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GLYCINE TRANSPORT BY HEMOLYSED AND RESTORED PIGEON RED CELLS

EFFECTS OF A DONNAN-INDUCED ELECTRICAL POTENTIAL ON ENTRY AND EXIT KINETICS

GEORGE A. VIDAVER*, SARA L. SHEPHERD, JOYCE B. LAGOW and KAREN J. WIECHELMAN

Department of Chemistry, University of Nebraska, Lincoln, Nebr. 68588 (U.S.A.)

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SUMMARY

The influence of a Donnan effect on the transport of glycine by hemolysed and restored pigeon red cells was examined. The Donnan effect was produced by replacing Cl^- with 2,4-toluenedisulfonate or glutamate. The effects of the associated membrane potential and inside-outside pH difference on glycine entry and exit rates were examined. The effects of pH on entry and exit rates in the absence of a Donnan effect were also examined.

In the absence of a Donnan effect, Na^+-dependent glycine entry requires the protonated form of a group with a pK_{app} of 7.9 and the deprotonated form of another group with a pK_{app} of 6.8. Neither of these are required for exit but the deprotonated form of a group(s) with a pK_{app} of 6.2 is required. The pK 7.9 group and pK 6.2 group probably react with H^+ at the inner face of the membrane and the pK 6.8 group probably reacts at the outer face.

The V for glycine entry was determined for cells with their Cl^- largely replaced by toluenedisulfonate and without such replacement. Between pH 6.1 and 7, the ratio of the respective V values, V_T/V_{Cl}, was 1.5–1.7. V_T/V_{Cl} rose above pH 7 to near 4 at pH 8.3. At pH 6.9, with glutamate replacing cell Cl^-, the analogous ratio (V_{Glu}/V_{Cl}) was 1.7. The increase of V_T/V_{Cl} above pH 7 could be quantitatively accounted for by the increase in cell $[H^+]$/medium $[H^+]$ caused by the Donnan effect together with the assumption that the pK 7.9 group reacts with H^+ at the inner face of the membrane.

When cell Cl^- was replaced by toluenedisulfonate or glutamate there was a drop in the term in the glycine K_m describing Na^+ dependence of glycine entry. When cell Cl^- was replaced by toluenedisulfonate there was a rise in the Na^+-independent term in the glycine entry K_m. By replacing varying amounts of cell Cl^- with either toluenedisulfonate or glutamate, plots were obtained of entry rates vs. the cell $[Cl^-]$/

* To whom reprint requests should be addressed.

medium [Cl^-] ratio consistent with the assumption that the Donnan-induced membrane potential acts on a "moving" charge. Glycine exit was only slightly accelerated by *trans*-toluenedisulfonate. The ratio, exit rate into toluenedisulfonate medium/exit rate into Cl^- medium rose with decreasing pH. This rise could be accounted for by a Donnan-induced inside-outside pH difference which affects a pK_{app} 6.2 group reacting with internal H^+.

The observed influences of the Donnan effect on V(glycine entry), on both components of K_m(glycine entry), on the shape of the plot of glycine entry rate vs. the cell [Cl^-]/medium [Cl^-] ratio and on glycine exit all fit the assumption that when the empty porter reorients, one unit of negative charge accompanies it "across" the membrane and that no other steps involve charge movement.

The properties of the system seem inconsistent with a translational ("ferry boat") mobile carrier.

INTRODUCTION

Pigeon red cells can be hemolysed and their selective impermeability to small molecules then restored. By lysis and restoration the composition of the solution inside the cell can be manipulated. Such lysed and restored cells actively transport glycine like intact cells [1–4]. Our lysis and restoration procedure equilibrates cell K^+ and glycine with the external solution and nearly equilibrates hemoglobin. This procedure gives a 6-fold dilution of small molecules and an approx. 5-fold dilution of hemoglobin. Intact cells at neutral pH show only a small Donnan effect; they have a high net permeability to Cl^- [5] and the cell [Cl^-]/medium [Cl^-] ratio is approx. 1.25 [6]. In the present work, cells lysed and restored with Cl^- as the major anion showed a still smaller Donnan effect (medium [Cl^-]/cell [Cl^-] = 1.1).

Glycine crosses the membrane of both intact and lysed and restored cells by two Na^+-dependent routes [1, 4, 7, 8] and a Na^+-independent route [1, 2]. Glycine and two Na^+ are co-transported by the main Na^+-dependent route [3, 5, 9, 10]. An anion is also required [11]. The system is highly specific for Na^+ and glycine [1, 8] but much less specific for the anion [11]. The Na^+ electrochemical gradient supplies most if not all of the energy for glycine "active" transport [2–5, 9, 10].

Previous work had yielded a model (Fig. 1) for glycine transport by the main Na^+-dependent route, rate equations describing the model, and some tests of the adequacy of the rate equations [1–4, 9, 11]. The equations describe a transport model which is purely passive if both Na^+ and glycine electrochemical potentials are considered. Coupling between Na^+ and glycine fluxes occurs because glycine cannot combine with the porter unless Na^+ has already combined, and the change of orientation ($ENa_2GCl_o \rightleftharpoons ENa_2GCl_i$) alters accessibility to both Na^+ and glycine simultaneously, producing co-transport of glycine and Na^+.

In the present work the central questions were: what step or steps in the translocation process involves movement of charge normal to the membrane plane and how much charge moves? The approach used was to impose an electrical potential across the membrane with a Donnan effect and measure the resulting glycine transport rates relative to those of the unperturbed system. Kinetic analyses were made to assign membrane potential effects to individual terms of the previously derived [4] rate

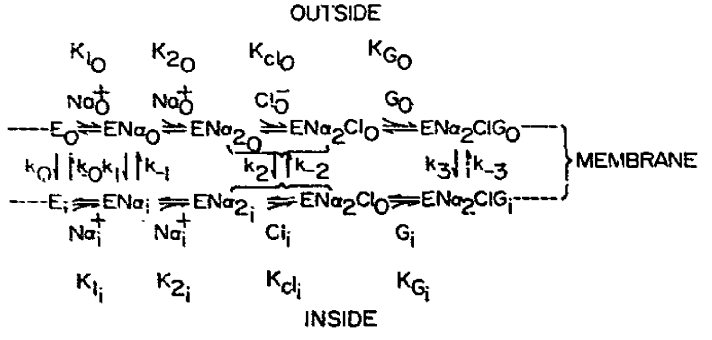

Fig. 1. The current model for Na^+-dependent glycine transport by hemolysed and restored pigeon red cells. This model was derived from kinetic studies. "G" is glycine. Capital K values designate equilibrium dissociation constants and small k values designate rate constants. Subscripts "o" and "i" distinguish constants for reactions with substrates outside and inside the cells, respectively. For Na^+-dependent glycine entry into cells with kinetically negligable Na_i^+ and saturating $[Cl_o^-]$ the rate equation [4] is:

$$1/v = \frac{1}{E_t}\left(\frac{k_{-o}+k_3}{k_{-o}k_3}\right)\left\{\left[\frac{K_{l_o}K_{2_o}K_{G_o}}{[Na_o^+]^2}\left(\frac{k_{-o}+k_o}{k_{-o}+k_3}\right)+K_{G_o}\left(\frac{k_{-o}}{k_{-o}+k_3}\right)\right]\frac{1}{[Gly_o]}+1\right\}.$$

The part of the equation enclosed in [ ] is $K_{m,o}$. The part outside { } is $1/V$. The above equation has been simplified from that in ref. 4 by omitting the imperceptible first-power Na^+ term from $K_{m,o}$ and omitting k_2, which is small compared to k_{-o}, from the K_{G_o} term in K_m.

equations. Since moving species might be proteins whose charges depend on pH, measurements were made at several pH values.

The Donnan effect was obtained by substituting 2,4-toluenedisulfonate or glutamate for Cl^- in the fluid compartment into which the glycine moved. The Donnan potential promotes co-transport of positive charge or counter-transport of negative charge. The imposed membrane potential was estimated from the cell $[Cl^-]$/medium $[Cl^-]$ ratio.

The membrane potential produced a pH-insensitive increase in V for glycine entry of approx. 70 %. This is ascribed to the movement of about one unit of negative charge accompanying the reorientation of the empty porter ($E_o \underset{k_{-o}}{\overset{k_o}{\rightleftharpoons}} E_i$, Fig. 1) with no charge movement at any other steps. The observed membrane potential effects on other kinetic parameters fit this interpretation. There were also pH-dependent membrane potential effects on both glycine entry and exit. These are attributed to the Donnan-induced shift of cell pH away from the pH of the medium affecting ionizable groups in toluenedisulfonate but not Cl^- cells.

Abbreviations and definitions

MES, 2 [*N*-morpholino]ethane sulfonic acid; TES, *N*-tris[hydroxymethyl]-methyl-2-aminoethane sulfonic acid.

Subscripts o and i, these distinguish the externally and internally oriented forms of the glycine porter, kinetic constants or substrates.

v_T, v_{Glu}, v_{Cl}: Na^+-dependent component of the glycine entry rate into cells with cell Cl^- (partially) replaced by toluenedisulfonate (v_T), by DL-glutamate (v_{Glu}), or not replaced (v_{Cl}), respectively.

V_T, V_{Glu}, V_{Cl}: the V components of v_T, v_{Glu}, v_{Cl} above.

$K_{m,T}$, $K_{m,Glu}$, $K_{m,Cl}$: The K_m components of v_T, v_{Glu}, v_{Cl} above.

v_T, v_{Cl}: exit rate coefficients. These are Na^+-dependent glycine exit rates divided by cell glycine concentrations when Cl^- of the medium is replaced by toluenedisulfonate (v_T) or not replaced (v_{Cl}).

K'_{Go}, $K_{1o}K_{2o}K'_{Go}/[Na^+]^2$: components of $K_{m,o}$ defined by $K_{m,o} = K'_{Go} + K_{1o}K_{2o}K'_{Go}/[Na^+]^2$. Primed K values are used for brevity. Thus K'_{Go} is $K_{Go}(k_{-0}+k_2)/(k_{-0}+k_3)$ and $K_{1o}K_{2o}K'_{Go}$ is $K_{1o}K_{2o}K_{Go}(k_{-0}+k_2)/(k_{-0}+k_3)$. K_{1o}, k_{-0} etc. are the constants of the model in Fig. 1. Subscripts T, Glu and Cl (e.g. $K'_{Go})_{Cl}$ are used to designate the trans-anion as for v etc. above.

Toluenedisulfonate, glutamate and Cl^- cells: cells with the indicated anions replacing internal Cl^-.

(Cl_o^-/Cl_i^-) and $(Cl_o^-/Cl_i^-)'$: (Cl_o^-/Cl_i^-) is the ratio of Cl^- concentration in the medium to that inside the cells. $(Cl_o^-/Cl_i^-)'$ is (Cl_o^-/Cl_i^-) for toluenedisulfonate or glutamate cells divided by (Cl_o^-/Cl_i^-) for Cl^- cells. (Cl_o^-/Cl_i^-) is a measure of the change in membrane potential produced by replacement of cell Cl^- with toluenedisulfonate or glutamate.

$\rho_\alpha \equiv K_{1i}K_{2i}K_{Gi}/K_{1o}K_{2o}K_{Go}$

MATERIALS AND METHODS

Chemicals. Inorganic chemicals were analytical reagent grade. ^3H- and ^{14}C-labelled glycine were obtained from New England Nuclear Corp. Unlabelled glycine was obtained from Fisher Scientific, bovine serum albumin from Sigma Chemical Co., D-glucose from Baker Chemical Co., and DL-alanine from General Biochemicals. The 2,4-toluenedisulfonate was prepared as before [6].

Preparation and handling of cells. Pigeon red cells were obtained as described before [1]. Lysed and restored cells were used for all experiments.

The experiments described were done in two groups. Extracellular space was measured in group 2 but not group 1 experiments with ^{22}Na. There were also slight modifications in procedure in group 2 to improve precision. The group is identified in the figures and tables.

The cell solute compositions were adjusted by lysis and restoration as previously described [2]. This involved (i) hypotonic hemolysis at 5–6 °C, (ii) restoration of tonicity at 5–6 °C, and (iii) incubation for 17 min at 39 °C ("annealing") to restore impermeability to small molecules. For glycine entry rate measurements three types of cells were used: (i) cells with part of their Cl^- replaced by 2,4-toluenedisulfonate (toluenedisulfonate cells), (ii) cells with part of their Cl^- replaced by glutamate (glutamate cells), and (iii) cells with no replacement (Cl^- cells). These cells also had [^3H]glycine inside. To replace most of the cell Cl^- with toluenedisulfonate we added a tonicity-restoring solution containing 0.30 M dipotassium toluenedisulfonate, 1.4 M sucrose, 0.0086 M $CaCl_2$ and 0.017 M $MgSO_4$.

0.4 ml was used per g original (unlysed) cells. For replacement of cell Cl^- with glutamate, the tonicity-restoring solution had 1.34 M potassium glutamate, 0.0097 M $CaCl_2$ and 0.0194 M $MgSO_4$ and was used at 0.35 ml/g. Chloride cells were prepared with the previously described restoring solution [2] at 0.85×strength (0.40 ml/g) or 0.97×strength (0.35 ml/g) according to whether the Cl^- cells were

controls for toluenedisulfonate cells or glutamate cells. Mixtures of restoring solutions were used for intermediate Cl^- replacements. After annealing, cell suspensions were chilled, diluted and centrifuged. To adjust pH, cells were suspended in buffer (38 ml/g) and held 30 min at 0–4 °C. The buffer solution composition was: 0.0016 M $CaCl_2$, 0.0032 M $MgSO_4$, 0.296 osM NaCl plus KCl plus buffer. Buffers were MES (2-(*N*-morpholino)ethane sulfonic acid), TES (*N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid) or *N,N*-bis(hydroxyethyl) glycine [12] with the minor component of the A^-/HA buffer pair at 0.010 M. After pH adjustment, cells were pelleted and the 0.3-g pellets suspended in 1.00 ml (group 2) or 2.00 ml (group 1) cold incubation media. All incubation media had 0.3 μCi/ml [^{14}C]glycine and the appropriate total glycine concentrations, 0.0013 M $MgSO_4$, 0.0023 M $CaCl_2$, 0.0083 M D-glucose, 0.010 M DL-alanine, 0.143 M KCl and/or NaCl and either 0.009 M of the buffer used for pH adjustment (toluenedisulfonate cells) or 0.006 M phosphate buffer (glutamate cells). Cells were incubated 4 min (group 2) or 9 min (group 1) at 39 or 0 °C, chilled 2 min, diluted with 7 or 6 ml cold unlabelled K^+ incubation medium and pelleted. The cold diluent contained ^{22}Na in group 2 experiments for extracellular space measurement. Picric acid extraction of pellets and supernatants, geiger counting procedures and glycine, Cl^- and Na^+ analysis were similar to procedures previously used [1, 6]. Picric acid deproteinization was with 2.00 ml/0.3 g for group 2 and 3.00 ml/0.3 g for group 1.

Extracts were also counted in a scintillation counter (Ansitron). The isotope inputs used gave pellet extract counts: $^3H > ^{14}C > ^{22}Na$. Non-contiguous windows were used and quench and spill correction factors were individually calculated for each sample. Pellet contents of all three isotopes could be determined but ^3H in the medium could not be determined.

For glycine exit measurements, Cl^- cells were prepared with (usually) 1.5 mM internal [^{14}C]glycine, 1 Ci/mol. pH adjustment was as described except that 15 ml buffer was used per g cells and the buffer contained 1 mg/ml bovine serum albumin. 0.3 g of cells were suspended in 5.00 ml incubation medium, incubated at 39 or 0° C for 7 min, chilled and centrifuged without dilution. Two kinds of incubation media were used. All had 0.0011 M $MgSO_4$, 0.0022 M $CaCl_2$, 0.0075 M D-glucose, 1.5×10^{-5} M unlabelled glycine, 1 mg/ml bovine serum albumin and were 0.0081 M in the buffer used for pH adjustment. Chloride media also contained 0.129 M KCl or NaCl, toluenedisulfonate media had instead 0.070 M dipotassium toluenedisulfonate plus 0.063 M sucrose. Thick sample plates of picric acid extracts were prepared and counted as previously described [1, 6].

pH measurements. For glycine exit experiments, the relationships between buffer composition and pH were determined in a separate experiment. For glycine entry experiments, pH values were measured directly.

Calculations. The glycine exit rate coefficient is the difference between the cpm in the media from incubated and unincubated cells divided by the average of the cpm in the incubated and unincubated pellets of a pair. The Na^+-dependent glycine exit rate coefficient is the difference between the coefficients from Na^+-filled and K^+-filled cells.

Entry rates were calculated several ways. For the simplest, the Na^+-dependent glycine entry rate was the difference between cpm/ml pellet water for incubated and unincubated cells from Na^+ medium, minus the corresponding difference for cells

from K^+ medium, divided by the cpm/μmol glycine added to the medium. With cpm/ml fixed, the cpm entering from K^+ media is independent of glycine concentration since Na^+-independent glycine entry is proportional to glycine concentration. More elaborate calculations were made with a minicomputer (DAC-512, Picker Nuclear). Corrections could be made for re-exit of ^{14}C and changes in external glycine concentration and specific activity during incubation. Average (i.e. half initial +half final) glycine concentrations were used for the double reciprocal plots. The simple and elaborate calculations gave similar results, showing that the conditions, chosen to keep corrections small, did so. Thus the calculated "rates" are good approximations of $\partial[Gly]/\partial t$ at the midpoint of the incubation time and average glycine concentration.

The above calculations gave entry rates in μmol/ml pellet water per incubation time. To compare entry rates for Cl^- cells with those for toluenedisulfonate or glutamate cells, an additional correction for "active fraction" was calculated. In early trials of restoration where dipotassium toluenedisulfonate isoosmotically replaced KCl, cells restored with toluenedisulfonate leaked more hemoglobin than KCl-restored cells. A mixture of dipotassium toluenedisulfonate and sucrose was found allowing restoration without much more hemoglobin loss than for KCl-restored cells. Since some difference in hemoglobin loss remained, we wanted a measure of the efficiency of restoration. Therefore, [^{3}H]glycine was included in the lysing solutions. Its concentration was the same for Cl^- and toluenedisulfonate cells through the annealing step. Subsequent steps removed external [^{3}H]glycine. Cells unable to retain the [^{3}H]glycine cannot show Na^+-dependent glycine uptake. During incubation, [^{3}H]glycine can be lost by lysis and by mediated exit. Glycine exit is calculable from Na^+-independent glycine entry since Na^+-independent glycine entry and exit coefficients are the same [1–3] and the cells were Na^+ poor. The ^{3}H/pellet after incubation plus the calculated loss of ^{3}H/pellet by exit should be proportional to the fraction of cells remaining sealed to glycine at the end of the incubation. This sum divided by (ml water/pellet) (^{3}H/ml of the original annealed suspensions) gave the "active fraction" estimates (ml ^{3}H space/ml pellet water) for the group 1 experiments. This "active fraction" for toluenedisulfonate cells averaged 69 % (range 80.5–61.2 %) of the Cl^- cell values for group 1 and 74 % for group 2. For each experiment in group 1 where V (entry) for toluenedisulfonate and Cl^- cells were compared, V_T/V_{Cl} (in μmol/ml pellet water) was divided by the ratio of "active fractions" for the two cell types in that experiment.

In the group 1 experiments, much of the effect of internal toluenedisulfonate on V (entry) arose from differences in "active fractions" as measured by ^{3}H retention. We wanted an independent estimate of the "active fraction". In the group 2 experiments extracellular space was determined before and after incubation by adding ^{22}Na to the chilled cell suspension just before centrifugation. The "active fraction" was also determined by ^{3}H retention and should have been equal to one minus extracellular space from ^{22}Na determination. For both toluenedisulfonate and glutamate cells it was smaller. The significance of this discrepancy is discussed later.

Medium chloride: cell chloride ratios. For the exit experiments, (Cl_i^-/Cl_o^-) values were estimated from the Cl^- contents of pellets and media and the extracellular space values. Extracellular space was estimated as before [1–4] by comparing the Na^+ contents of otherwise identical cell pellets pelleted from Na^+-rich and Na^+-poor media. For the group 1 entry experiments, a slight change in this procedure produced

20–40 % errors in extracellular space values which grossly distorted the (Cl_o^-/Cl_i^-) ratios. Therefore for group 1 entry experiments, (Cl_o^-/Cl_i^-) ratios were calculated from individual pellet and media Cl^- contents and the average value for extracellular space of Cl^- cells (35 %) obtained in earlier experiments. Since pellet Cl^- did not vary with pH, an average (Cl_o^-/Cl_i^-) ratio was used for group 1. This is 3.61 ± 0.43 (S.D., $n = 9$), the average of the group averages for unincubated cells (4.73) and incubated cells (2.05). This (Cl_o^-/Cl_i^-) value agrees with the more accurate group 2 estimates where ^{22}Na space was used to calculate cell [Cl^-].

Relative Cl^- and cation permeability of hemolysed and restored cells. The Cl^- permeability must be much greater than Na^+ and K^+ permeabilities for the (Cl_o^-/Cl_i^-) ratio to be used to measure the membrane potential. Cl^- permeabilities of lysed and restored Cl^- and toluenedisulfonate cells were measured by the modification of Azzone's method [13] previously described [5]. Cell suspensions were treated [5] with 15 μg/g gramicidin D for 3 min at 39 °C and chilled. At zero time 0.10-ml aliquots were mixed with 3.00-ml portions of various prewarmed media and $A_{650\,nm}$ followed with a Spectronic 20 spectrophotometer. The $A_{650\,nm}$ changes with cell volume. With appropriate solute gradients, cell volume changes at a rate limited by the Cl^- permeability since the gramicidin removes the barrier to Na^+ and K^+ movements.

Permeability to K^+ and Na^+ was assessed from changes in wet weight of toluenedisulfonate and Cl^- cells on incubation. Since these cells were not treated with gramicidin, Cl^- permeability was much greater than cation permeabilities and the latter limit the rate of volume change.

RESULTS

Relative Cl^- and cation permeabilities of hemolysed and restored cells

Gramicidin makes cells permeable to Na^+ and K^+. The dose used here was 15 times that sufficient to equilibrate Na^+ and K^+ in 3 min [5]. Such K^+-permeable cells take up or lose KCl at rates limited by their Cl^- permeability. The "×" curve in Fig. 2A (pH 6.1) shows gramicidin-treated toluenedisulfonate cells swelling in hypertonic KCl medium (falling $A_{650\,nm}$) from their volume in hypertonic choline chloride (●) to their volume in hypotonic choline chloride (■) in 3.5 min. This volume change corresponds to an uptake of 130 mM Cl^-. Chloride cell Cl^- permeability is illustrated by Fig. 2B (pH 8.3). Here gramicidin-treated cells in isotonic choline chloride (■) shrink to the volume of untreated cells in hypertonic KCl (▲) (corresponding to a loss of 72 mM Cl^-) in less than 3 min. Figs. 2A and 2B are representative of results obtained at pH 6.1, 7.1 and 8.3 with both types of cells. The longest time to double or halve volume was 5 min. Note that the permeability measured is net Cl^- permeability, not anion exchange.

Net K^+ and Na^+ permeabilities were assessed from changes in pellet wet weights on incubation in the absence of gramicidin. The observed weight changes were too small to be governed by Cl^- permeability, so they reflect cation permeability. The ratio of incubated to unincubated pellet wet weights of Cl^- cells (approx. 1) was subtracted from the corresponding ratio for toluenedisulfonate cells to give the relative weight changes. In the pH range 6.1–7.85 with 9 min incubation at 39 °C in K^+ media, the relative weight change was 0 ± 1 % $(S.D.)_M$, $n = 9$. At pH 8.1–8.2,

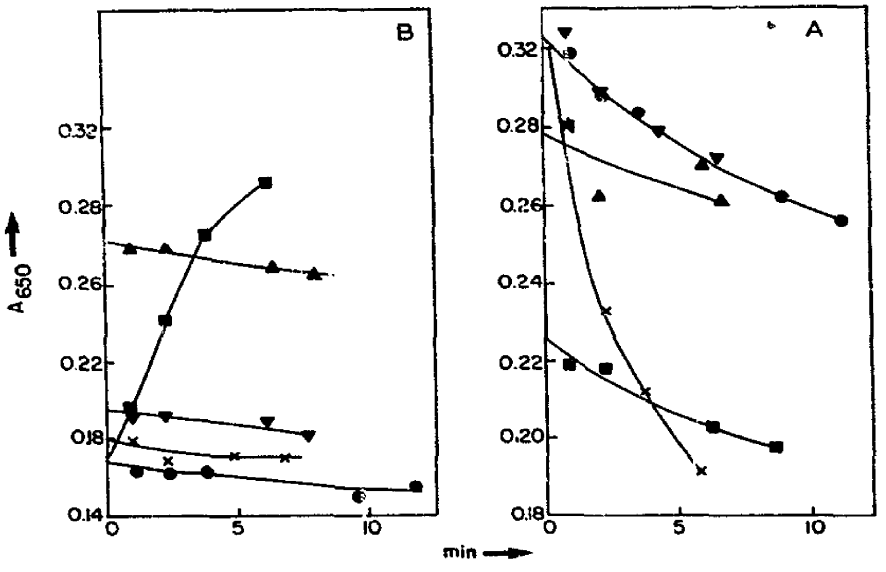

Fig. 2. Plots of $A_{650\,am}$ vs. time for gramicidin-treated and control lysed and restored cells in various media. A: Toluenedisulfonate cells, pH 6.1, were prepared, treated with gramicidin or control solution and their $A_{650\,am}$ vs. time measured as described in Materials and Methods. MES was used as buffer. ×, gramicidin-treated cells in a medium with 1.6 times the normal KCl concentration (1.6×145.4 mM); ●, gramicidin-treated cells in 1.6 times normal choline chloride medium; ▼, control cells in 1.6 times normal KCl medium; ▲, control cells in 1.6 times normal choline medium; ■, gramicidin-treated cells in 0.84 times normal choline chloride medium. B: Cl^- cells, pH 8.3, were prepared, treated with gramicidin or control solution and their $A_{650\,am}$ vs. time measured as described in Materials and Methods. *N,N'*-bis (2-hydroxyethyl)glycine was used as buffer. ■, gramicidin-treated cells in medium with the normal concentration (145.4 mM) of choline chloride; ●, gramicidin-treated cells in medium with the normal concentration of KCl; ×, control cells in normal choline medium; ▼, control cells in medium with the normal concentration of KCl; ▲, control cells in medium with twice the normal KCl concentration.

the corresponding value was $-2.3\pm0.15\,\%$, $n = 2$. With cells incubated in Na^+ media, the Na^+ permeability limits the swelling rate. The relative weight changes in 9 min were $+3.7\pm0.94\,\%$ (S.D.), $n = 9$ (pH 6.1–7.85) and $+0.45\pm1\,\%$ $(S.D.)_M$, $n = 2$ (pH 8.1–8.2). Assuming only 50 % of the pellet water is intracellular, correcting for dry weight, and assuming that all wet weight change is due to cell volume change, the percentage changes in cell volumes in 9 min at 39 °C were for K^+ media (pH 6.1–7.85), $0\pm2.3\,\%$ and for Na^+ media (pH 6.1–7.85), $+8.5\pm2.1\,\%$. The largest change, +8.5 %, corresponds to a cation uptake of 13 mM in 9 min, compared to the slowest Cl^- transfer, 70 mM in less than 3 min. From Eqn. 4.18 of ref. 14, if P_{Cl} were as little as four times P_K or P_{Na}, the membrane potential of toluenedisulfonate or glutamate cells would correspond to a Donnan ratio 2/3 of that when P_{Cl} is infinitely greater than P_K or P_{Na}. If P_{Cl} is 10 times P_K or P_{Na}, the equivalent Donnan ratio is 80 % of the limiting value.

Experiments on glycine entry

(1) ***Effect of pH on glycine entry in the absence of a Donnan effect.*** The glycine entry rate (v_{Cl}) vs. pH curve (Fig. 3) indicates that two ionizable groups govern entry,

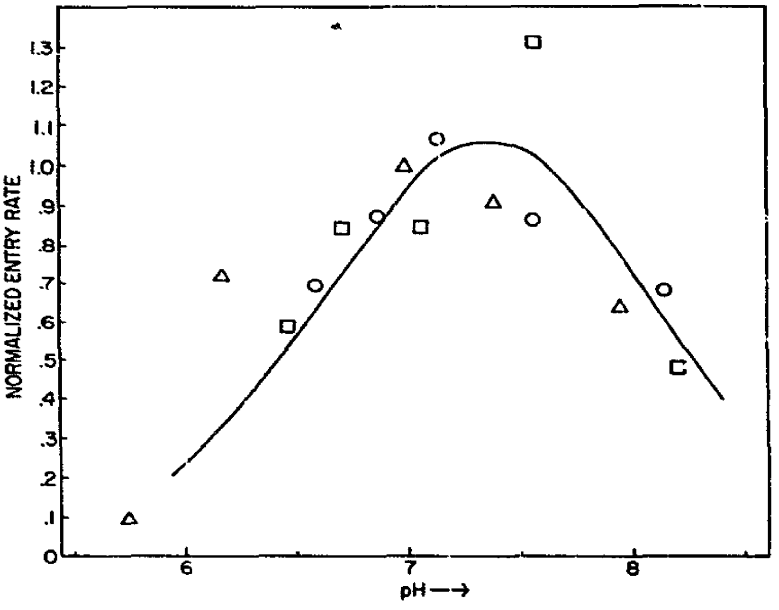

Fig. 3. Influence of pH on the glycine entry rate in the absence of a Donnan effect. The Na^+-dependent glycine entry rate, v_{Cl}, into Cl^- cells is plotted against the pH of the medium. The simple calculations method was used (see text). Three experiments have been normalized to the average pH 7 v_{Cl} value for presentation on a single graph. (Different symbols are used for different experiments.) The curve is that calculated for two groups, p*K* values 6.8 and 7.9, required in the deprotonated and protonated forms, respectively. For one of the experiments (△) the pH values were known to be slightly underestimated at the higher pH values so that the p*K* estimate of 7.9 comes from the other two experiments. External Na^+: 143 mM, external glycine: 0.5 mM. These experiments are from group 1.

TABLE I

EXTRACELLULAR SPACE DETERMINED FROM ^{22}Na ENTRAINMENT AND [^3H]GLYCINE RETENTION

^{22}Na and ^3H space data from the group 2 experiments are tabulated here.

Cell type	^{22}Na space (percent of pellet water ±S.D.)$_M$	One minus ^3H space (percent of pellet water ±S.D.)$_M$	One minus ^3H space $-^{22}Na$ space	*N*
Toluenedisulfonate	37.2±1.6	48.7±1.1	11.5	4
Glutamate	29.1±1.8	42.0±2.8	12.9	4
Cl^-	25.4±1.0	30.8±1.6	5.4	8

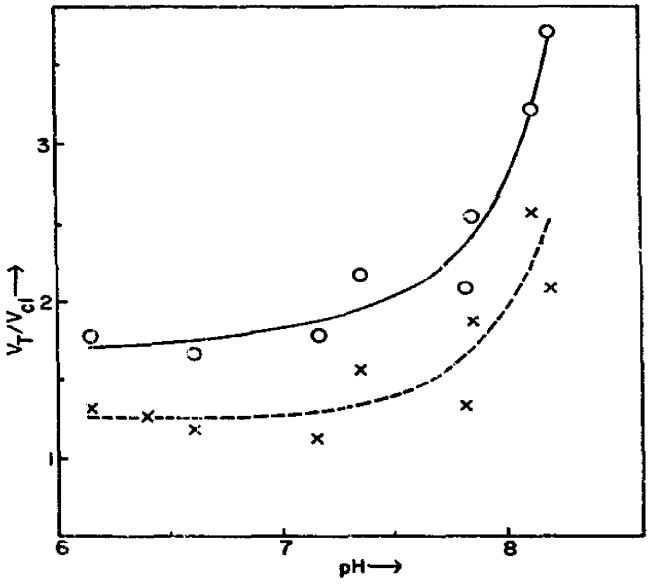

Fig. 4. The influence of a Donnan effect on the maximal velocity for glycine entry as a function of pH. The ratio, V_T/V_{Cl}, is plotted against the pH of the medium. V_T is the V for the Na^+-dependent component of glycine entry into toluenedisulfonate cells (Donnan effect present) and V_{Cl} the V into Cl^- cells (no Donnan effect). Solid line and "0" points, computer calculations with ^3H space "active fraction" correction (see text); dotted line and "X" points, simple calculations without "active fraction" correction. Each point is the ratio of V value determined in one experiment. The V values were obtained from double reciprocal plots. Incubation was for 9 min at 39 °C. In order to avoid subjective errors in calculating V and K_m, two lines were drawn for each plot, one through the highest glycine point and just between the other two and another through the lowest glycine point and just between the other two. The averages of the two V and K_m values were used. In most experiments both Geiger and scintillation plots showed reasonably low scatter. In those cases, V estimates from both sets of counts were averaged. Compositions for these experiments were: Na^+ media, $[Na_o^+]$ = 143 mM, $[Cl_o^-]$ = 148 mM; cells in Na^+ media, initial: toluenedisulfonate cells, Na_i^+ = 14.5 ± 1.8 (S.D.) μmol/ml cell water; average (initial/2 + final/2) Cl^- in toluenedisulfonate cells 41.0 ± 4.4 (S.D.)$_M$ μmol/ml cell water; in Cl^- cells Cl_i^- was 120.1 ± 5.3 (S.D.)$_M$ μmol/ml cell water; initial glycine in toluene-disulfonate and Cl^- cells: approx. 0.8 μmol/ml cell water. Final cell Na^+ values were not routinely determined, but in two experiments at pH 7.6 toluenedisulfonate and Cl^- cells took up 16.1 and 18.3, 23.6 and 17.0 μmol/ml cell water, respectively. From entry rate values, cell glycine changed by a few tenths of a μmol/ml or less during incubation. These experiments are from group 1.

a deprotonated group, pK_{upp} 6.8 and a protonated group, pK_{app} 7.9. Fig. 3 shows v_{Cl} vs. pH rather than V_{Cl}, but v_{Cl} here is approximately proportional to V_{Cl} since [glycine]/K_m is ≥ 2.5 in this pH range (data not shown).

(2) *Calculation of "active fractions" from cell space retaining [^3H]glycine and cell space excluding ^{22}Na.* In group 1 experiments [^{3}H]glycine trapped during restoration was used to estimate the "active fractions" of toluenedisulfonate and Cl^- cells. In group 2 experiments, extracellular space was also measured with ^{22}Na. One minus ^{3}H space was larger than ^{22}Na space for both toluenedisulfonate and glutamate cells (Table I). For Cl^- cells, the difference between one minus ^{3}H space and ^{22}Na space was small, and by either measure the Cl^- cell extracellular space was smaller than that of toluenedisulfonate or glutamate cells. The difference between

the glutamate cell and Cl^- cell ^{22}Na spaces is not significant. The significance of the different "active fraction" values obtained with the two methods is considered in Discussion.

(3) *Effects of internal toluenedisulfonate or glutamate on glycine entry rates.* Fig. 4 shows the ratio, V for toluenedisulfonate cells/V for Cl^- cells (V_T/V_{Cl}) plotted against the pH of the medium. The solid line is through points calculated with the computer and corrected for "active fraction" from ^3H space (Materials and Methods), the dotted line is through points calculated by the simple method and lacking "active fraction" corrections. The latter are included to show that only the magnitude, not the sign of the toluenedisulfonate effect depends on the "active fraction" correction, to allow inclusion of an experiment where tritium data was lacking, and to show the small effect of all but the "active fraction" corrections. Below pH 7, V_T/V_{Cl} is 1.7 and rises with increasing pH. The rise in V_T/V_{Cl} above pH 7 is ascribed to the cell-medium pH difference resulting from the Donnan effect (Discussion). If this effect is subtracted, V_T/V_{Cl} is 1.7 over the whole pH range (Fig. 5). Correcting V_T/V_{Cl} using the one minus ^{22}Na space value obtained in group 2 experiments (Discussion) gives a V_T/V_{Cl} value of 1.5.

Estimates of V_{Glu}/V_{Cl} were made at pH 6.9. The values were 1.60 ± 0.16 ($\pm$S.D.$_M$, $n=4$) with no "active fraction" correction applied, 1.70 ± 0.14 corrected for ^{22}Na space and 1.96 ± 0.17 corrected for ^3H space. The V_{Glu}/V_{Cl} values show more clearly than the V_T/V_{Cl} values (where "active fraction" corrections are larger) that the Donnan-induced increase in V is real and not an artifact due to the corrections. This increase in V is similar whether glutamate or toluenedisulfonate is used to produce the Donnan effect.

(4) *Donnan effects on $K_{m,o}$ and its component terms*: $K_{m,o} = (K_{1o}K_{2o}K'_{Go})/[Na_o^+]^2+K'_{Go}$. $K_{m,Cl}$ ($[Na_o^+] = 143$ mM) varied little with pH; $K_{m,Cl}$ was 0.1–0.2 mM over the pH range 6.1–8.3. The Donnan effect on K_m was small; there appeared

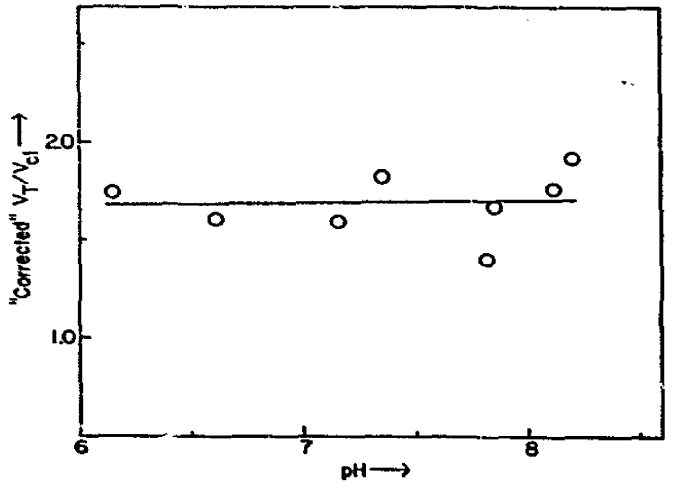

Fig. 5. The influence of a Donnan effect on the maximal velocity (V) for glycine entry as a function of pH "corrected" for the inside-outside pH difference. The points of Fig. 4 were adjusted as described in the text.

TABLE II

THE INFLUENCE OF A DONNAN EFFECT ON THE COMPONENT TERMS IN K_m FOR GLYCINE ENTRY

Experiment No. and cell type	External pH	K'_{GoT}/K'_{GoCl}[a]	$(K_{1o}K_{2o}K'_{Go})_T/(K_{1o}K_{2o}K'_{Go})_{Cl}$[a]
Toluenedisulfonate			
23	7.6	1.30	0.98
26	7.6	1.48	0.49
27	7.6	1.71	0.44
7	7.6	1.44	0.63
Average		1.48 ± 0.085 (S.D.)$_M$	0.63 ± 0.12 (S.D.)$_M$
4	6.5	1.23	0.63
24	7.1	1.61	0.78
		K'_{GoGlu}/K'_{GoCl}[b]	$(K_{1o}K_{2o}K'_{Go})_{Glu}/(K_{1o}K_{2o}K'_{Go})_{Cl}$[b]
Glutamate			
5	6.9	1.30	0.74
6	6.9	0.97 (1.28)[c]	0.58
Average		1.11 (1.29)[c]	0.66

[a] Measurements were made of Na^+-dependent glycine entry rates at three (non-zero) Na^+ concentrations with both toluenedisulfonate and Cl^- cells in each experiment. The reciprocal entry rate ($1/v$) was taken to be: $1/V \cdot \{(K_{1o}K_{2o}K'_{Go}/[Na^+]^2 + K'_{Go})/[Gly] + 1\}$. The slopes and intercepts of plots of $1/v$ vs. $1/[Na^+]^2$ were measured. As a first approximation, K'_{Go} was taken to be K_m at 143 mM Na^+ at the appropriate pH. Using this value, an estimate of $1/V$ was made from the intercept ($= K'_{Go}/V[Gly] + 1/V$) and then an estimate of $K_{1o}K_{2o}K'_{Go}$ was made from the slope ($= K_{1o}K_{2o}K'_{Go}/V$ [Gly]). This estimate was used to obtain a new estimate for K'_{Go} from K_m ($= K_{1o}K_{2o}K'_{Go}/[Na^+]^2 + K'_{Go}$). The process was repeated until recalculation produced no significant change. The ratios of values from the last literation are shown.

[b] The $K_{1o}K_{2o}K'_{Go}$ and K'_{Go} values were calculated from the equations: $K_{1o}K_{2o}K'_{Go} = \{K_m + [Gly]\}/\{(1/[Na^+]^2) + (1/[Na^+]_{\frac{1}{2}}^2)\}$ and $K'_{Go} = \{(K_{1o}K_{2o}K'_{Go}/[Na^+]_{\frac{1}{2}}^2) - [Gly]\}$. K_{1o} was obtained from a plot of $1/V$ vs. $1/[Gly]$ at a fixed $[Na^+]$ (133 mM] which is the "$[Na^+]$" in the above equations. $[Na^+]_{\frac{1}{2}}^2$ was obtained from a $1/v$ vs. $1/[Na^+]^2$ plot at fixed [Gly] (0.20 mM) which is the "[Gly]" in the above equations. $[Na^+]_{\frac{1}{2}}^2$ is the $[Na^+]^2$ value giving $1/v$ equal to twice the intercept. Both plots had four points and data for both plots were obtained in the same experiment.

[c] The $K_{m,Cl}$ and $K_{m,Glu}$ values obtained here differed slightly from each other. When these values were used, the K'_{GoGlu}/K'_{GoCl} values listed to the left of the parentheses were obtained. However, these K_m values were not significantly different and the values in parentheses are calculated assuming $K_{m,Cl} = K_{m,Glu}$. Toluenedisulfonate experiments were made in group 1, glutamate experiments in group 2.

to be a trend in $K_{m,T}/K_{m,Cl}$ from approx. 0.8 at pH 6.1 to approx. 1.2 at pH 7–8 (data not shown).

The Donnan effects on $K_{1o}K_{2o}K'_{Go}$ and K'_{Go} were also measured. Values of $(K_{1o}K_{2o}K'_{Go})_T/(K_{1o}K_{2o}K'_{Go})_{Cl}$ and $(K'_{Go})_T/(K'_{Go})_{Cl}$ are given in Table II with toluenedisulfonate cells for external pH 7.6. Also listed are single values at pH 7.1 and 6.5, and the analogous ratios obtained with glutamate cells at pH 6.9. The Donnan effect lowered $(K_{1o}K_{2o}K_{Go})$ 1.5–1.6-fold; $\{(K_{1o}K_{2o}K'_{Go})_T/(K_{1o}K_{2o}K'_{Go})_{Cl}\}^{-1} = 1.59$, $\{(K_{1o}K_{2o}K'_{Go})_{Glu}/(K_{1o}K_{2o}K'_{Go})_{Cl}\}^{-1} = 1.52$). These ratios are similar to V_T/V_{Cl} (1.5, corrected for ^{22}Na space) and V_{Glu}/V_{Cl} (1.7, corrected for ^{22}Na space).

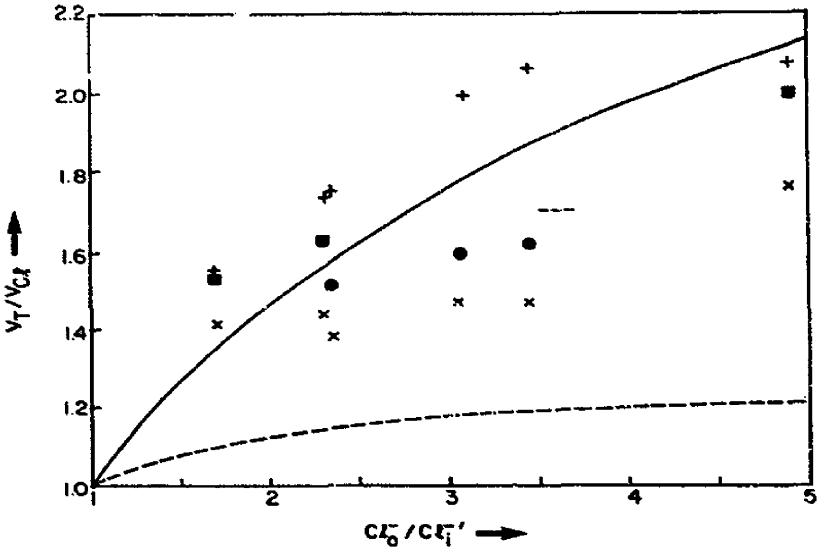

Fig. 6. Effect of varying internal toluenedisulfonate on glycine entry rate. v_T/v_{Cl} is the ratio, (Na^+-dependent entry rate into toluenedisulfonate cells)/(Na^+-dependent entry rate into Cl^- cells). The averages of incubated and unincubated cell Cl^- values were used and from these, cell Cl^- concentrations were calculated using the ^{22}Na spaces. $(Cl_o^-/Cl_i^-)'$ is the medium [Cl^-]/cell [Cl^-] ratio for toluenedisulfonate cells divided by the corresponding ratio for Cl^- cells in the same experiment. $(Cl_o^-/Cl_i^-)'$ reflects the change in membrane potential produced by replacing cell Cl^- with toluenedisulfonate. ×, v_T and v_{Cl} are μmol of glycine/ml pellet water entering in 4 min with no "active fraction" corrections used; ●, ■, the × points "corrected" for ^{22}Na space, i.e. multiplied by (one minus ^{22}Na space)$_{Cl}$/(one minus ^{22}Na space)$_T$; ● and ■, points from different experiments; +, the × points corrected for ^3H space; i.e. multiplied by (^3H space)$_{Cl}$/(^3H space)$_T$. The solid curve is the calculated one for the interpretation given in Discussion. The dashed curve is the calculated contribution of the inside-outside pH difference alone at external pH 7.4. The short dashed line at v_T/v_{Cl} = 1.7 is the average V_T/V_{Cl} corrected for ^3H space from the group 1 experiments. In the experiments shown in this figure, glycine was 0.46 mM and Na_o^+ 133 mM. Since K_m is only slightly affected by internal toluenedisulfonate, and the glycine concentration is approx. 2.5 times K_m, V_T/V_{Cl} and v_T/v_{Cl} are approximately equal. These experiments are from group 2.

With toluenedisulfonate cells, the Donnan effect on K'_{Go} was opposite to that on $K_{1o}K_{2o}K'_{Go}$; the former was raised 1.48-fold while the latter was lowered 1.6-fold. With glutamate cells the effect on K'_{Go} was less clear though probably similar.

(5) *Effects of varying* $(Cl_o^-/Cl_i^-)'$ *on glycine entry rates.* $(Cl_o^-/Cl_i^-)'$ was varied by replacing cell Cl^- with toluenedisulfonate or glutamate. Fig. 6 shows the results with toluenedisulfonate cells, a plot of v_T/v_{Cl} vs. $(Cl_o^-/Cl_i^-)' \equiv (Cl_o^-/Cl_i^-)_T/(Cl_o^-/Cl_i^-)_{Cl}$. Fig. 7 shows the analogous plot for glutamate cells. The filled points are the v ratios corrected for ^{22}Na space. The solid lines show the calculated effect on the v ratios of the Donnan-induced membrane potential plus the effect of the Donnan-induced inside-outside pH difference. The dashed line in Fig. 6 shows the calculated effect of the pH difference alone. Calculations were made according to the interpretation of these effects given in Discussion. The experiments shown in Fig. 7 were made at pH 6.9 so the effect of the pH difference alone is insignificant. The plots of the entry rate ratios against $(Cl_o^-/Cl_i^-)'$ are consistent with our

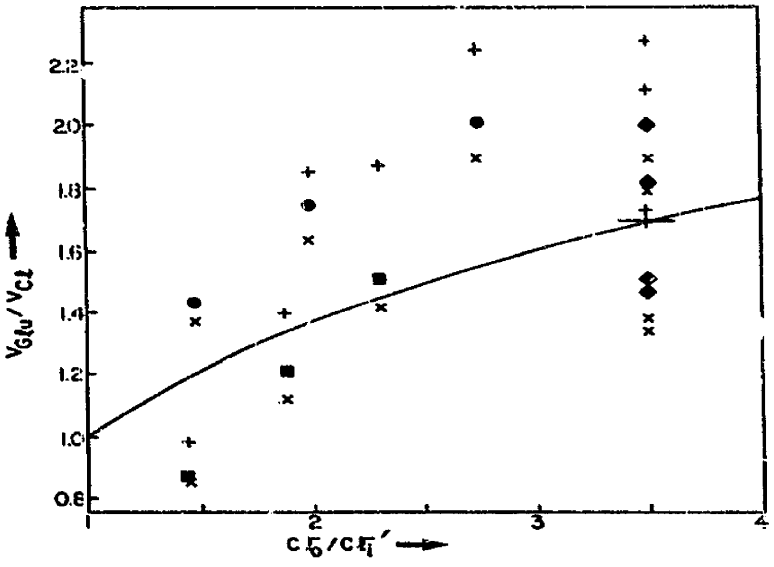

Fig. 7. Effect of varying internal glutamate on glycine entry rate. v_{Glu}/v_{Cl} is analogous to v_T/v_{Cl} (Fig. 6) and $(Cl_o^-/Cl_i^-)'$ was obtained the same way. As in Fig. 6, "×" points are uncorrected, solid points (different solid symbols show data from different experiments) are the "×" points corrected for ^{22}Na space, and "+" points are the "×" points corrected for ^3H space. The horizontal bar at $v_{Glu}/v_{Cl} = 1.7$ is the average of the "×" points. The solid line is again the theoretical curve but, since the pH of the medium was 6.9, the effect of the inside-outside pH difference is negligible and is not shown. There was no significant difference in cell Na^+ for unincubated glutamate and Cl^- cells. This cell Na^+ was 12.7 μmol/ml cell water ±1.9 (S.D.); ±0.7 $(S.D.)_M$, $n = 8$. For incubated glutamate cells, cell Na^+ was 23.4±8.1 (S.D.); ±4.0 $(S.D.)_M$ $n = 4$ and for incubated Cl^- cells, 17.4±3.2 (S.D.); ±1.6 $(S.D.)_M$ $n = 4$. The difference is small and not statistically significant. These experiments are from group 2.

interpretation in that (i) the calculated line falls within the envelope of data points corrected for ^{22}Na space and (ii) the points are better represented by a convex curve than by a concave or linear one.

Experiments on glycine exit

(1) *Effect of external toluenedisulfonate on Na^+-dependent glycine exit.* Measurements were made over the external pH range of 6.1–7.7. Within each experiment, Na^+-dependent and Na^+-independent exit rate coefficients with and without external toluenedisulfonate were measured at two pH values. Fig. 8 shows the ratio of Na^+-dependent exit rate coefficients with and without *trans*-toluenedisulfonate, v_T/v_{Cl}, plotted against external pH. Values obtained in the same experiment are connected by solid lines. External toluenedisulfonate increased v_T/v_{Cl} nearly 2-fold at the lowest pH, but only by about 1.2-fold at higher pH. The average of the v_T/v_{Cl} values between pH 6.4 and 7.0 was 1.224±0.021 ($S.D._M$, $n = 5$). The interpolated value at pH 6.85 from the lowest line is 5.6 S.D. away from the mean. However, its inclusion gives $v_T/v_{Cl} = 1.176 \pm 0.052$ ($S.D._M$, $n = 6$). The pH-insensitive *trans*-toluenedisulfonate effect on exit appears much less than the effect on entry despite the larger Donnan ratio, 11 vs. 3.6.

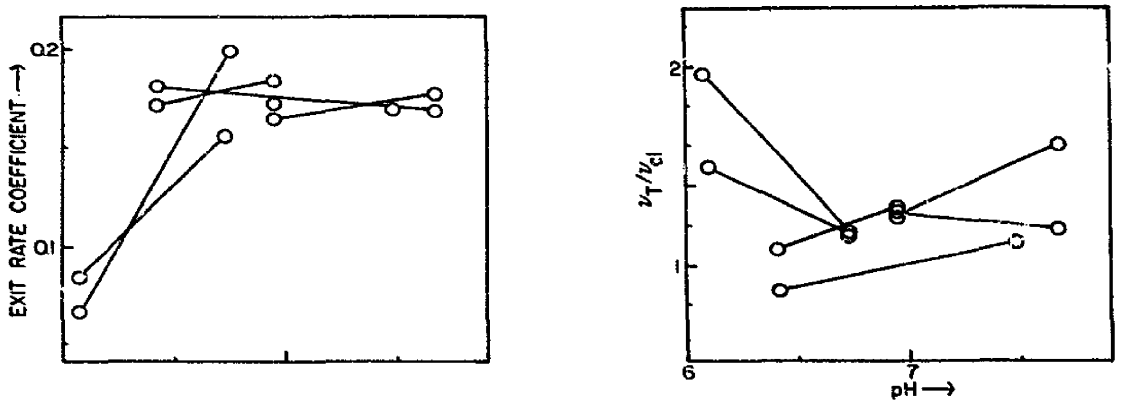

Fig. 8. The influence of a Donnan effect on the Na^+-dependent glycine exit rate coefficient. The ratio, v_T/v_{Cl}, is plotted against the pH of the medium. v_T is the Na^+-dependent glycine exit rate coefficient into toluenedisulfonate medium (Donnan effect present) while v_{Cl} is the corresponding exit coefficient into Cl^- medium (no Donnan effect). Determination of v_T/v_{Cl} was always made at two widely separated pH values within each experiment and the members of such pairs are shown connected by the solid lines. Internal Na^+, 126 mM; external Na^+, approx. 0 mM. These experiments are from group 1.

Fig. 9. Na^+-dependent glycine exit rate as a function of external pH in the absence of a Donnan effect. The Na^+-dependent glycine exit rate coefficient v_{Cl} (no Donnan effect) is plotted against the pH of the medium. Values of v_{Cl} at two widely separated pH values were obtained in each experiment and the plot shown was constructed by normalizing the segments from different experiments to the overlap regions. Data are from the experiments whose v_T/v_{Cl} ratios are shown in Fig. 8.

The effect of external toluenedisulfonate on v (exit) was measured with cell Na^+ = 25 mM instead of 126 mM. The v_T/v_{Cl} values from two experiments, external pH 7.05, were (expt. 1), 1.53 and (expts. 2a and 2b) 1.19 and 1.00; average: 1.25±0.16 ($S.D._M$, n = 3). This value is similar to v_T/v_{Cl} at high internal Na^+.

(2) *Effects of pH on Na^+-dependent glycine exit in the absence of a Donnan effect.* A plot of exit rate coefficients (v_{Cl}) vs. external pH was constructed (Fig. 9) from data for exit in the absence of external toluenedisulfonate by normalizing the (overlapping) pairs of points. There is an abrupt drop at low pH corresponding in position and size to the rise in v_T/v_{Cl} seen in Fig. 8. This exit curve is quite different from the curve of entry vs. pH (Fig. 3). The low pH drop (exit) appears at a different pH than that for entry and no drop in exit is seen at high pH.

Low pH might lower v_{Cl} because of changes in V (exit), K_m (exit) or both. We tried to measure K_m and V for exit at two pH values, 7 and 6, within the same experiment. The points were too badly scattered to give reliable values for K_m and V, but the ratios of exit rates at the two pH values appeared independent of glycine concentration (i.e. K_m (exit) was similar at the two pH values). Thus much of the low pH inhibition of exit is probably due to inhibition of V (exit).

DISCUSSION

There are several effects of *trans*-toluenedisulfonate or glutamate to be considered. One, of primary interest, is the interaction between the Donnan potential

and charge moving across the membrane. An effect could also arise from the Donnan-induced difference between internal and external pH. A third effect comprises actions of toluenedisulfonate or glutamate as reactive chemical entities. A fourth effect is a response of glycine transport to the necessary lowering of *trans*-Cl^- upon its replacement by toluenedisulfonate or glutamate.

Direct effects of cell Cl^-. The increase of glycine entry upon replacing cell Cl^- with toluenedisulfonate or glutamate is not directly due to lowered cell Cl^-. High $[Cl_i^-]$ would then have to inhibit entry. From earlier studies [11] on anion dependence of glycine entry into intact cells with no Donnan effect ($[Cl_o^-] = [Cl_i^-]$), Cl^- behaved as a pure co-substrate giving linear 1/v vs. $1/[Cl^-]$ plots from 148 to 4 mM $[Cl^-]$. A superimposed inhibitory effect of $[Cl_i^-]$ should have produced concavity. Also, in the present studies, replacing cell Cl^- raised V whereas in the previous studies [11] where $[Cl_i^-]$ and $[Cl_o^-]$ varied together, only K_m was affected. Finally, in the present studies $K_1K_2K'_{Go}$ and K'_{Go} were oppositely affected by Cl_i^- replacement, but changing $[Cl_i^-]$ should alter k_{-o} which should change $K_1K_2K'_{Go}$ and K'_{Go} in the same direction.

Effects of glutamate and toluenedisulfonate as reactive chemical entities. Direct effects are unlikely because toluenedisulfonate lacks reactive groups, chelating ability and large hydrophobic regions. Also, the quite different anions, toluenedisulfonate and glutamate, have similar effects. Further, with the impermeant anion *cis* to glycine, the Donnan effect should oppose glycine movement; such inhibition of entry was seen [6] when either toluenedisulfonate or mucate replaced Cl^- in the medium. Part of this "mucate inhibition" can now be ascribed to lowered *cis* $[Cl^-]$[11] but V(entry) was also lowered and $[Cl^-]$ does not effect V. Thus there is similar *trans*-stimulation of V(entry) by toluenedisulfonate or glutamate and similar *cis*-inhibition of v(entry) by toluenedisulfonate and mucate. The common element is the associated Donnan potential. Another possibility, that toluenedisulfonate or glutamate cells had higher cell Na^+ than Cl^- cells is also eliminated since they did not. Cell Na^+ contents are listed in the legends for Figs. 4 (toluenedisulfonate) and 7 (glutamate).

Effects of pH. Trans-Toluenedisulfonate raised V(entry) at all pH values tested (Fig. 4) but this rise was pH dependent only above pH 7. This pH-dependent rise mirrored the drop in v_{Cl} at high pH in the absence of toluenedisulfonate (Fig. 3) governed by a group of pK_{app} 7.9. This rise in V_T/V_{Cl} at high external pH might result from the Donnan-induced reduction of internal pH if entry requires the protonated form of the pK_{app} 7.9 group at the internal face of the membrane. This assumption fits the data. When V_T/V_{Cl} is corrected for the effect of pH seen on v_{Cl} (Fig. 3) by multiplying V_T/V_{Cl} by $[HA/(HA+A^-)]_{pH_o-0.556}$ for a group of pK 7.9, the pH dependence is entirely removed (Fig. 5). The average pH-corrected V_T/V_{Cl} value is 1.698 ± 0.057 $(S.D.)_M$, $n = 8$ compared with the value of 1.7 at low pH values (Fig. 4). Since cells are quite permeable to H^+ (unpublished titration data), $[H_i^+]/[H_o^+]$ should be equal to $[Cl_o^-]/[Cl_i^-]$.

The data of Fig. 3 indicates that entry requires another group with a pK_{app} of 6.8 in the deprotonated form. Since V_T/V_{Cl} is pH independent in this range, entry presumably requires the deprotonated form of the pK 6.8 group at the external face of the membrane.

Similar relationships appear for exit. Exit drops at low external pH in the absence of a Donnan effect (Fig. 9). The rise in v_T/v_{Cl} (Fig. 8) mirrors this drop,

suggesting that a group with a pK_{app} of 6.2 is required for exit in the deprotonated form at the inner face. When toluenedisulfonate replaces Cl_o^-, the rise in internal pH deprotonates the pK 6.2 group which raises v_T relative to v_{Cl}.

Effects of membrane potential. (a) Assessment of the role of the "active fraction" corrections in estimating V_T/V_{Cl}. Such corrections must be considered because both toluenedisulfonate and glutamate cell pellets have more extracellular space than Cl^- cell pellets. In group 1 ^3H space was used for the "active fraction" correction. However, ^3H space was smaller than one minus ^{22}Na space for toluenedisulfonate cells (Table 1). One minus $^{22}Na^+$ space is a better measure than ^3H space of the space retaining toluenedisulfonate or glutamate and excluding Cl^-. We conclude this because $[Cl_i^-]$ values calculated from the total Cl^- in the original unlysed cells and the lysing and restoring solutions agree better with $[Cl_i^-]$ values determined from pellet Cl^- and extracellular space when ^{22}Na space rather than one minus ^3H space is used. Some $[Cl_i^-]$ values obtained using ^3H space were less than zero. Also, ^{22}Na space is equal to the space occupied by [^{14}C]glycine in the cold (data not shown).

When the data of Fig. 4 is corrected using one minus ^{22}Na space instead of ^3H space the pH-independent part of V_T/V_{Cl} is 1.5 instead of 1.7. The ^{22}Na space correction seems valid since V_T/V_{Cl} and V_{Glu}/V_{Cl} corrected for one minus ^{22}Na space are similar (1.5 and 1.7, respectively) and where the correction is smaller (glutamate cells), the uncorrected V ratio is higher. (Uncorrected V_T/V_{Cl} and V_{Glu}/V_{Cl} values are, respectively, 1.25 (Fig. 4, pH 6–7) and 1.6.) We conclude that the pH-insensitive component of the acceleration factor for V arising from a Donnan effect, whether produced by toluenedisulfonate or glutamate, is 1.5–1.7 at a Donnan ratio of approx. 3.5. We prefer the value of 1.7 since less correction is used for V_{Glu}/V_{Cl} than for V_T/V_{Cl} and the higher ionic strength or the sucrose in toluenedisulfonate cells might inhibit transport.

This pH-insensitive component of acceleration can hardly arise from the inside-outside pH difference. If the lowering of the cell pH by the Donnan effect increased glycine entry, lowering cell pH in the absence of a Donnan effect should also have increased entry instead of lowering it. The 1.7-fold increase is therefore ascribed to interaction of the Donnan potential with a "moving" charged group. "Movement" means relative movement between charge and field gradient; the latter rather than the former might move relative to the membrane.

(b) Assignment of membrane potential effects to steps in the translocation model. The next questions are, which step(s) in $V(\text{entry}) = E_i k_3 k_{-0}/(k_3+k_{-0})$ are affected and how much charge moves. The step could be that governed by k_3 for $ENa_2GCl_o \xrightarrow{k_3} ENa_2GCl_i$, and involve inward movement of positive charge, or that governed by k_{-0} for $E_i \xrightarrow{k_{-0}} E_o$, involving outward movement of negative charge, or some combination.

Before proceeding we must consider what effects on kinetic parameters to expect from the action of a membrane potential on moving charge. Regardless of the mechanism of transport, each step in the transport process where charge moves parallel to the potential gradient must obey Eqn. 1:

$$(k_{+i}/k_{-i})_{\Delta E} = (k_{+i}/k_{-i})_{\Delta E=0} \cdot e^{zF\Delta\Gamma/RT} \qquad (1)$$

ΔE is the potential difference between the positions, not necessarily the full trans-membrane potential. The equilibrium concentration ratios of all of the transported species must obey Eqn. 2:

$$\prod_1^n (S_j)_o / \prod_1^n (S_j)_i = e^{zF\Delta E/RT} \tag{2}$$

$S_1 \cdots S_n$ includes all species co- or countertransported (not just those known to be), ΔE is the full trans-membrane potential and z is the sum of charges transported. $\prod_1^n (S_j)_o / \prod_1^n (S_j)_i$ is equal to the product of the rate constant ratios over the reaction path. The reaction path is the sequence of steps necessary for one transport event. The reaction path of Fig. 1 gives Eqn. 3:

$$\prod_1^n (S_j)_o / \prod_1^n (S_j)_i = \frac{(Na_o^+)^2(Cl_o^-)(G_o)}{(Na_i^+)^2(Cl_i^-)(G_i)} = \frac{k_0 k_{-3}(K_{1o}K_{2o}K_{Clo}K_{Go})}{k_{-0}k_3(K_{1i}K_{2i}K_{Cli}K_{Gi})} = e^{zF\Delta E/RT} \tag{3}$$

with $z = 2\cdot(+1)+1\cdot(-1)+0$ if two Na^+, one Cl^- and one glycine are co-transported and no other species are co- or countertransported. If $\Delta E = 0$, $k_0 k_{-3}(K_{1o}K_{2o}K_{Clo}K_{Go}) = k_{-0}k_3(K_{1i}K_{2i}K_{Cli}K_{Gi})$ as required by microscopic reversibility.

Eqns. 1–3 must be obeyed by any transport mechanism because their violation allows perpetual motion machines to exist. We can construct a simple passive transport model, e.g.

$$E_o + S_o \overset{K_{s,o}}{\rightleftharpoons} ES_o,\ ES_o \overset{k_2}{\underset{k_{-2}}{\rightleftharpoons}} ES_i,\ E_i + S_i \overset{K_{s,i}}{\rightleftharpoons} ES_i,\ E_o \overset{k_4}{\underset{k_{-4}}{\rightleftharpoons}} E_i,\ \frac{S_o}{S_i} = \frac{K_{s,o}k_{-2}k_4}{K_{s,i}k_2k_{-4}}$$

as can be shown either by deriving the rate equations and solving for $v(\text{entry}) = v(\text{exit})$ or by recalling that free energy is a state function so that $\Delta F = 0$ going from, e.g. E_o around the reaction path back to E_o. Such a model can be constructed where all steps do obey Eqn. 1 and some unique equilibrium value for S_o/S_i obtained. If Eqn. 1 could be violated for any step, the resulting equilibrium S_o/S_i would be different and coupling the two "equilibria" would produce perpetual motion. For simplicity here, S is assumed uncharged, so no current flows and no energy is drawn from the source of the imposed potential. Eqns. 2 and 3 follow because the equilibrium value for S_o/S_i produced by passive transport and by simple diffusion cannot be different or again a perpetual motion machine could be constructed by coupling a simple diffusion leak and a passive transport mechanism.

To assign membrane potential effects to steps in the model we start with the kinetic equation (Fig. 1, legend) [4]. V and the two terms in K_m, K'_{Go} and $K_1K_2K'_{Go}$, all contain reorientation rate constants $k_{-0}\cdots k_3$. $V = E_t k_3 k_{-0}/(k_{-0}+k_3)$, $K'_{Go} = K_{Go}k_{-0}/(k_{-0}+k_3)$ and $K_1K_2K'_{Go} = K_{1o}K_{2o}K_{Go}(k_{-0}+k_0)/(k_{-0}+k_3)$.

K_{Go}, $K_{1o}K_{2o}K_{Go}$ etc. describe reactions with no vectorial components normal to the cell surface so the membrane potential should affect K'_{Go} and $K_1K_2K'_{Go}$, as well as V, by altering the reorientation rate constants. The rate constants in V and K'_{Go} differ by the factor k_3 in the numerator of V. Since V and K'_{Go} were similarly increased by the membrane potential by factors of 2 and 1.5, respectively, the membrane potential effect on k_3 is small and k_{-0} is the

affected constant. That is, the empty but not the fully loaded mechanism is charged and, since V is increased, the charge is negative. The same charge location and sign was obtained, together with an estimate of the size of the charge by calculating the membrane potential effects on $K_1K_2K'_{Go}$, V and K'_{Go} expected from various assumptions about the size and location of the charge. To do this, values were needed for k_{-0}/k_3 and k_0/k_{-3}. From previous work [4], these values depend on the value chosen for "ρ_α" $= K_{1i}K_{2i}K_{Gi}/K_{1o}K_{2o}K_{Go}$. With $\rho_\alpha = 1$ we calculated the effects on V, K'_{Go} and $K_1K_2K'_{Go}$ with one and two units of charge distributed in all possible ways over the steps described by k_{-0}, k_0, k_{-3} and k_3 (Table III). For example, with one unit of negative charge on E and no charge on ENa$_2$ClGly, k_{-0}/k_0 would be increased (Eqn. 1) by a factor of 3.6 (the Donnan ratio). If k_{-0} and k_0 were equally affected they would be, respectively, increased and decreased 1.9-fold. This gives $V_T/V_{Cl} = 1.79$, $K'_{GoT}/K'_{GoCl} = 1.79$ and $K_1K_2K'_{GoT}/K_1K_2K'_{GoCl} = 0.69$ vs. the observed values of 1.7, 1.48 and 0.63. All other charge assignments gave poorer fits. If, e.g. the charge on E is $-\frac{1}{2}$ and that on ENa$_2$ClGly is $+\frac{1}{2}$, V_T/V_{Cl}, K'_{GoT}/K'_{GoCl} and $K_1K_2K'_{GoT}/K_1K_2K'_{GoCl}$ are respectively 1.38, 1.00 and 0.60. Although the calculations of Table III depend somewhat on ρ_α, the dependence is weak. Thus for line 1a the range for $\rho_\alpha = 100$ to $\rho_\alpha = 0.1$ is from 1.9 to 1.39 for K'_{Go}, 0.54 to 0.84 for $K_{1o}K_{2o}K'_{Go}$, and from 1.9 to 1.39 for V.

The same charge assignment explains the small observed effect of the membrane potential on exit; the calculated v_T/v_{Cl} is 1.15 ($\rho_\alpha = 1$), the observed pH-independent part of v_T/v_{Cl} is 1.2 (Fig. 8). For $\rho_\alpha = 100$, $v_T/v_{Cl} = 1.02$ and for

TABLE III

CALCULATED EFFECTS OF MEMBRANE POTENTIAL ON PARAMETERS OF THE ENTRY RATE EQUATION

	f(k_3)[a]	f(k_{-0})[a]	f(k_0)[a]	$\frac{V_T}{V_{Cl}}$	$\frac{K'_{GoT}}{K'_{GoCl}}$	$\frac{(K_{1o}K_{2o}K'_{Go})_T}{(K_{1o}K_{2o}K'_{Go})_{Cl}}$
1a	1	1.9	1/1.9	1.79	1.79	0.69
2a	1.38	1.38	1/1.38	1.38	1.00	0.60
3a	1.9	1	1	1.03	0.54	0.54
4a	3.6	1	1	1.05	0.29	0.29
5a	1	3.6	1	3.06	3.06	1.18
6a	1	1	1/3.6	1.00	1.00	0.39
1b	1	3.6	1/3.6	3.06	3.06	0.66
2b	1.9	1.9	1/1.9	1.90	1.00	0.39
4b	12.96	1	1	1.067	0.08	0.08
5b	1	12.96	1	7.13	7.13	1.54
6b	1	1	1/12.96	1.00	1.00	0.22

[a] The "f" factors are the factors by which the indicated rate constants of Fig. 1 would be affected by a membrane potential giving a Donnan ratio of 3.6 under various assumptions as to how much charge (z) moves at each step and how the effect is distributed between the forward and reverse rate constants of the affected steps. The letter "a" in the left hand column indicates $z = \pm 1$ and "b" indicates $z = \pm 2$. Thus for 4a, $z = +1$ and the full effect is on k_3, so k_3 is increased by a factor of 3.6; for 1a, $z = -1$ and the effect is equally distributed between k_{-0} and k_0; for 2a, $z = +\frac{1}{2}$ for the k_3, k_{-3} pair (k_{-3} does not appear in the listed entry parameters) and $-\frac{1}{2}$ for the k_{-0}, k_0 pair and is equally distributed among all four constants. 3b would be the same as 4a and so is not listed.

$\rho_\alpha = 0.1$, $v_T/v_{Cl} = 1.49$. In view of the experimental errors and simplifying assumptions, the agreements are at least as good as can be expected. All effects fit the interpretation that reorientation of E moves about one unit of negative charge and reorientation of fully loaded mechanism moves virtually none.

Role of Cl^-: The appearance of a $[Cl^-]$ term in K_m [11] suggests that ENa_2GCl rather than ENa_2G is the productive complex. If Cl^- is cotransported, the membrane crossing step involving E carries only one unit less positive charge than that for ENa_2GCl (Fig. 1). This rationalizes the Cl^- kinetics with the Donnan effects. This is the basis for the inference that Cl^- is cotransported.

pH effects in the absence of toluenedisulfonate: The pH effects on exit differ markedly from those on entry (Fig. 9 vs. Fig. 3). However, $V(\text{exit}) = E_t k_0 k_{-3}/(k_0 + k_{-3})$, and is dominated by k_{-3}. With $\rho_\alpha = 1$, $k_0/k_{-3} = 2.3$. $V(\text{entry}) = E_t k_{-0} k_3/(k_{-0} + k_3)$ and is dominated by k_{-0}; $k_{-0}/k_3 = 0.073$ with $\rho_\alpha = 1$. If different steps limit entry and exit, the different effects of pH are plausible. We could assign the pK 6.2, 6.8 and 7.9 groups to individual steps in the exit and entry processes so as to account for the observed pH effects. Since these assignments are not necessarily unique they are not given. Since consistent assignments can be made, the pH effects do not conflict with our model or interpretations.

Comparison of expected behavior of various transport models with observed behavior. The glycine transport system is unlikely to be a translational mobile carrier ("ferry boat"). First, it is probably too large. The homotropic interactions between the two Na^+ and the heterotropic interactions between Na^+ and glycine [1, 4] and between anions and glycine [11] as well as the large increase in the trans-membrane transfer rate constant after the disodium form of the porter combines with glycine [4], are standard kinetic signs of an allosteric protein. This implies at least four allosterically interacting binding sites. From the compilation of Klotz et al. [15], proteins that complex have molecular weights of 96 000 or more. The known transport proteins are not particularly small compared to proteins in general [14, 16–28]. A protein with a molecular weight of 10^5 would simply span a bilayer. Second, the slight, pH-independent charge transport is not expected for a translational mobile carrier. Both transport proteins and hydrophobic membrane proteins have normal contents of histidine and nearly normal contents of charged residues [14, 17, 19–23, 29–40]. A 10^5 dalton protein should have 100–200 charged residues. Unless these just balance by chance, there should be a sizable net charge at neutral pH. There should also be several histidine residues and N-terminal amino groups making charge transport pH dependent. Note that rate constant ratios are altered by the Donnan ratio to the z^{th} power (z = charge) and so rates are very sensitive to charge.

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