghosts that were pretreated before ^{36}Cl loading with 16 nmol DIDS/ml packed ghosts, enough to inhibit Cl-Cl exchange by 55–75%.

$\times 10^6$ to 0.5×10^6 ions/cell (left). Therefore, exchange of ^{36}Cl with an impurity can represent at most a small fraction of the rapid ^{36}Cl loss.

DISCUSSION

Our interpretation of the results in Figs. 4–6 is that the 10^6 Cl ions that leave each ghost in the first 10 s of the experiments represent one half-turnover of the catalytic cycle, i.e., binding to intracellular sites, outward translocation, and release. This temporal separation of two portions of the catalytic cycle is direct evidence that, as predicted by the ping-pong model, the influx and efflux events do not take place simultaneously.

The number of Cl ions transported outward in the half-turnover experiments is only slightly less than the number of band 3 monomers (Fairbanks et

² This corresponds to a nominal DIDS dose of ~800,000 molecules/ghost, using total phosphate as the measure of ghost number and correcting for the NaCl contaminant in the DIDS.

al., 1971). This indicates that most of the transport systems face inward in the absence of a large Cl gradient, and that the stoichiometry of a complete catalytic cycle is one pair of anions exchanging per band 3 monomer. This is the first direct estimate of the stoichiometry of band 3 function, and is consistent with the well-known stoichiometry of one H₂DIDS binding site per monomer (Lepke et al., 1976; Ship et al., 1977; Jennings and Passow, 1979). Nonetheless, band 3 is apparently a dimer in the membrane (Yu and Steck, 1975), and the present results do not exclude the possibility of functional interactions between subunits of a dimer.

The finding that most of the transport systems face inward in the absence of a large Cl gradient is in agreement with three kinds of steady-state flux

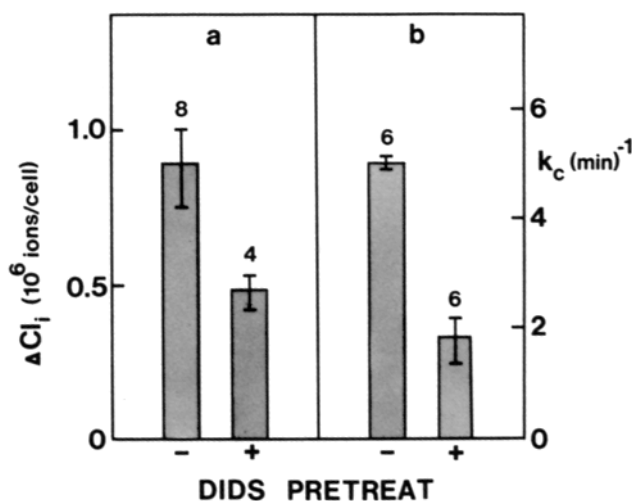


FIGURE 6. (a) Effect of partial DIDS pretreatment (16 nmol/ml packed ghosts) on the "half-turnover" Cl loss from low Cl ghosts, measured by linear extrapolation to zero time of data such as that in Figs. 4 and 5. (b) The rate constant for Cl-Cl exchange for ghosts prepared and DIDS-treated exactly as in a, and subsequently loaded with 1.5 mM ³⁶Cl. The Cl-Cl exchange rate was measured at 0°C in a 30-mM Na₂SO₄ medium containing 2 mM Cl. The number of flux experiments is indicated above each bar. At least two separate ghost preparations were used for each kind of experiment. Error bars indicate extent of data.

data. First, the net SO₄ influx into Cl-loaded cells is much larger than the SO₄ self-exchange flux in the same medium, especially at low pH (Fig. 3; Jennings, 1980). Second, the effect of an outward Cl gradient on H₂DIDS potency suggests that most transporters face inward when Cl_i = Cl_o = 10 mM (Knauf et al., 1980). Finally, the apparent affinity for extracellular Cl is much higher than for intracellular Cl (Gunn and Fröhlich, 1979). This could result from a true difference in affinity or from a more rapid inward than outward translocation rate constant for Cl; either situation would cause the majority of the transporters to be inward-facing in a low Cl medium.

It should be noted that the quantitative asymmetry in the distribution of

inward- and outward-facing transport systems, in the absence of any anion gradients, may depend on which anion is present. For example, for red cells at Donnan equilibrium in a 100-mM SO_4 medium, there are six to eight times more inward-facing than outward-facing transporters at pH 6 (Fig. 3), but the distribution is more nearly symmetric at pH 7 (Jennings, 1980). The distribution may have a completely different pH dependence in a Cl medium, since the translocation rate constants for Cl and for SO_4 would be expected to have different dependences on pH (e.g., Schnell et al., 1977). Possible asymmetries in binding to inward- and outward-facing transport sites may also vary among substrate anions.

The supposition that 10^6 Cl ions leave the cell without being replaced does not imply any net charge transfer across the membrane. The translocation event may involve Cl movement as an electrically neutral ion pair with a protein-bound positive charge. The release at the outer surface should not cause a large change in surface potential because of the high ionic strength and because SO_4 can bind to the outward-facing transport site vacated by Cl (Schnell et al., 1977). The net loss of 10^6 Cl ions therefore does not necessarily cause a large change in either the transmembrane potential or the surface potential at the outer membrane surface.

The outward recruitment of transport systems and the concomitant loss of Cl apparently take <0.2 min at 0°C (Fig. 4). However, the use of the inhibitor stop method may make it appear that the recruitment (half-turnover) is faster than it actually is. The inhibitor used is DIDS, and there is evidence that the related molecules DNDS (Fig. 3) and H_2DIDS (Knauf et al., 1980) bind preferentially to the outward-facing form of the transport system. It may be, then, that DIDS stops the transport only after the initial outward recruitment. For example, in the experiment in Fig. 4, all tubes were centrifuged at $t = 1.6$ min. (Waiting until $t = 5$ min gives the same result.) If the DIDS does not stop the first half-turnover, but only subsequent events, then the half-turnover may *appear* to be over at $t = 0.2$ min, but in reality it may have continued to completion in the time between exposure to DIDS ($t = 0.2$ min) and centrifugation. This would cause no error in the stoichiometry of the half-turnover, but it would make the process appear to be complete sooner than it actually is. Therefore, we are not certain that the half-turnover is complete in 0.2 min. It *should*, however, have a half-time smaller than that of Cl-Cl exchange at 2 mM Cl (0.14 min; Fig. 6), so the observed rapid time-course is not inconsistent with the model.

The theoretical time-course of the half-turnover can also be estimated from the turnover number of band 3-catalyzed Cl transport at 0°C (15,000/min, from data in Gunn and Fröhlich, 1979). The unimolecular rate constant for the outward translocation event must be at least as fast as the turnover number, and at 70 μM intracellular Cl, $\sim 0.1\%$ of the inward-facing transport sites should be occupied (see $K_{1/2}$ values in Gunn and Fröhlich, 1979, and Knauf, 1979). Therefore, the probability per unit time of an outward translocation event in a given transporter is at least 15/min. The half-turnover should therefore be $>90\%$ complete in 0.2 min.

It is important to point out that the measured 0.9×10^6 ions/cell transported outward in a half-turnover of band 3 is subject to several sources of error. Uncertainties in the specific activity of the ^{36}Cl , in the number of ghosts per milliliter packed ghosts, and in the fraction of the ^{36}Cl that is initially extracellular could introduce a 10–20% error in the half-turnover stoichiometry. The true uncertainty in the stoichiometry is thus considerably larger than the range in the data in Fig. 6 indicates. We believe that the data are sufficiently reliable to indicate that the number of inward-facing transport sites in the low Cl ghosts is between 0.5 and 1 times the number of band 3 monomers. The data do not, however, constitute a measure of the precise inward vs. outward distribution of transport proteins.

Finally, we believe that the half-turnover experiments constitute the final proof that the band 3 protein is indeed the catalyst of anion transport. In all previous chemical labeling or reconstitution experiments, the possibility existed that a minor protein component, distinct from the bulk of band 3, actually catalyzed the transport. Here we have shown that a partial catalytic cycle of the transport involves nearly 10^6 ions per cell. If a minor protein component were the catalyst, it must have a catalytic cycle that involves many Cl ions—this is not consistent with the known kinetic features of the anion transport (see Knauf, 1979).

Although the above discussion has concerned only the half-turnover experiments, we wish to emphasize that the steady-state flux data in Fig. 2 also constitute strong support for the ping-pong mechanism. The apparent K_c^i for Cl- P_i exchange is lower than that for Cl-Cl exchange by at least 30-fold. This is what is expected from the ping-pong model, because the catalytic cycle should be limited by the P_i influx rather than the Cl efflux, even at intracellular Cl concentrations well below the true dissociation constant for the inward-facing site. In contrast, a simple sequential model, which requires a ternary complex among intracellular Cl, extracellular P_i , and the protein, does not predict such a low K_c^i .

APPENDIX

The following is a derivation of the implications of the ping-pong model with regard to the inhibitory potency of a nonpenetrating extracellular inhibitor, I, which binds only to the outward-facing state. The inhibition is measured on tracer SO_4 influx from a 100-mM Na_2SO_4 medium into either Cl-loaded cells (J_{c-s}) or SO_4 -loaded cells (J_{s-s}). The ping-pong model predicts that if J_{c-s} is larger than J_{s-s} by a factor Y in the absence of inhibitor, then the inhibitor should be more potent (lower K_I) toward J_{c-s} than toward J_{s-s} by the same factor Y .

Let E_i represent all inward-facing forms of the transport protein (e.g., occupied with an anion or unoccupied, protonated or unprotonated). Let E_o represent all outward-facing forms of the transporter *except* those occupied by inhibitor. The inhibited transporters are represented by X . Depending on the intrinsic symmetry of the transport system and on the anion gradients present, E_o may be larger or smaller than E_i . For any steady state, though, there must exist some ratio between E_i and E_o . Call this ratio N :

$$N \equiv E_i/E_o. \quad (1)$$

Assume that the inhibitor I can bind only to the outward-facing state. Whether this inhibition is fully competitive or not, it is clear that in a given medium *some* concentration of I can be found at which half of the outward-facing transport systems are occupied. This concentration, K_I , is not a true dissociation constant because of effects of competition. From the definitions of K_I and X , it must be true that:

$$(I \cdot E_o)/X = K_I. \quad (2)$$

Conservation of the total number of transport systems (normalized) requires that:

$$X + E_o + E_i = 1. \quad (3)$$

Eqs. 1-3 can be solved for the fractional inhibition X of the transport as a function of inhibitor concentration.

$$X = I/(I + K_I[1 + N]). \quad (4)$$

Therefore, the inhibition is 50% at $I = K_I(1 + N)$.

Now consider a Donnan equilibrium in an all-SO₄ medium. There will be some equilibrium distribution ratio (N^{eq}) of E_i and E_o , and some fraction ($1/[1 + N^{eq}]$) of the transport systems face outward. The unidirectional SO₄ influx (J_{s-s}) is proportional to this fraction, because influx requires an outward-facing transporter. The initial SO₄ influx (J_{c-s}) from the same medium, but into Cl-loaded cells, should therefore be $(1 + N^{eq})$ times as large as J_{s-s} , because all, rather than $1/(1 + N^{eq})$, of the transporters should be recruited outward, and all other conditions (pH, extracellular SO₄ concentration) are constant:

$$J_{c-s}/J_{s-s} = 1 + N^{eq}. \quad (5)$$

Finally, let the K_I in Eq. 4 refer to the 100-mM SO₄ medium in which J_{c-s} and J_{s-s} are measured. Since $N = 0$ for Cl-SO₄ exchange, inhibition is clearly 50% at $I = K_I \equiv K_I^{c-s}$. For SO₄-SO₄ exchange, however, $N \neq 0$, and 50% inhibition occurs at:

$$I = K_I^{s-s} = K_I^{c-s} (1 + N^{eq}). \quad (6)$$

Combining Eqs. 5 and 6,

$$J_{c-s}/J_{s-s} = K_I^{s-s}/K_I^{c-s}. \quad (7)$$

Eq. 7 says that, if the uninhibited J_{c-s} is twice as large as J_{s-s} (as would be true if $N^{eq} = 1$), then I should be twice as good an inhibitor of Cl-SO₄ as of SO₄-SO₄ exchange in the same medium. Similarly, if J_{c-s} is eight times J_{s-s} , as in Fig. 3, then K_I^{s-s} should be eight times K_I^{c-s} .

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