Na- and Cl-dependent Glycine Transport in Human Red Blood Cells and Ghosts

A Study of the Binding of Substrates to the Outward-Facing Carrier

PATRICIA A. KING and ROBERT B. GUNN

ABSTRACT Na- and Cl-dependent glycine transport was investigated in human red blood cells. The effects of the carrier substrates (Na, Cl, and glycine) on the glycine transport kinetics were studied with the goal of learning more about the mechanism of transport. The $K_m$ was 100 μM and the $V_{max}$ was 109 μmol/kg Hb·h. When cis Na was lowered (50 mM) the $K_{m,cis}$ increased and the $V_{max,cis}$ decreased, which was consistent with a preferred order of rapid equilibrium loading of glycine before Na. Na-dependent glycine influx as a function of Na concentration was sigmoidal, and direct measurement of glycine and Na uptake indicated a stoichiometry of 2 Na:1 glycine transported. The sigmoidal response of glycine influx to Na concentration was best fit by a model with ordered binding of Na, the first Na with a high $K_a$ (>250 mM), and the second Na with a low $K_a$ (<10.3 mM). In the presence of low Cl (cis and trans 5 mM), the $K_{m,cl}$ increased and the $V_{max,cl}$ increased. The Cl dependence displayed Michaelis-Menten kinetics with a $K_{m,cl}$ of 9.5 mM. At low Cl (5 mM Cl balanced with NO₃), the glycine influx as a function of Na showed the same stoichiometry and $V_{max,cl}$ but a decreased affinity of the carrier for Na. These data suggested that Cl binds to the carrier before Na. Experiments comparing influx and efflux rates of transport using red blood cell ghosts indicated a functional asymmetry of the transporter. Under the same gradient conditions, Na- and Cl-dependent glycine transport functioned in both directions across the membrane but rates of efflux were 50% greater than rates of influx. In addition, the presence of trans substrates modified influx and efflux differently. Trans glycine largely inhibited glycine efflux in the absence or presence of trans Na; trans Na largely inhibited glycine influx and this inhibition was partially reversed when trans glycine was also present. A model for the binding of these substrates to the outward-facing carrier is presented.

INTRODUCTION

Several carrier systems for amino acid transport have been identified and characterized according to Na dependence, competition and inhibitor studies, structural dis-
crimination, and substrate affinities (Christensen, 1984). For some of the amino acids, transport appears to proceed by a number of different pathways, making quantitative assessment of one pathway difficult. As a consequence, investigations have largely concentrated on identifying and categorizing the transport pathways in a variety of tissues. However, information concerning cotransported substrates and their binding affinities, stoichiometry, order of binding, and symmetry of transport is needed to elucidate their molecular mechanisms and their possible regulation.

The human red blood cell membrane provides a convenient system for the study of amino acid transport mechanisms. The composition of the medium on both sides of the membrane can be controlled with the native membrane still intact, unlike membrane vesicle preparations (Bodemann and Passow, 1972); low metabolic activity allows amino acid flux to be measured with minimal interference from metabolism; initial rates of transport can be measured enabling an accurate derivation of kinetic constants. In the red cell, the maintenance of amino acid transport systems is important for providing the amino acids necessary for the production of glutathione (GSH). GSH accounts for almost all of the red blood cell nonprotein thiol, and is responsible for maintaining the oxidation/reduction state of the cell proteins, particularly hemoglobin, which cannot carry O\textsubscript{2} if the heme iron becomes oxidized (Eaton and Brewer, 1974). GSH is synthesized in the red cell from cysteine, glycine, and glutamate, which are transported into the cell from the plasma. The importance of these transport systems is exemplified by a breed of Finnish Landrace sheep whose red blood cells have decreased survival, and are characterized by low GSH and decreased cysteine and glycine transport (Young et al., 1976, 1980, 1982).

In human red blood cells, Ellory et al. (1981) have identified five components of glycine transport. The predominant pathway is a high-affinity, Na- and Cl-dependent flux (glycine system; see Christensen, 1984), while the other components include a Na-dependent, Cl-independent flux characterized by a higher $K_{\text{m,gly}}$ (the ASC system; see Christensen, 1984), a Na-independent, SITS (4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid)-sensitive flux thought to be transported by band 3 (Young et al., 1981), a Na-independent flux inhibited by leucine (system L; see Christensen, 1984), and a residual Na-independent component of transport. Glycine transport has been studied in a variety of other cell types, including avian erythrocytes (Vidaver, 1964a, b; Eavenson and Christensen, 1967; Vidaver and Shepard, 1968), sheep reticulocytes (Weigensberg and Blostein, 1985), rabbit reticulocytes (Winter and Christenson, 1965), rat hepatocytes (Christensen and Handlogten, 1981), Ehrlich ascites cells (Johnstone, 1978), renal tubule cells (Barfuss et al., 1980), and rat synaptosomes (Kuhar and Zarbin, 1978), among others. In general all studies have demonstrated the major pathway of transport to be the high-affinity Na-dependent system. Where anion effects have been examined (Imler and Vidaver, 1972; Kuhar and Zarbin, 1978; Ellory et al., 1981; Weigensberg and Blostein, 1985), this transport system has been shown to be dependent on Cl, but the exact mechanism of the dependence is not clear. Studies of the kinetic properties and symmetry of Na-dependent glycine transport have revealed evidence for both asymmetric (Vidaver and Shepard, 1968; Johnstone, 1978) and symmetric (Weigensberg and Blostein, 1985) transport. The stoichiometry of Na and glycine transport has been reported as 2:1 in pigeon red blood cells (Vidaver, 1964a), sheep reticulo-
cyte vesicles (Weigensberg and Blostein, 1985), and rat hepatocytes (Christensen and Handlogten, 1981).

This paper examines the Na-dependent, Cl-dependent glycine transport in the human red blood cell. We found that Na and glycine are cotransported in a 2:1 stoichiometry. The first Na appears to bind with a low affinity and the second with a high affinity, and the binding of Na requires the prior binding of Cl. The effects of Na and Cl on the kinetic constants for glycine were investigated as well as the effects of trans substrates on glycine influx and efflux. From these data a model is presented for the order of binding to the outward-facing carrier.

METH O DS AND MATERIALS

Preparation of Cells
Fresh heparinized human blood was centrifuged at 10,000 g in a Sorvall RC-5B centrifuge. The plasma and buffy coat were removed and the cells were washed three times in 150 mM KCl and 10 mM HEPES, at 0°C and pH 7.8. The packed cells were stored at 0–4°C until use (usually within 1 h).

Anion Replacements
In some experiments Cl salts were replaced with isotonic NO₃ or SO₄ salts. To replace internal chloride, the cells were incubated in the replacement anion solution (pH 7.4) at a 10% hematocrit at 37°C for 15 min. The suspension was then centrifuged, the supernatant was removed, and the cells were resuspended in the replacement anion solution, as before, to repeat the incubation. This procedure was repeated three times and the final packed cells were stored at 0–4°C until use (usually within 1 h).

Influx Studies
The influx of glycine was measured by following the uptake of [¹⁴C]glycine (2-[¹⁴C]glycine, 47.3 mCi/mmol, New England Nuclear, Boston, MA) at 37°C. In general, the incubation medium contained 150 mM NaCl or 150 mM KCl, 10 mM HEPES, and 100 μM [¹⁴C]glycine (5–6 μCi/μmol glycine), pH 7.4; replacement of anions, cations, and the addition of inhibitors are indicated in the figure and table legends. The specific activity of glycine was determined from a medium sample taken before the start of the flux, and the influx was started by the addition of packed cells to the prewarmed medium (5% final hematocrit). At time intervals of 2, 5, 10, and 15 min, samples of the cell suspension were withdrawn and transport was stopped by dilution into 5 ml of isotonic MgCl₂ (120 mM) at 0°C, pH 7.8. The cells were centrifuged and the cell pellet was washed three times with 5 ml of the stopping solution; the final washed pellet was lysed with 1 ml of distilled water. An aliquot of the lysate was removed and diluted with a modified Drabkin’s reagent (VanKampen and Zijlstra, 1961) for hemoglobin analysis (optical density recorded at 540 nm). The lysate proteins were then precipitated with 100 μl of 70% perchloric acid (PCA) and a sample of the supernatant was counted for radioactivity (Ecoscint; National Diagnostics, Inc., Somerville, NJ) using a Tri-Carb (Packard Instrument Co., Downers Grove, IL) scintillation counter. The rate of transport was calculated from the slope of the line of glycine uptake/kg Hb vs. time and expressed as micromoles glycine per kilogram hemoglobin per hour. Uptake was linear for at least 30 min in preliminary experiments. The y intercept of the time course for uptake was slightly above zero and varied systematically between batches of [¹⁴C]glycine. In view of the low glycine flux rates, the nonzero intercept could be due to contamination of the [¹⁴C]glycine. This problem
(points out the necessity of measuring multiple time points in determining the initial flux rate rather than a single time point and assuming a zero y intercept.

**Definition of Flux Components**

The results are discussed in terms of three components of transport: Na-dependent flux; Na-independent, DNDS (4,4'-dinitrostilbene-2,2'-disulfonate)-sensitive flux; and Na-independent, DNDS-insensitive flux. The Na-dependent flux is defined as the rate of transport in NaCl medium minus the flux in KCl medium. The Na-independent DNDS-sensitive influx is defined as the rate of transport in KCl minus the rate of transport in KCl plus DNDS. The remaining flux (transport in KCl plus DNDS) is the Na-independent, DNDS-insensitive flux.

**Nystatin-treated Cells**

In one series of experiments, nystatin was used to load cells with 295 mM KNO₃ and 5 mM KCl, and glycine influx as a function of Na concentration up to 300 mM Na was measured. Washed cells were incubated in 295 mM KNO₃, 5 mM KCl, 30 mM sucrose, with 50 µg/ml nystatin at a 5% hematocrit, at 0°C for 10 min. The cells were then centrifuged and washed eight times in the KNO₃, KCl, sucrose medium (no nystatin) at room temperature. Glycine influx was assayed as before, while Na was varied (0–300 mM) at constant anion concentration (295 mM NO₃, 5 mM Cl).

**Effects of Membrane Potential**

The effects of membrane potential (inside negative) on the components of glycine influx were qualitatively studied. Cells were incubated in low K medium (150 mM NaCl and 5 mM KCl, or 150 mM N-methyl-D-glucamine chloride [NMG-Cl] and 5 mM KCl) and the influx was measured in the presence and absence (ethanol only) of valinomycin. Valinomycin was present at a final concentration of 20 µM, added in ethanol with the final ethanol concentration <0.5% (Fröhlich et al., 1983). Under these conditions, there is an outward-directed K gradient which, in the presence of valinomycin, produces an inside negative membrane potential.

**Effect of pH on Glycine Influx**

In some experiments, glycine influx was measured over a pH range of 6.4 to 8.8 at 37°C. In all cases the pH was in equilibrium with pHₐ. To titrate cells to pH values below 7.4, cells were suspended in 150 mM KCl at a 50% hematocrit and gently bubbled with CO₂ to lower pH. To titrate cells to values above pH 7.4, cells were incubated in a mixture of 150 mM KCl and 150 mM KHCO₃ adjusted with KOH to the desired pH. In all cases cells were centrifuged, resuspended in 150 mM KCl, and the pH was measured. The titration procedures were repeated until the desired pH upon resuspension was attained. At pH values outside the useful buffer range of HEPES, either MES (2[N-morpholino] ethanesulfonic acid) (pH < 6.5) or PIPES (piperazine-N,N'-bis[2-ethanesulfonic acid]) (pH > 8.5) was used as the medium buffer.

**Na Influx Measurements**

In a few experiments the influx of Na was measured as a function of glycine in the incubation medium by following the uptake of ²²Na (Amersham Corp., Arlington Heights, IL). The influx medium included bumetanide (0.1 mM), ouabain (0.1 mM), and amiloride (1.0 mM) to minimize the background Na flux. Influx procedures were as above; details of the experiment are in the table legend.
Influx and Efflux Measurements Using Red Blood Cells Ghosts

Red blood cell ghosts were prepared by the method of Bodemann and Passow (1972). Briefly, the procedure was as follows. The blood was washed twice in 165 mM KCl to remove the plasma and buffy coat and once in medium similar to the desired ghost composition (150 mM KCl, NaCl, KNO₃, or 25 mM K₃-citrate, 200 mM sucrose plus 10 mM HEPES). The cells were then hemolyzed in 3.8 mM acetic acid and 4 mM Mg acetate at 0°C. After 5 min, a concentrated restoring solution was added and the mixture was stirred on ice (0°C) for another 10 min. The cells were then resealed by incubation at 37°C for 45 min. At the end of this period, the ghosts were washed two times with medium identical to the inside ghost composition and packed at 17,000 g.

Glycine influx was measured using methods similar to those described for influx with whole cells. The packed ghosts were held on ice and the influx started by adding ghosts to the incubation medium. Samples were withdrawn at timed intervals, diluted into ice-cold stopping solution, centrifuged, and the ghost pellet was washed three times. The pellet was lysed and analyzed for [¹⁴C]glycine and hemoglobin. Due to the low hemoglobin content, the hemoglobin analysis was performed at a wavelength of 419 nm. At the end of the experiment, a sample of the original packed ghosts (saved on ice) was analyzed for ghost number (Coulter counter model ZB; Coulter Electronics Inc., Hialeah, FL) and hemoglobin. Results were calculated as influx equals micromoles per unit of ghosts per hour; and for clarity they are expressed here as micromoles per kilogram of cell solids per hour. One unit of ghosts is the number of ghosts (3.1 × 10¹⁳) corresponding to 1 kg cell solids in normal intact cells. Since the cell solids are 90% hemoglobin, the flux units in ghosts and cells refer to approximately the same number of cells or cell surface area. When flux rates for ghosts and cells are directly compared, the cell fluxes have been converted to flux units of micromoles per kilogram of cell solids per hour.

For efflux studies, ghosts were resealed to contain 100 µM glycine at a specific activity of ~3-4 µCi/µmol glycine. At the end of the resealing period, the ghosts were not washed but instead centrifuged and the supernatant was removed to bring the ghosts to approximately a 50% hematocrit. The ghosts were then resuspended and packed in nylon tubes by centrifugation (Sorvall RC-5B) at 17,000 g for 15 min. The tubes were cut to remove the supernatant which was saved for determination of the specific activity. The pellets were stored at 0–4°C until the efflux studies, usually within 1 h.

The efflux from ghosts was started by the addition of the cells to 5 ml of incubation fluid (composition indicated in the legends). At different time points (2, 7, 12, and 17 min), 1-ml samples were withdrawn and immediately centrifuged in a microcentrifuge at maximum speed (model 59A; Fisher Scientific Co., Pittsburgh, PA) for 2 min; the supernatant was quickly removed and saved for liquid scintillation counting. At the end of the flux period, the remaining ghost suspension was placed on ice. An aliquot of this suspension was diluted with isotonic KCl, pH 7.4, and analyzed for the number of ghosts present. Another aliquot was precipitated with PCA and a sample of the supernatant was counted to obtain a(∞). The initial rate coefficient, −b, was determined from the slope of a graph of ln[1 − a(t)/a(∞)] vs. time, where a(t) is the cpm/0.2 ml of supernatant of sample taken at time t, and a(∞) is the cpm/0.2 ml supernatant of the precipitated suspension, corrected for the added PCA volume. The final efflux was calculated as micromoles per unit ghosts per hour by the following equation,

\[ M = b \left( \frac{\text{µmol glycine}}{\text{cpm}} \right) \times 3.1 \times 10^{13} \frac{\text{ghosts}}{\text{unit}} \times \left( \frac{\text{cpm}}{\text{ghost}} \right) \]  

(1)

and expressed as micromoles per kilogram of cell solid per hour.
Glycine Metabolism

The metabolism of glycine during the time course of the influx studies was measured both by following conversion to CO₂ and the recovery of ¹⁴C label as glycine. To measure conversion of glycine to CO₂, cells (0.35 ml packed cells) were incubated in 150 mM NaCl, 10 mM HEPES, and 100 μM ¹⁴C glycine at pH 7.4 and 37°C. The flask was capped with a self-sealing rubber stopper fitted with a center well cup that extended into the flask (Kontes Glass Co., Vineland, NJ). After 15 min, 0.3 ml of 2 M KOH was injected into the center well to trap CO₂, and 0.6 ml of 6 N H₂SO₄ was added to the medium. The flasks were incubated an additional hour at 37°C. The rubber stopper was then removed and the contents of the cap were counted by liquid scintillation counting. In control incubations, the formation of ¹⁴CO₂ from ¹⁴C glycine in the absence of red blood cells was measured. The conversion of glycine to CO₂ by the cells was calculated as the difference of ¹⁴CO₂ production in experimental (plus cells) vs. control flasks and expressed was as micromoles CO₂ per kilogram of cell solids per hour. In another set of experiments, the recovery of ¹⁴C label as glycine inside the cell was measured. The influx study was performed as usual, the cell pellet was lysed, and the proteins were precipitated. An aliquot of the supernatant (10 μl) plus nonlabeled glycine and serine were spotted on thin-layer chromatography plates (cellulose, Baker-flex; J. T. Baker Chemical Co., Phillipsburg, NJ), which were run in a solvent of pyrimidine and water (10:3). On additional plates, a sample of supernatant plus nonlabeled glycine and serine were spotted and run in a solvent of butanol, acetic acid, and water (60:15:25). When the solvent front had moved 15 cm, the plates were dried and sprayed with ninhydrin to identify the glycine and serine spots. The plates were cut into 1-cm segments and analyzed by liquid scintillation counting. The Rᵣ value for glycine was 0.21 and for serine 0.34.

Statistical Analyses

Values are expressed as means ± SE. Means were compared using the Student t test, paired t test, or analysis of variance, and significant differences are indicated by P values. V₅₅Na is the V₅₅ of the Na dependence of glycine influx at set concentrations of glycine and Cl, Na₀.₅ is the Na concentration at which influx is one-half the V₅₅Na, and n is apparent number of Na transported with each glycine. V₅₅Na, Na₀.₅, and n were determined by a fit of the data to the Hill equation: v = (V₅₅Na(Na*)/(Na₀.₅ + Na*)). V₅₅Cl and K₅₅Cl are the V₅₅ and K₅₅ values for glycine influx at set concentrations of Na and Cl. K₅₅Na is the K₅₅ for the Cl dependence of glycine influx at set concentrations of Na and glycine. The values (V₅₅Cl, K₅₅Cl, K₅₅Na) were derived from a nonlinear least-squares fit of the data.

RESULTS

The Components of Glycine Transport

The components of glycine transport by human red blood cells are shown in Table I. At a glycine concentration of 100 μM, the Na-dependent influx averaged 47 μmol/kg Hb·h and ranged from 35 to 60 flux units. The Na-independent stilbene-sensitive flux averaged 14 μmol/kg Hb·h (range, 10.5–17), and the Na-independent stilbene-insensitive influx was 16.0 μmol/kg Hb·h (range, 11–19). The Na-independent fluxes were the same with potassium, NMG, or choline as the cation (not shown). The flux values reported here are similar to those obtained by Ellory et al. (1981) under comparable experimental conditions. When the data of Ellory et al. are expressed as micromoles per kilogram of hemoglobin per hour, their values are
52, 13, and 15 for the Na-dependent, Na-independent stilbene-sensitive, and Na-independent stilbene-insensitive fluxes, respectively.

The effects of a number of compounds on glycine flux were tested to identify potential inhibitors and determine if the Na-dependent and Na-independent stilbene-sensitive pathways had common inhibitors. Table I shows that these two components of transport could be separated not only by Na dependence but also by the effects of DNDS and the sulfhydryl-reactive agents N-ethylmaleimide (NEM) and p-chloromercuribenzenesulfonate (pCMBS). The Na- and Cl-dependent flux was inhibited 91% by NEM and 93% by pCMBS, but was unaffected by DNDS or DIDS (4,4'-disothiocyanostilbene-2,2'-disulfonate) (not shown). The stilbene-sensitive flux was unaffected by the sulfhydryl-reactive agents.

### Table I

| Medium                  | Glycine transport  |
|-------------------------|--------------------|
|                         | Total | Na-dependent | Na-independent, DNDS-sensitive | Na-independent, DNDS-insensitive |
| NaCl                    | 77 ± 2.4 (9)      | 47 ± 2.6     |                      |
| NaCl + DNDS             | 62 ± 0.4 (4)      | 46 ± 1.52    |                      |
| NaCl + NEM              | 30 ± 1.5 (5)      | 4.4 ± 2.1    |                      |
| NaCl + pCMBS            | 25 ± 1.1 (5)      | 3.2 ± 1.4    |                      |
| KCl                     | 50 ± 1.1 (9)      | 14 ± 1.6     |                      |
| KCl + NEM               | 26 ± 1.5 (4)      | 12 ± 1.7     |                      |
| KCl + pCMBS             | 20 ± 0.8 (5)      | 14 ± 0.8     |                      |
| KCl + DNDS              | 16 ± 1.2 (5)      | 16 ± 1.2     |                      |
| KCl + DNDS + NEM        | 14 (2)            | 14           |                      |
| KCl + DNDS + pCMBS      | 6 (2)             | 6            |                      |

The values are the means ± SE from the number of experiments indicated in parentheses. The glycine concentration was 100 µM and the experiments were performed at 37°C, pH 7.4. When indicated, DNDS was present at a concentration of 100 µM, NEM at 1.0 mM, and pCMBS at 100 µM. The Na-dependent flux is the flux in NaCl minus the flux in KCl; the Na-independent, DNDS-sensitive flux is the flux in KCl minus the flux in KCl plus DNDS; the Na-independent, DNDS-insensitive flux is the flux in KCl plus DNDS.

**Metabolism of Glycine by Human Red Blood Cells**

The metabolism of glycine by red cells was examined to verify that the influx studies reflected rates of transport and not metabolism. Glycine metabolism was measured as conversion to CO₂ as well as recovery of transported ¹⁴C as glycine. Over the time course of the influx experiments, the rate of ¹⁴CO₂ formation by the cells was only 0.27 µmol/kg cell solid. h, or <1% of the rate of glycine transport. In addition, 85–90% of the cellular ¹⁴C was recovered as glycine and 10% as serine. There was no detectable incorporation of label into glutathione during the 15-min incubation. We therefore believe that membrane transport is the rate-limiting step for influx and that the calculated flux rates are initial unidirectional transport rates.
Na- and Cl-dependent Glycine Transport

Na dependence at high Cl. The Na dependence of glycine influx was studied by investigating the transport of glycine as a function of Na in the incubation medium at high (150 mM) Cl. As Na in the medium was increased, glycine influx increased in a sigmoidal fashion (Fig. 1). When the data were fit to the Hill equation, the kinetic constants were $65 \pm 4 \mu$mol/kg Hb-h for $V_{\text{maxNa}}$, $56 \pm 5$ mM for $Na_{0.5}$, and $2.1 \pm 0.3$ for $n$ (means ± SE), which indicates at least two binding sites for Na. The kinetic constants for $V_{\text{maxNa}}$ and $Na_{0.5}$ were similar when derived by Eadie-Hofstee ($v/s$ vs. $v$) or double reciprocal ($1/v$ vs. $1/s$) plots (not shown).

Stoichiometry. The transport of $^{22}$Na was then measured and compared with the concomitant transport of glycine in two experiments (Table II). The presence of glycine (200 $\mu$M) in the incubation medium significantly stimulated the $^{22}$Na (50 mM Na) influx ($P < 0.05$, see Table II); the influx rate was increased 60-76 $\mu$mol/kg Hb-h. At the same time, the presence of 50 mM Na increased the $[^{14}C]$glycine

| Table II |
|---|
|**Na-stimulated Glycine Influx and Glycine-stimulated Na Influx**|
| Experiment | 0 Na | 50 mM Na | $\Delta$ |
| Glycine (200 $\mu$M) influx |  |  |  |
| 1 | 54 | 82 | 28 |
| 2 | 54 | 83 | 28 |
| Na (50 mM) influx |  |  |  |
| 1 | 1,528 | 1,542 | 14 |
| 2 | 1,518 | 1,594 | 76 |

The data represent the results of two experiments in which $[^{14}C]$glycine influx was measured in the absence (150 mM KCl) and presence (50 mM NaCl, 100 KCl) of Na, and $^{22}$Na influx was measured in the absence and presence (0.2 mM) of glycine. The fluxes were performed in the presence of 0.1 mM bumetanide, 0.1 mM ouabain, and 1.0 mM amiloride to minimize background Na fluxes. $^{22}$Na influx was significantly greater in the presence vs. absence of glycine ($P < 0.05$) as tested by a paired t test (one-tailed) or by a two-way analysis of variance.
influx 28–40 μmol/kg Hb·h as compared with influx in the absence of Na. The ability of either solute to stimulate the transport of the other is an essential characteristic of cotransport. In addition, the measured ratio of the stimulated fluxes taken together with the calculated slope of the Hill plot provide strong evidence for a stoichiometry of 2 Na:1 glycine transported on this pathway.

Na dependence at low Cl. In the presence of low Cl (5 mM) the Na dependence of glycine influx was altered. Over the range of 0–150 mM Na, the glycine flux was significantly reduced compared with the transport rates in high Cl, and they appeared to increase linearly with increasing Na. Glycine influx was then measured as a function of Na up to 300 mM Na, using cells loaded with 295 mM KNO₃ and 5 mM KCl by the nystatin technique (Fig. 2). Over this larger range of Na, a sigmoidal response of glycine influx was observed, with a fit of the data to the Hill equation giving the kinetic parameters of $V_{\text{max}} = 78 \pm 23$ μmol/kg Hb·h, $N_{0.5} = 256 \pm 80$ mM Na, and $n = 2.0 \pm 0.4$ (means ± SE). Values for glycine influx vs. Na into normal cells or nystatin-treated cells were identical over the range of Na concentrations testable with normal cells, 0–150 mM. Therefore, under low Cl conditions, the Na

![Figure 2](image2.png)

**Figure 2.** Na-dependent glycine influx as a function of Na concentration in the presence of low Cl (5 mM with NO₃ substituted) and 100 μM glycine, pH 7.4 at 37°C. The cells were loaded with 295 mM KNO₃ and 5 mM KCl using nystatin as described in the Methods. Na was varied by substituting K. The values are the rates of transport in NaNO₃/KNO₃ and 5 mM KCl minus the rate in KNO₃ and 5 mM KCl.

![Figure 3](image3.png)

**Figure 3.** Na-dependent glycine influx as a function of Cl. Cl was varied by substitution with NO₃ and the cells' anions were equilibrated with media of the designated anion concentration before the experiments. The values are the Na-dependent rates of transport (influx in NaCl/NaNO₃ minus influx in KCl/KNO₃) from which the Na-dependent influx in the absence of Cl (influx in NaNO₃ minus influx in KNO₃) has been subtracted.
dependence shows no change in the $V_{\text{max}, Na}$ or stoichiometry but a large increase in $N_{a_{0.5}} (P < 0.05)$, which indicates that the effect of Cl is to increase the affinity of Na for the carrier.

$Cl$ dependence. The Na-dependent uptake was found to be greatest with Cl as the anion (37 $\mu$mol/kg Hb-h) as compared with either NO$^-_3$ (16 $\mu$mol/kg Hb-h) or SO$_4^-_2$ (7 $\mu$mol/kg Hb-h), which is in agreement with the data of Ellory et al. (1981). The Cl dependence was further investigated by measuring glycine uptake as a function of increasing Cl ($Cl_i - Cl_0$) with constant Na (150 mM). As Cl was increased, glycine influx increased hyperbolically (Fig. 3). A nonlinear least-squares fit to the chloride-stimulated flux indicates a single binding site for Cl with an apparent dissociation constant, $K_{W, Cl}$, of 9.5 ± 1.7 mM.

**Kinetic Constants for Na- and Cl-dependent Glycine Uptake**

Glycine influx was assayed as a function of glycine concentration and compared under conditions of normal Na and Cl, normal Na and low Cl, and low Na and normal Cl. Low Cl was 5 mM Cl, slightly below the $K_{W, Cl}$ for Cl stimulation, and low Na was 50 mM, approximately the $K_{W, Na}$ for Na stimulation. Because some glycine flux has been reported to occur via the ASC system (Ellory et al., 1981), 2 mM alanine was included in all the incubations to block influx through this pathway.

| Influx medium | $K_{1/2, e}$ (µM) | $V_{\text{max}, e}$ (µmol/kg Hb·h) |
|---------------|------------------|-------------------------------|
| 150 mM NaCl   | 100 ± 15         | 109 ± 6.3                     |
| 145 mM NaN$O_3$, 5 mM NaCl | 388 ± 45 ($P < 0.05$) | 135 ± 7.6 ($P < 0.05$) |
| (low Cl)      |                  |                               |
| 50 mM NaCl, 100 mM KCl | 313 ± 60 ($P < 0.05$) | 85 ± 6.7 ($P < 0.05$) |
| (low Na)      |                  |                               |

The values are the mean ± SE of the kinetic constants derived from a nonlinear least-squares fit of the Na- and Cl-dependent uptake vs. glycine concentration (Wilkinson, 1961). The fluxes were performed in the presence of 2.0 mM alanine to eliminate any flux of glycine via the ASC system (Ellory et al., 1981). The P values indicate significant differences compared with the value for $K_{1/2, e}$ or $V_{\text{max}}$ at 150 mM NaCl.
However, it should be noted that in our experiments, at 100 μM glycine there appeared to be insignificant influx via the ASC system; in high-Cl media (150 mM Cl) there was no effect of 2 mM alanine on the Na-dependent glycine influx and in zero Cl medium (150 mM NO₃) there was only a slight inhibition (19%). The $K_{\text{m, glycine}}$ for glycine uptake in 150 mM NaCl was 100 μM and the $V_{\text{max, glycine}}$ was 109 μmol/kg Hb·h (Fig. 4, Table III). Ellory et al. (1981) reported a lower $K_{\text{m, glycine}}$ of 29 μM, and a lower $V_{\text{max, glycine}}$ of 80 μmol/kg Hb·h. The differences between these data and those of the present study are most likely due to differences in procedure for measuring the kinetic constants. Ellory et al. (1981) measured the kinetic constants of the flux component corresponding to the flux in NaCl minus the flux in sodium methylsulfate and the kinetic constants were derived from a double reciprocal plot ($1/v$ vs. $1/s$). In addition, the flux was measured over a smaller glycine range and fluxes were calculated from a sample at a single time point. Both studies, however, are consistent with a high glycine affinity characteristic of the Na- and Cl-dependent glycine transport (glycine system) as compared with glycine transport by the ASC system (Ellory et al., 1981; Christensen, 1984). When medium Na or Cl was lowered, both $K_{\text{m, glycine}}$ and $V_{\text{max, glycine}}$ were altered. Under conditions of low Cl (5 mM), the $K_{\text{m, glycine}}$ increased 3.4-fold to 338 μM and the $V_{\text{max, glycine}}$ increased 1.3-fold to 135 μmol/kg Hb·h (Fig. 5, Table III). In a low Na medium (50 mM), the $K_{\text{m, glycine}}$ increased...
3.1-fold to 313 μM, and the $V_{max-gly}$ decreased 20%, to 85 μmol/kg Hb·h (Fig. 6, Table III).

**Effect of pH on glycine influx.** The effects of pH on Na- and Cl-dependent glycine uptake were examined to address the question of whether the zwitterionic or anionic form of glycine is transported. The pK of the amino group of glycine is 9.4 and the pK of the carboxyl group is <3.00. As a result, over the pH range 6.4–8.7, the amount of glycine present as the anion rises dramatically from 0.1 to 17 μM, while by comparison there is little change in zwitterion concentration (99.9–83 μM). Fig. 7 shows that over the pH range 7.4–8.7, the Na- and Cl-dependent glycine flux is relatively constant, suggesting that the zwitterion is the transported species. At pH 6.4 flux is decreased, possibly due to H⁺ inhibition of the transport mechanism.

**Effect of membrane potential on glycine influx.** Since Na-dependent glycine flux appeared to involve cotransport of the zwitterion and Na and possibly Cl, we were interested in evaluating the effect of membrane potential on this process. An inside negative membrane potential generated by a K gradient (Kₒ = 5 mM and Kᵢ = 145 mM) in the presence of valinomycin, stimulated Na-dependent glycine uptake from 45 to 68 μmol/kg Hb·h (Table IV). Further increasing the K gradient (Kₒ = 2 mM, Kᵢ = 145 mM), and therefore the membrane potential, resulted in an additional increase in the Na-dependent glycine flux (not shown). Assuming the constant field equation, this increase is consistent with a single cationic charge moving through

![Figure 7](image_url)

**Figure 7.** The pH dependence of Na- and Cl-dependent glycine influx. Values are the rates of transport in NaCl minus the rates in KCl at the same pH. In all cases, pHi is equilibrated with pHₒ. Glycine was present at a concentration of 100 μM.

### Table IV

**Effect of Membrane Potential on Na-dependent Glycine Influx**

| Medium                  | Total flux (μmol/kg Hb·h) | Na-dependent flux (μmol/kg Hb·h) |
|-------------------------|---------------------------|---------------------------------|
| NaCl                    | 77, 80                    | 44, 47                          |
| NMG-CI                  | 55, 52                    |                                 |
| NaCl plus valinomycin   | 99, 98                    | 69, 68                          |
| NMG-CI plus valinomycin | 29, 32                    |                                 |

The results represent duplicate fluxes from one experiment. Membrane potential was varied by the addition of valinomycin in the presence of a K gradient with Kᵢ = 5 mM. The incubation media were 150 mM NaCl, 5 mM KCl, 100 μM [³⁵]glycine, and 10 mM HEPES (pH 7.4 at 37°C), or 150 mM NMG-CI, 5 mM KCl, 100 μM [³⁵]glycine, and 10 mM HEPES (pH 7.4 at 37°C). Valinomycin was added in ethanol to bring the final concentration to 20 μM. An equal volume of ethanol only was added to the controls (0.3% final concentration).
only 12% of the constant electric field or higher-valence cationic complexes moving through a smaller percentage of the field.

**Symmetry of Na- and Cl-dependent transport.** Glycine influx and efflux were studied using red blood cell ghosts to investigate the operation of Na-dependent transport in both directions across the membrane. For efflux studies, we used ghosts containing 150 mM NaCl or 150 mM KCl and 100 μM [14C]glycine, and we measured efflux into mM 150 KCl. For influx studies, we used ghosts resealed to have a final concentration of 150 KCl, and we measured [14C]glycine influx from 150 mM NaCl or 150 mM KCl medium. Na-dependent efflux is defined as efflux from Na-containing ghosts minus efflux from KCl-containing ghosts. Na-dependent glycine influx is, as previously, influx in NaCl medium minus influx in KCl medium. Under these conditions, Na-dependent transport operated in the direction of efflux as well as influx, but the efflux rate (38 ± 3.5, mean ± SE) was greater than the rate of influx.

### TABLE V
**Effects of Trans Membrane Substrate on Na-dependent Glycine Influx and Efflux in Red Blood Cell Ghosts**

| Medium → Ghost contents | Glycine influx | Total flux | Na-dependent flux |
|-------------------------|---------------|-----------|------------------|
| NaCI KCl                | 59 ± 8.8 (4)  | 25 ± 1.9  |
| KCl                     | 34 ± 7.5 (4)  |
| NaCI NaCl               | 15 ± 1.0 (4)  | 3.7 ± 1.3, P < 0.05 |
| KCl                     | 15 ± 1.1 (3)  |
| NaCI NaCl + glycine     | 36 ± 7.8 (4)  | 11 ± 2.8, P < 0.05 |
| KCl                     | 26 ± 5.6 (4)  |
| NaCI KCl + glycine      | 51 ± 10 (4)   | 18 ± 0.45, P < 0.05 |
| KCl                     | 35 ± 9.4 (4)  |

| Ghost contents↑ → Medium | Glycine efflux | Total flux | Na-dependent flux |
|--------------------------|----------------|-----------|------------------|
| NaCl KCl                 | 82 ± 1.2 (4)   | 35 ± 3.7  |
| NaCl NaCl                | 68 ± 2.9 (4)   | 21 ± 4.5, P < 0.05 |
| NaCl KCl + glycine       | 53 ± 5.1 (4)   | 6.0 ± 4.2, P < 0.05 |
| NaCl NaCl + glycine      | 54 ± 1.8 (4)   | 7.0 ± 3.3, P < 0.05 |
| KCl KCl                  | 47 ± 2.8 (4)   |
| KCl KCl + glycine        | 47 (2)         |

The values are the means ± SE of the number of experiments indicated in parentheses. For influx, ghosts contained 150 mM NaCl, or KCl and, where indicated, glycine at 100 μM. The media were 150 mM NaCl or KCl, 100 μM [14C]glycine, and 10 mM HEPES (pH 7.4 at 37°C). Na-dependent influx was calculated separately in each experiment as influx rate in NaCl minus influx rate in KCl. For efflux, ghost contents were 150 mM NaCl or KCl and 100 μM [14C]glycine. The media were 150 mM NaCl or KCl, 10 mM HEPES, and, where indicated, 100 μM glycine. The Na-dependent efflux was calculated as the efflux from NaCl ghosts minus the efflux from KCl ghosts. The P values indicate significant differences compared with transport (influx or efflux) with no trans substrates.
(25 ± 1.0, mean ± SE (Table V). The anion dependence of Na-dependent glycine efflux was also investigated. When glycine efflux into 150 mM KNO₃ was measured from ghosts containing 150 mM NaNO₃, 100 μM [¹⁴C]glycine or 150 mM KNO₃, and 100 μM [¹⁴C]glycine, the flux rates were 50 ± 3.5 and 41 ± 3.6 μmol/kg cell solid·h, respectively (mean ± SE, n = 4). The Na-dependent flux from NaNO₃ ghosts was calculated as 9 μmol/kg cell solid·h, only 26% of the Na-dependent efflux from NaCl ghosts, indicating that Cl stimulation is characteristic of the Na-dependent efflux as well as influx.

It is possible that the procedure for the ghost preparation loses important cytoplasmic factors from the system that modulates transport. It is difficult to evaluate the effects of possible cytoplasmic modulators on efflux by comparing glycine efflux from intact cells and ghosts. The slow rate of glycine uptake would necessitate long incubations at 37°C to load intact cells with [¹⁴C]glycine, and the subsequent efflux rates could be complicated by transport of [¹⁴C]glycine metabolic products. On the other hand, we can compare glycine influx into whole cells and ghosts. The results in Tables I and V show that Na-dependent glycine uptake by the ghosts (25 μmol/kg cell solid·h) is less than the average uptake for whole-cell studies (46 μmol/kg cell solid·h). If the transporter itself is unaffected by the ghosting procedure, these data suggest that cytoplasmic factors stimulate glycine influx.

In the same series of experiments the effects of cotransported substrates on the trans side of the membrane were investigated for Na-dependent glycine influx and efflux in red blood cell ghosts (Table V). The rate of Na-dependent influx with no trans Na or glycine was 25 μmol/kg cell solid·h. The presence of trans Na most significantly decreased the Na-dependent influx; transport was decreased to 3.7 μmol/kg cell solid·h, an 85% inhibition. When trans glycine was added, i.e., trans Na and glycine, influx was stimulated relative to rates with trans Na alone with inhibition falling to 56%. Trans glycine alone inhibited influx much less (28%). The rate of Na-dependent efflux in the absence of trans substrates was 35 μmol/kg cell solid·h (Table V). Compared with influx measurements, trans Na had a smaller effect on Na-dependent efflux (40% inhibition) while trans glycine most significantly inhibited efflux (83% inhibition) and this inhibition was unchanged when both glycine and Na were present on the trans side (80% inhibition).

DISCUSSION

The present study investigates the Na- and Cl-dependent glycine transport across the human red blood cell membrane. This component of transport can be separated from the Na-independent, stilbene-sensitive flux by the differences in Na dependence, stilbene sensitivity, inhibition by sulfhydryl reactive agents, and pH dependence.

The Na-dependent glycine flux was demonstrated to be Na and glycine cotransport as both glycine and Na stimulated the transport of the other solute. The direct measurements of Na and glycine transport indicated a minimal stoichiometry of two Na per glycine transported, which is consistent with the value for n obtained when the data for glycine uptake vs. Na concentration were fit to the Hill equation. Multiple Na ions transported by the carrier may bind in either a random or ordered...
scheme. Using the phenomenologically observed kinetic constants for \( V_{\text{max},Na} \) and \( Na_{0.5} \), we attempted to model random and ordered schemes for equilibrium binding and we compared these results with those of our experimental data. The binding of substrates is in rapid equilibrium and, therefore, the rate-limiting step is elsewhere: translocation, unloading, or return of the empty carrier. In each case only the fully loaded carrier was considered to be translocated. The simplest model is a random binding scheme having equal \( K_u \)’s. The rate equation for this reaction is

\[
v = \frac{V_{\text{max},Na}}{K^2 + 2KNa + Na^2}
\]

where \( K \) is equal to the \( K_u \) for Na binding to either site. When \( V_{\text{max}} \) is 65 \( \mu \text{mol/kg Hb-h} \) and \( Na_{0.5} \) is 56 mM, \( K \) for this equation is 23 mM. With these kinetic constants, the random binding model results in a profile for glycine uptake vs. Na concentration shown in Fig. 8 (open triangles). Compared with the experimental data (solid line), this model shows less sigmoidicity and appears to approach \( V_{\text{max}} \) more slowly. If random binding is modeled with unequal \( K_u \)’s, the rate equation is

\[
v = \frac{V_{\text{max},Na}}{K_1K_2 + NaK_1 + NaK_2 + Na^2}
\]

where \( K_1 \) and \( K_2 \) are the Na binding constants for sites 1 and 2. Knowing the \( V_{\text{max},Na} \) and \( Na_{0.5} \), we can calculate \( K_1 \) for a range of \( K_2 \)’s. Fig. 8 shows the profile of glycine flux vs. Na concentration that is obtained when one of the \( K \)’s is 50 mM and the other 3.2 mM (open circles). We see that as the \( K_u \)’s are set to be unequal, the random model moves farther away in its fit to the experimental data, appearing more similar to Michaelis-Menten kinetics. Thus these two random schemes for Na binding to the carrier do not provide a good model for our data. In the case of an
ordered binding scheme, the rate equation for the reaction is

\[
v = \frac{V_{\text{max}} \text{Na}^2}{\text{Na}^2 + \text{NaK}_1 + K_1 K_2}
\]

where \(K_1\) is the \(K_u\) of the first Na binding site and \(K_2\) is the \(K_u\) of the second Na binding site. We can again calculate \(K_1\) for a range of \(K_1\)'s and obtain the corresponding profiles of glycine flux vs. Na concentration. Comparing these profiles, the experimental data is best fit with schemes having a high \(K_1\) and a low \(K_2\). At \(K_1 = 250 \text{ mM}\), \(K_2\) is calculated to be 10.3 mM and the model using these kinetic constants (\(V_{\text{max}} = 65 \mu\text{mol/kg Hb-h}, K_1 = 250 \text{ mM}, \text{ and } K_2 = 10.3 \text{ mM}\)) results in a profile (open squares) of glycine influx nearly identical to that of the experimental data. When the system is modeled with decreasing \(K_1\)'s, \(K_2\) increases (\(K_1 = 50, K_2 = 28; K_1 = 35, K_2 = 35\)) and the fit to the experimental data retrogresses (not shown). Such an ordered binding scheme (high \(K_1\) and low \(K_2\)) is very similar to a random binding model with high positive cooperativity between two similar binding sites. The rate equation for the latter reaction is

\[
v = \frac{V_{\text{max}} \text{Na}^2}{\text{Na}^2 + 2K_2 \text{Na} + K_1 K_2}
\]

in which \(K_1\) is the \(K_u\) for both sites (1 and 2) with no Na bound to the carrier, and \(K_2\) is the \(K_u\) for the remaining Na site (1 or 2) after one Na has bound. When \(K_1\) is high (300 \text{ mM Na}) and \(K_2\) is low (7.7 mM), the profile for glycine influx vs. Na concentration is almost identical to that for the ordered binding scheme above (\(K_1 = 250 \text{ mM Na}, K_2 = 10.3 \text{ mM Na}\)). Thus, we find that the data best fit a model in which the first Na binds to a low affinity site (\(K_u > 250 \text{ mM}\)) and the second Na binds to a high affinity site (\(K_u < 10.3 \text{ mM}\)) in either an ordered binding sequence or a random binding sequence with high positive cooperativity. The presence of order in the binding of the other substrates (see below) prejudices us to an ordered scheme for Na also.

The Cl-dependence of Na-glycine cotransport was found to be characterized by a single binding site for Cl with a \(K_u\) of 9.5 mM, a value similar to the \(K_u\) for Cl stimulation of Na-dependent glycine transport in pigeon erythrocytes (Imler and Vidaver, 1972). For the non–red cell tissues, the reported \(K_{\text{u-Cl}}\) values are higher (22 mM for fish intestine). A Cl dependence for amino acid transport is unusual, having been noted for only a few other transport systems, such as β-amino acid (taurine and β-alanine) transport in fish (King et al., 1982) and rat kidney (Chesney et al., 1985; Turner, 1986) and α-amino isobutyrate transport in fish intestine (Bogé and Rigal, 1981). In none of these tissues is the mechanism of Cl stimulation clear. Although the Na glycine cotransport is voltage-dependent and thus probably rheogenic, it is unlikely that the requirement for Cl is for an accompanying anion to dissipate electrical gradients; the high anion conductance, including nitrate and sulfate, of the red blood cell (Fröhlich, 1984) effectively clamps the membrane potential close to the equilibrium potential for these anions, which is near zero. In addition, while other anions have been shown to support a reduced rate of transport (with a selectivity of Cl > Br > SCN > NO₃), there appears to be an absolute anion requirement,
as transport is not supported by I, MeSO₄, or acetate (Imler and Vidaver, 1972; Ellory et al., 1981; Turner, 1986).

The present study demonstrates that the mechanism of the Cl stimulation involves an interaction with Na binding, which is seen by comparing the graphs of glycine influx vs. Na at low and high Cl (Figs. 1 and 2). At low Cl, glycine uptake in response to Na shows an increased Na₀.s with no change in Vₘₐₓₙ Na or the Na/glycine stoichiometry. The modifying effect of Cl is to increase the affinity of Na for the carrier, which suggests that Cl binds to the carrier before Na.

This analysis does not answer the question of whether Cl is cotransported; the ordered binding of Cl to the transporter, facilitating the binding of Na, does not necessitate that the anion be transported. Unfortunately, it is impossible to assess directly whether Cl is cotransported with Na and glycine on the carrier because of the rapid chloride fluxes by other pathways. Even in the presence of stilbene inhibitors, the residual Cl flux across the red cell membrane is great enough to preclude the detection of a Na- and glycine-stimulated Cl flux. Another approach is to look at Cl transport indirectly, reasoning that if Cl is transported then the influx of glycine may be dependent on the chloride gradient across the membrane as well as the glycine and Na gradients. We tested this idea in an experiment in which cells were pre-equilibrated in 145 mM KNO₃ and 5 mM KCI (decreasing the CI to about the KCI), or in 150 mM KCl, normal Cl. For both cell types, influx was measured in (a) 145 mM NaCl, 5 mM KCl plus valinomycin and DNDS, and in (b) 145 NMG-Cl, 5 mM KCl plus valinomycin and DNDS. The Na-dependent flux was calculated as the difference in uptake rates in these two media. With the membrane potential clamped at Eₖ by the presence of valinomycin and DNDS (to block the Cl conductance), the Na-dependent influx under normal conditions (CIᵢ = 110 mM, Clₒ = 150 mM) was 52 µmol/kg Hb·h (n = 2), and under conditions of a CI gradient (CIᵢ = 5 mM, CIₒ = 150 mM), was not significantly different, 55 µmol/kg Hb·h. Unfortunately, the absence of trans inhibition in this one type of experiment does not rule out the cotransport of Cl (with a possible explanation being that the inward-facing transporter is saturated at Clᵢ = 5 mM, and, thus, raising Clᵢ further has no effect). Turner (1986) has recently examined the possible cotransport of Cl with Na and β-alanine in rat kidney vesicles. He reported that the presence of an infinite CI gradient alone (Na at equilibrium) increased the rate of Na-dependent β-alanine uptake and results in an accumulation of the amino acid against its concentration gradient.

We studied the mechanism for Na and Cl stimulation of glycine transport by investigating the effects of these ions on the glycine kinetic constants (Table III). Increasing Na resulted in a decrease in Kᵢₚₛₓᵧ and an increase in Vₘₐₓᵧ, while increasing Cl resulted in a decrease in Kᵢₚᵧ and a decrease in Vₘₐₓᵧ. In both cases the changes in Kᵢₚᵧ values were larger than the changes in Vₘₐₓ. Similar findings have been reported for pigeon erythrocytes (Imler and Vidaver, 1972). Varying Na, Imler and Vidaver found that when Naᵢ was increased from 44 to 130 mM, the Kᵢₚᵧ decreased almost three-fold and the Vₘₐₓ increased 20%. For Cl, increasing the anion decreased the Kᵢₚᵧ 2.5- to 25-fold depending on the substituting anion, while the Vₘₐₓ was either slightly decreased (30% decrease with F⁻ substituted) or slightly increased (40% increase with NO₃ substituted). If the Na and Cl and glycine binding
are in rapid equilibrium so that transport is rate-limiting, the effects of Na and Cl on the glycine kinetic constants can be used to evaluate their order of binding to the carrier in relation to the amino acid (Segel, 1975). Again, the assumptions made are that binding of substrates is very fast compared with the translocation of the loaded carrier or some subsequent step, and that we are considering only the transport of the fully loaded carrier. If the ion (Na or Cl) binds first and is followed by glycine, then the effect of raising that ion should be to decrease $K_{m-g}$ with no effect on the $V_{max-g}$. If glycine binds before the ion, then the effect of raising the ion should be to decrease $K_{m-g}$ and increase $V_{max-g}$. If analyzed by these criteria, which follow from the law of mass action, the effects of Na on the glycine kinetic constants indicate that glycine and Na binding is ordered, with glycine binding first. The effects of Cl on glycine flux (Table III), on the other hand, are not diagnostic of either order of binding. As a result, we must suppose that either Cl and glycine bind in random order, or that there is an obligatory order between the binding of glycine and Cl but the Cl effect on $K_{m-g}$ is due to allosteric changes in the transporter rather than being the consequence of mass action alone; or, alternatively, we must suppose that either the assumptions of rapid binding reactions or the assumption that forbids transport of the partially loaded transporter is false. We suppose that Cl and glycine binding is ordered, but consider both ordered sequences. These data together with those for the effects of Cl on Na binding suggest a scheme for binding to the outward-facing carrier in which glycine binds first with a $K_{m-g}$ of 100 mM followed by Cl ($K_{m-Cl} = 9.5$ mM), Na ($K_{m-Na} > 250$ mM), and Na ($K_{m-Na} < 10.3$ mM) in order (Fig. 9), or, alternatively, a scheme in which chloride binds first with a $K_{m-Na}$ of 9.5 mM followed by glycine ($K_{m-g} = 100$ mM), Na ($K_{m-Na} > 250$ mM), and Na ($K_{m-Na} < 10.3$ mM) in order. These models can be tested by evaluating the scheme in terms of the effects of trans substrates on glycine efflux (Table V), data that also reflect binding to the outward-facing carrier. We found that trans glycine in the presence or absence of trans Na largely inhibits the Na-dependent glycine efflux (82%), while trans Na alone has a much smaller inhibiting effect (37%). These results are consistent with our proposed model if the inward translocation of the unloaded carrier is faster than that of the loaded forms (complete or partially loaded; Fig. 9 with $c > a$). In this way, trans glycine would inhibit maximally with or without trans Na since it binds to the carrier independent of whether Na is bound. Likewise trans Na alone would be expected to show a smaller inhibition since its binding requires prebinding of glycine to the unloaded carrier.

The Na gradient hypothesis (Crane, 1962; Curran et al., 1970) predicts that the cotransporter should be able to couple the efflux of the Na and amino acid just as it does the influx. The physiological direction of net transport therefore depends on the amino acid and cation electrochemical gradients with transport in both directions. The resealed red blood cell ghost provides a convenient system for testing the symmetry of transport; there are no ambiguities regarding the fractional sidedness as with membrane vesicle preparations and, unlike other intact cells, internal Na and amino acid can be varied without extended preloading incubations and ouabain treatment. Studies of the symmetry of glycine transport in different systems are equivocal. In pigeon red blood cells, Na-dependent glycine transport was found to be asymmetrical, with the $K_m$ and $V_{max}$ for efflux greater than for influx (Vidaver
and Shepherd, 1968). Similar properties of asymmetry have been shown for Ehrlich ascites tumor cells (Johnstone, 1978). In contrast, studies with sheep reticulocyte inside-out and right-side-out vesicles demonstrated the affinity constants for both Na and glycine to be symmetrical as well as the Na/glycine stoichiometry (Weigensberg and Blostein, 1985).

The present study demonstrated that Na-dependent glycine transport can operate in both directions across the membrane and in both cases requires Cl for full activity. The measurements of glycine influx and efflux, however, indicate an asymmetry in the carrier. First, in red blood cell ghosts, Na-dependent glycine transport at 100 μM glycine and 150 mM NaCl (zero trans Na) was greater for efflux than for influx. Second, trans substrates affected influx and efflux differently. Efflux was strongly inhibited by trans glycine and only slightly by trans Na, while influx was strongly inhibited by trans Na and only slightly by trans glycine. These data indicate a difference in the order of loading and unloading of the outward-facing vs. the inward-facing carrier, and are consistent with a reversed binding order at opposite sides of the membrane (first on, first off, or glide symmetry; Turner, 1981). The trans effects also indicate a difference in the relative rates of the inward and outward carrier translocations. As discussed above, the effects of trans substrates on efflux are consistent with a more rapid inward translocation of the unloaded vs. loaded carrier (Fig. 9 c > a) as efflux was maximally inhibited in the presence of trans Na and glycine together. For influx, on the other hand, the trans inhibition by Na was partially reversed when trans glycine was also present, which suggests that the outward translocation of the fully loaded carrier is equal to or greater than the outward translocation of the unloaded carrier (b = d). The absence in Table V (top) of full reversal

![Figure 9. A scheme for the loading of glycine, Na, and Cl to the outward-facing transporter incorporating experimental data for the substrate affinities and order of binding. T" is the inward-facing transporter and T' is the outward-facing transporter. Gly or G is glycine.](image-url)
or stimulation of influx to above control levels by the addition of Na and glycine to the ghosts, may have been due to our experimental conditions, i.e., if 100 μM trans glycine is not saturating for the inward-facing carrier. These characteristics describe an asymmetry in the inward and outward translocation rates of the unloaded and/or loaded carrier, which has implications for the recruitment of the unloaded carrier. The relative rates of translocation, \( c > a \) and \( b > d \), indicate that the ratio of the unloaded inward/unloaded outward translocation rate coefficients is greater than the ratio of the loaded inward/loaded outward translocation rate coefficients: \( (c/d) > (a/b) \). If the inward and outward translocation rate coefficients of the loaded carrier are similar, the unloaded carriers will favor recruitment to the inward-facing state.

When comparing transmembrane fluxes for whole red blood cells and red blood cell ghosts, the effects of cytoplasmic components must be considered. Carruthers and Melchior (1983) (Carruthers, 1986) have reported that, for glucose, the symmetry of transport is regulated by cytoplasmic factors. Such factors may or may not be removed by the ghosting procedure. In the present study, we observed a difference in the Na-dependent glycine influx for whole cells vs. ghosts, with influx rates being lower for the red blood cell ghosts. A similar effect of ghost preparation on Na-dependent glycine influx was observed for pigeon erythrocytes (Vidaver and Shepherd, 1968). Whether this decrease in transport activity results from the loss of an important cytoplasmic factor or from an effect of the ghosting procedure on the transporter directly is unknown. It should be noted, however, that the ghost procedure has no effect on the DNDS-sensitive uptake of glycine, which indicates that the membrane proteins in general are not adversely affected by this technique.

In summary, the Na- and Cl-dependent glycine transport in human red cells is an asymmetrical system with a preferred order of loading on the outside with either zwitterionic glycine or Cl first, followed by the other, then Na, then a second Na. 60% of the glycine influx is Na-dependent while 20% is by a stilbene-sensitive mechanism and 20% is both Na- and stilbene-insensitive.

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