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Transport of Glycine by Pigeon Red Cells*

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Glycine entry into pigeon red cells can be analyzed into two components, a sodium-independent one which is linear with glycine concentration, and a sodium-dependent one which fits Michaelis-Menten kinetics. A double reciprocal plot of the sodium-dependent component of glycine entry against $(Na^+)^2$ also gives a straight line, as though two sodium ions are cosubstrates with glycine at some step in the entry process. The K_m term (glycine concentration giving half-maximal entry rate) in the entry equation, rather than the V_{max} term (maximum entry rate), is governed by Na^+, implying a sodium effect on glycine binding by the transport mechanism rather than on its transfer across the membrane. The sodium dependence implies the existence of a complex containing two sodium ions and one glycine. The exit process also has a sodium dependent component. Glycine inside the cells does not appear to be bound. Glycine accumulation seems to be an energy-utilizing ("active") process arising from a sodium-accelerated glycine entry, not balanced by a corresponding glycine exit.

Many types of cells can accumulate amino acids (Christensen, 1962). The entry process could be considered as the result of two components, one obeying Michaelis-Menten kinetics and one resembling simple diffusion (Heinz, 1957; Akedo and Christensen, 1962; Helmreich and Kipnis, 1962). Amino acid influx was inhibited by metabolic inhibitors (Heinz, 1957). Amino acid loss from cells followed the kinetics of a passive process (Heinz and Walsh, 1958; Helmreich and Kipnis, 1962). Evidence has been presented that amino acid inside the cells is "free" (Christensen *et al.*, 1952c; Heinz, 1957).

Amino acid accumulation by Ehrlich ascites cells was inhibited when Na^+ in the medium was replaced by K^+ (Christensen *et al.*, 1952b) and it has recently been reported that Na^+ is required for glycine entry into Ehrlich ascites cells (Kromphardt *et al.*, 1963).

Studies so far made on amino acid transport have been hampered by the difficulty of modifying the composition of the cell interior. The reversibility of hemolysis of red cells suggested the use of lysed and restored red cells to circumvent this limitation, since during hemolysis materials exchange freely between the inside of the cells and the lysing solution (Hoffman, 1958).

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Pigeon red cells were chosen for the present study of active transport of glycine since avian red cells accumulate glycine much more strongly than mammalian red cells (Christensen *et al.*, 1952a; Lietz *et al.*, 1958).

Some experiments, however, can be done more easily with intact cells than with lysed and restored ones. After first establishing that hemolysis of pigeon red cells, like that of mammalian red cells, could be reversed, and that "hemolyzed and restored" pigeon red cells could pump glycine, the behavior of the intact red-cell system was investigated. It is described in this paper.

It was found that the overall glycine-transport process is sodium dependent. Sodium appears to govern the binding of glycine to the transport mechanism rather than the actual transit process. In the entry process two sodium ions behave as cosubstrates with the glycine. Internal glycine appears to be free and the inequality of Na^+ on the two sides of the cell membrane is probably responsible for the glycine accumulation. Entry from a high-sodium medium is more rapid than exit from the low-sodium cell interior.

Materials and Methods

Preparation of Cells.—Adult commercial pigeons were used. Blood was obtained by severing the spinal column in the neck and bleeding from the cervical vessels. The gullet, which was not cut, was held shut during bleeding. Blood from two or three birds was collected in a 40-ml centrifuge tube held in an ice bath. The tube contained *ca.* 15 ml cold saline and 20 mg (100 units/mg) heparin sodium. The blood was filtered through cheesecloth and centrifuged in the cold. The cells were washed once with cold saline and once with cold KRPG,[1] and then a weighed portion was suspended in KRPG and held in the cold until use. The saline and KRPG Na^+ and K^+ contents were chosen to minimize the unwanted ion in subsequent incubation media.

Incubation Procedure.—The appropriate volumes of red-cell suspension were placed into a series of weighed 12-ml heavy-walled Pyrex "Servall" centrifuge tubes and centrifuged 10 minutes in the cold at 9000 rpm in a Servall SSl head. The pellets, 0.3–0.4 g wet weight, were suspended with 2.00 ml of the appropriate incubation medium (usually a supplemented or substituted KRPG). Even-numbered tubes were incubated at 38.5–39.5°, usually for 15 minutes. They were stirred for the first 2 minutes of incubation. After incubation, they were chilled in an ice bath with stirring during the first 2 minutes of chilling. The half-time for temperature equilibration in these tubes (without stirring) is 25 seconds. Each odd-numbered tube (unincubated control) had the same contents as the succeeding even-numbered one, and was treated in the same way except that it was not incubated. If entry of ^{14}C-glycine was being measured, the samples were diluted with 6 ml of cold saline or KRPG just before centrifugation. This dilution reduced the ^{14}C in the extracellular space to tolerable levels and replaced a washing step. The samples were then centrifuged in the cold for 10 minutes at 9250 rpm in a Servall SSl head. The supernatants were poured off and the pellets were drained by inverting the tubes and blotting the mouths with tissue, placing the tubes mouth down on a rack at a shallow angle from the horizontal and, after a few minutes, again blotting the mouths with tissue. The unincubated control samples were processed while the others were being incubated. The tubes plus pellets were then weighed.

Analysis of Samples.—Cells and media were deproteinized with picric acid (Lietz *et al.*, 1958); base was added to the extracts (Christensen and Streicher, 1949), and glycine was chemically determined by the method of Alexander *et al.* (1945) as modified by Christensen *et al.* (1951). Sodium and potassium were determined by flame photometry of the picric acid extracts.

Radioactivity was determined by plating and counting. Two different plating procedures were used. In the simplest ("thin-sample" plating), used for most of the experiments, duplicate 0.10_0-ml aliquots of pellet extracts were spread over all but the outer annular space of 1 × $^5/_{16}$-inch ringed stainless steel planchets (Atomic Accessories SP-132) and allowed to air dry. The radioactivity on each planchet was counted for 1600 counts. In the second procedure ("thick-sample" plating), for pellet samples, 1.00 ml picric acid extract was mixed with 0.50_0 ml 2.50 M unlabeled glycine and the glycine was precipitated with *ca.* 6.4 ml acetone. The precipitate was air dried overnight, dissolved in 0.6 ml H_2O, reprecipitated with 5 ml acetone, washed with 5 ml acetone, again air dried, and then spread uniformly on weighed 1 × $^5/_{16}$-inch flat-bottomed stainless steel planchets with the aid of three *ca.* 0.5-ml portions of acetone. After air drying, the radioactivity on each planchet was counted for a total of 3200 counts and the planchets were again weighed. In the thick-sample procedure as applied to supernatant samples, a suitable aliquot of supernatant was mixed with 0.50_0 ml 2.50 M glycine plus 0.2 ml 10% picric acid in acetone plus H_2O to give a total volume of 1.7 ml. After at least 30 minutes at room temperature the precipitated protein was removed and the samples then processed as pellet extracts. The total cpm obtained from thick-sample plates were corrected to a standard sample weight from a standard curve of cpm vs. mg glycine/planchet. With the thick-sample plating method, ^{14}C radioactivity in both supernatant and pellets was counted with the same efficiency (within 2–5%). Reproducibility was ±2.4%. This was the greatest range within four groups of replicates of 3, 4, 4, and 4 members each. The thin-sample plating method gave fairly reproducible results for pellet extracts (standard error, *ca.* ±5%). Thin-sample plating of untreated supernatants gave unreliable results and use of picric acid extracts of supernatants involved inconveniently great dilutions of radioactivity. Therefore supernatant radioactivities were always determined from thick-sample plates.

Calculation of Entry and Exit Rates.—

Entry rate/ml pellet H_2O
= Δcpm/(sp act$_s$ × ml H_2O/pellet × incubation time)

Entry rate/ml cell H_2O
= (entry rate/ml pellet H_2O)/0.9

[1] Abbreviations used in this work: KRPG is Krebs Ringer Phosphate Glucose solution, slightly modified from that given in Umbreit *et al.* (1949). The composition is: $Na^+ + K^+$ = 146 mM, Ca^{2+} = 2.5 mM, Mg^{2+} = 1.2 mM, Cl^- = 136 mM, SO_4^{2-} = 1.2 mM, HPO_4^{2-} = 6 mM, $H_2PO_4^-$ = 3 mM, Glucose = 150 mg/100 ml. These media are stable at 5–7° for *ca.* 12 hours. The subscript "*o*" or "*i*" after a symbol for or name of a substance means the substance represented is present in the medium or cell, respectively. Tetrakis is tetrakis(β-hydroxyethyl)ammonium chloride, $(HOCH_2CH_2)_4N^+Cl^-$ (Wurtz, 1862). K_m and V_{max} are, respectively, the glycine concentration giving half-maximal entry rate and the maximum entry rate, both as obtained from a Lineweaver-Burk plot. Although these are terms of enzyme kinetics, their use is meant to imply only an analogy in kinetic behavior, not necessarily a detailed analogy of mechanism. "Saline" is 154 mM NaCl or KCl, or a mixture of the two.

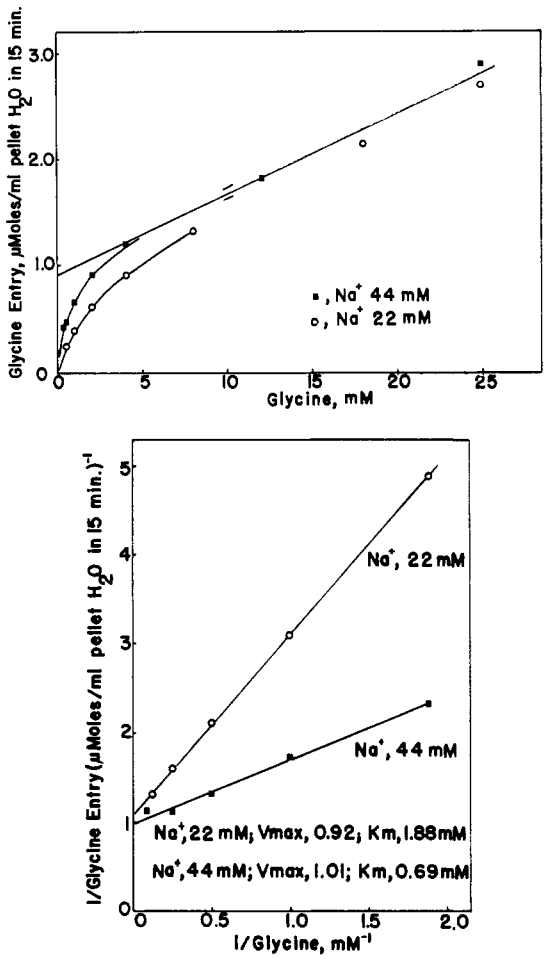

FIG. 1.—Cells were prepared, incubated, and processed as described under Methods. Thin-sample plates were used. In (a) glycine entry from media of two different Na^+ concentrations is plotted against the glycine concentration in the medium. The limiting slopes of the individual sets of points are indicated by the short lines flanking the line of the average limiting slope for the two sets. All three are drawn to have intercepts at V_{max}. In (b) the glycine-entry values are "corrected" by subtracting the linear component (the average slope was used) and plotted against glycine concentration in double-reciprocal plots.

where

Δcpm/pellet = (cpm in whole incubated pellet) − (cpm in whole unincubated pellet)

sp act_s = average of the glycine specific activities in the media from incubated and unincubated samples

ml H_2O/pellet = ml H_2O in the unincubated control pellet. This was taken to be 0.675 times the wet weight of the pellet. This, rather than the actual H_2O content, was used in order to eliminate the effect of changes in H_2O/cell which may occur during incubation in some media.

The factor 0.9 arises from an extracellular H_2O value for pellets of intact cells of *ca.* 10%. Exit rates were calculated in a similar fashion, e.g.,

Exit rate/ml cell H_2O

$= \Delta$ cpm $_{e\ \text{total medium}}$/(sp act $_{\text{cell}}$ × ml H_2O/pellet × 0.9 × incubation time)

The Na^+ and glycine concentrations, pellet volumes, and incubation times were chosen to minimize changes during incubation of the glycine concentration and specific activity from which movement occurred. The largest such change in any point in any experiment reported here is less than 16%. Most were much less. The changes that did occur were partially compensated

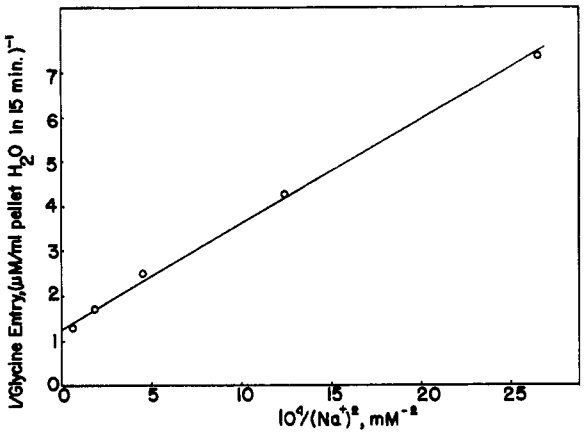

FIG. 2.—This shows a double-reciprocal plot of glycine entry, from which the linear component has been subtracted, against $10^4/(Na^+)^2$. Glycine in the medium was 0.53 mM. Cells were prepared, incubated, and processed as described under Methods. Thin-sample plates were used. Values for sodium in the media were obtained by determining Na^+ in media from incubated and unincubated samples which had been suspended in originally Na^+-free medium, and then adding the average of the amounts found to the amounts added to the Na^+-containing media in their preparation. All media were also directly analyzed for Na^+, but the analytical scatter was larger than the scatter in the values obtained as described above, so the latter were used.

for by using average values of these quantities for the calculations. Since only a small part of the ^{14}C-glycine, which was taken up in 15 minutes, can escape during this period, the error caused by this loss of ^{14}C-glycine is presumably small.

Note that these entry and exit rates are proportional to rates per unit dry weight and therefore, within a given experiment, proportional to rate per unit of cell surface area.

Extracellular space of such pellets was determined from time to time. In some cases, this was done by comparison of the Na^+/ml pellet H_2O in pellets from a pair of duplicate samples where one member of the pair had been diluted with a Na^+-free solution and the other with a high Na^+ one just before centrifugation. In others, ^{14}C-glycine was used instead of Na^+. The value for fresh intact cells is around 10% of the total pellet H_2O.

RESULTS

In Table I are shown the effects on glycine entry of various substitutions for NaCl or KCl in the medium. Only Na^+ is active. Choline$^+$, Li^+, K^+, and tetrakis neither antagonize nor substitute for Na^+.

The curve obtained by plotting glycine entry against the glycine concentration of the medium can be considered as the result of two component curves, one linear with concentration (Figs. 1a, 4a) and one obeying Michaelis-Menten kinetics (Fig. 1b). Comparison of the linear component of glycine entry with the entry from Na^+-free medium (both measurements made within the same experiment) showed the two to be substantially the same. Thus in one experiment the linear entry component (Na_o^+ = 114 mM) was 0.11_8 μmole per ml pellet H_2O per 15 minutes per mM $glycine_o$, and the entry rate from Na^+-free medium was 0.11_4. In another experiment the values were (for Na_o^+ = 130 mM) 0.12_4 and (for Na_o^+ = 0) 0.11_7. In other experiments the linear components at different (but not zero) Na_o^+ values were quite similar. The Na^+-

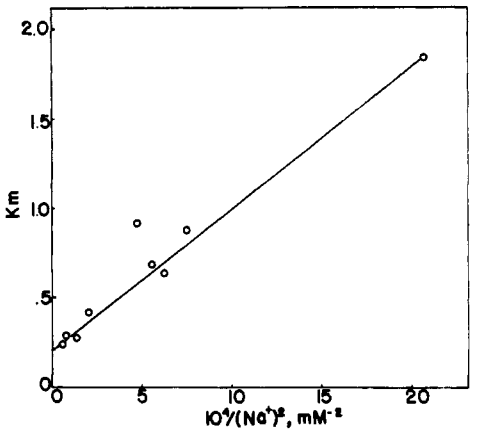

FIG. 3.—Values for K_m were collected from experiments done over a period of many months and are plotted against $10^4/(Na^+)^2$. The values used for Na_o^+ are simply the input values.

TABLE I

CATION SPECIFICITY OF THE GLYCINE ENTRY PROCESS[a]

Expt.	KCl_o (mM)	$NaCl_o$ (mM)	Other Additions (mM)	Glycine Influx (μmole/ml pellet H_2O in 15 min)
1	51	95	None	0.59
	20	95	$Sucrose_o$, 62	0.57
	6	95	$Sucrose_o$, 90	0.52
	106	40	None	0.37
	20	40	$Tetrakis\ chloride_o$, 86	0.39
	6	40	$Tetrakis\ chloride_o$, 100	0.40
2	146	0	None	0.050
	116	0	$Choline\ chloride_o$, 30	0.040
	116	0	$LiCl_o$, 30	0.042
	116	30	None	0.22
3	146	0	None	0.041
	106	40	None	0.23
	12	40	$LiCl_o$, 94	0.26
	12	40	$Choline\ chloride_o$, 94	0.25

[a] Cells were prepared, incubated, and processed as described under Methods. Thin-sample plates were used. The incubation media were KRPG solutions containing 0.52–0.58 mM ^{14}C-glycine with other additions and substitutions as indicated in the table. The last column lists the glycine entry during incubation. In experiment 1 the slope of the linear component of glycine entry (see Results, on Figure 1) was determined rather than the entry from Na^+-free medium. In experiment 1 this value is 0.053.

free (or linear component) entry-rate values varied from experiment to experiment in the range 0.06–0.12 μmole per ml pellet H_2O per 15 minutes per mM $glycine_o$.

From Figure 1b it can be seen that the glycine entry dependence on Na^+ is due primarily to an effect of Na^+ on the K_m, not the V_{max}. In this figure, the glycine entry plotted is the total entry minus that due to the linear component.

Figure 2 is a double-reciprocal plot of glycine entry, corrected for the linear component, against $(Na^+)^2$. The linear relation would be expected if two sodium ions were "cosubstrates" of glycine at some step in the entry process. Figure 3 shows K_m values collected from a number of experiments and plotted against $10^4/(Na^+)^2$. Values for V_{max} in these same experiments ranged from 0.42 μmole/ml pellet H_2O in 15 minutes to 1.20 μmoles. The average was 0.83, with a standard deviation of ± 0.25. The K_m value extrapolates to *ca.* 0.20 mM at infinite Na_o^+.

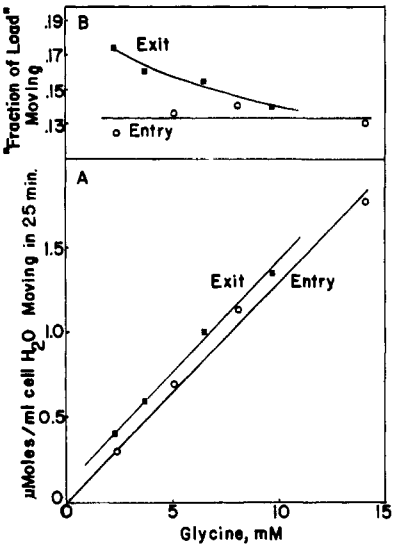

FIG. 4A, B.—Glycine exit and entry are plotted against the (average) glycine concentration from which movement occurs. Cells were prepared as described under Methods. They were then divided into aliquots and incubated 128 minutes at 38.5° in K-KRPG (KRPG with all Na^+ replaced by K^+) containing different amounts of glycine, with or without ^{14}C-glycine, depending on whether efflux or influx was subsequently to be measured. The cells were washed, and then incubated (for 25 minutes instead of the usual 15 minutes) and processed as described under Methods. Thick-sample plating was used. The (unlabeled) glycine in the cells into which entry was measured was 3.4 mM; the (unlabeled) glycine in the media from aliquots used for measurement of exit was 5.2 mM. All media were Na^+ free. Values for "glycine concentration" on the graphs are averages of incubated and unincubated samples as determined by analysis, with correction for extracellular space. In (B), entry and exit are plotted as "fraction of load" moving. This is: cpm leaving or entering cells during incubation ÷ the average cpm in the cells or in an equivalent volume of medium.

Entry of glycine can be described by a Michaelis-Menten equation. As K_m, but not V_{max}, is sensitive to Na^+ (Fig. 1b), the glycine entry-rate dependence on $1/(Na^+)^2$ (Fig. 2) must be due to a dependence of K_m on $1/(Na^+)^2$. Since the plot of K_m vs. $10^4/(Na^+)^2$ (Fig. 3) does not go through the origin, a sodium-independent term, as well as a $(Na^+)^2$-dependent term, must be included in the expression for K_m. The sodium-dependent glycine entry rate can therefore be represented by an equation of the form: $V_{max}/V = \{[K_1/(Na^+)^2] + K_2\}(1/G) + 1$, where (G) = glycine concentration. In the same experiment in which the data of Figure 2 were obtained, determinations of V_{max} and K_m at Na_o^+ = 130 mM were also made. These were 1.2 μmoles/ml pellet H_2O in 15 minutes and 0.24 mM, respectively. The intercept of Figure 2 is 1.2_7. From the equation, the intercept in Figure 2 should be: $1/V_{max} + 1/[V_{max} \times (1/K_2) \times glycine_o]$; taking $K_2 = K_m$ (at Na_o^+ = 130 mM), the calculated intercept is $1/1.2 + 1/(1.2 \times 4.17 \times 0.53)$ or 1.2_1. Thus the double-reciprocal plots of glycine entry vs. $glycine_o$ and glycine entry vs. $(Na_o^+)^2$ are both consistent with the equation and with each other.

Freshly prepared pigeon cells contain little Na^+. Determinations on four different preparations gave values of $13._3$–$22._1$ (avg $17._5$) μmoles/ml cell H_2O. K^+ was 122–136; avg 130 μmoles/ml cell H_2O (4 determinations). Pilot experiments indicated that Na_i^+ was compartmentalized so that the Na^+ content per ml H_2O cannot be taken as the effective concen-

tration inside the cell and a change in Na^+ content can only be taken as a qualitative indication of the effective Na^+ change. However glycine exit is also partially Na^+ dependent. The data of Table II show that the fraction of ^{14}C-glycine leaving Na^+-"loaded" cells is greater than that leaving Na^+-"depleted" ones, and also that the percentage of loss from Na^+-loaded cells is greater at low than at high internal glycine concentration. The latter is a characteristic of a saturatable exit route.

As shown in Figure 4, in the absence of Na^+ in the medium the glycine entry rate is small and approximately equal to the exit rate at equal glycine concentrations (see discussion). That is, in Na^+-free medium the cells will not accumulate glycine.

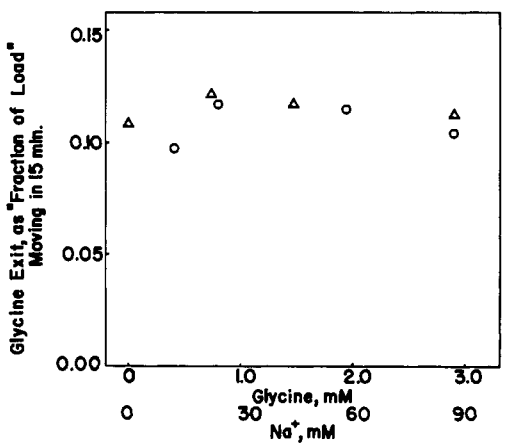

FIG. 5.—Glycine exit is plotted against the glycine and Na^+ concentration in the medium (i.e., vs. concentration *to* which movement occurs). Cells were prepared as described under Methods. They were then incubated with 6.3 mM ^{14}C-glycine for 107 minutes at 38.5° in KRPG in which all Na^+ was replaced by K^+. They were washed and then incubated and processed as described under Methods. Thick-sample plating was used. Where glycine was varied Na_o^+ was 44 mM, and where Na_o^+ was varied $glycine_o$ was 0.8 mM. Glycine exit with $glycine_o$ varied is shown by circles; exit with Na_o^+ varied is shown by triangles. The data are given as "fraction of load" moving. This is: cpm leaving cells ÷ average cpm in cells.

As Figure 5 shows, glycine exit is not changed significantly by changes in the medium glycine or Na^+ singly or in combination. Since the entry rate is strongly Na_o^+ dependent, Na^+ in the medium will shift the system from a "nonpumping" state to one of glycine accumulation, this shift being due to the increase in entry rate rather than a decrease in exit rate.

A few other observations may be mentioned. Glycine entry was inhibited about 50% by lowering the *p*H to 6.5 or by the presence of 10 mM β-phenylethylamine hydrochloride. In single trials the following changes in the medium had no significant effect on glycine entry rate: omission of the Ca^{2+} and Mg^{2+}, substitution of Tris or bicarbonate buffer for the phosphate buffer, addition of 90 mM sucrose (making the medium hypertonic), or addition of 10 μg/ml K-strophanthin. In addition, omission of Ca^{2+} and Mg^{2+} had no effect on K_m. Comparison of K_m values from Ca^{2+}- and Mg^{2+}-free media of various Na^+ concentrations with the corresponding K_m values calculated from Figure 3 (KRPG medium) gave the following: observed K_m values (four experiments): 0.64, 0.28, 0.26, 0.42; calcd: 0.71, 0.27, 0.24, 0.36 mM. The V_{max} values also fell well within the range found with KRPG.

TABLE II

THE PARTIAL SODIUM DEPENDENCE OF GLYCINE EXIT[a]

$Glycine_i$ (μmoles/ml pellet H_2O)	Na_i^+ (μmoles/ml pellet H_2O)	Glycine Exit (as % of average ^{14}C load leaving cells in 15 min)	Na_i^+ in Cells
1.1_1	28	$11._3$	Depleted
1.2_3	34	$15._2$	Loaded
1.6_6	34	$13._4$	Loaded
2.3_3	34	$14._2$	Loaded
5.5_8	35	$11._8$	Loaded

[a] Glycine exit is given as "% of average ^{14}C load." This is: 100 × cpm leaving cells ÷ average of the cpm in cells before and after incubation. The cell Na^+ and glycine were adjusted by dialyzing 51 ml of a 25% (w/v) suspension of washed cells in phosphate-buffered isotonic NaCl (total PP_i = 6.6 mM, *p*H 7.8) against 4 liters of the same solution containing (as did all subsequent solutions and media) 0.5 μg/ml K-strophanthin at 5–7° for 22 hours. The dialysis tubing was strung on a slowly rotating frame for mixing during dialysis. Sodium dihydrogen phosphate was added to the outer solution to bring it to *p*H 7.2 and 9 mM in P_i (as were all subsequent solutions), and dialysis continued for 2 more hours. The suspension was diluted (to 20%, w/v) with fresh phosphate-buffered NaCl containing 0.022 mM ^{14}C-glycine and incubated for 30 minutes at 39°. The suspension was then diluted with phosphate-buffered KCl to bring Na_o^+ to 50 mM and incubated a further hour. At this point the cells were depleted in glycine, contained ^{14}C-glycine, and were fairly rich in Na^+. Aliquots were centrifuged, and suspended and incubated for 1 hour in buffered isotonic saline media containing 0 or 50 mM Na^+ and various concentrations of unlabeled glycine. This made the final adjustment of Na_i^+ and $glycine_i$. The cells were washed and then incubated and processed as described under Methods. The incubation medium was KRPG with Na^+ partially replaced by K^+ to give 50 mM Na_o^+ and containing 0.5 mM unlabeled glycine. Thick-sample plates were used.

DISCUSSION

The two-component entry curve (Fig. 1a) is most simply explained by supposing that glycine crosses the cell surface both by pure passive diffusion and also by a Na^+-dependent route. However, it is possible that the Na^+-independent entry is due to a second facilitated entry route with a K_m well above the highest glycine concentration used (in Fig. 4 this is 14.1 mM).

As mentioned, the Na^+-dependent entry can be described by an equation: $V_{max}/V = \{[K_1/(Na^+)^2] + K_2\}(1/G) + 1$. This expression must be considered empirical only. The lack of a first power term in Na^+ implies a reaction: $E + 2\,Na^+ \rightleftarrows ENa_2^+$, where E stands for substance or binding site assumed to combine with the Na^+ and glycine. If a "carrier" exists in this system it might be identical with E. A trimolecular collision of E with two sodium ions is very unlikely. Therefore the empirical equation should at least be modified to contain a first-power term in Na^+, with this first-power term being too small to be seen in the plot of Figure 2. If the reactions are assumed to be, in order,

$$(1)\quad E + Na^+ \rightleftarrows ENa^+ \qquad K_1 = \frac{(E)(Na^+)}{(ENa^+)}$$

$$(2)\quad ENa^+ + Na^+ \rightleftarrows ENa_2^+ \qquad K_2 = \frac{(ENa^+)(Na^+)}{(ENa_2^+)}$$

$$(3)\quad ENa_2^+ + G \rightleftarrows ENa_2{}^+G \qquad K_G = \frac{(ENa_2^+)(G)}{(ENa_2G)}$$

(4) $(ENa_2G)_o \xrightarrow[k]{} (ENa_2G)_i$

the equation describing glycine entry is:

$$\frac{V_{max}}{V} = \left(\frac{K_1K_2K_G}{(Na^+)^2} + \frac{K_2K_G}{(Na^+)} + K_G\right)\frac{1}{G} + 1$$

If the second reaction were $ENa^+ + G \rightleftarrows ENaG$ and the third $ENa^+ G + Na^+ \rightleftarrows ENa_2G$, the equation would be:

$$\frac{V_{max}}{V} = \left(\frac{K_1K_2K_G}{(Na^+)^2} + K_2K_G\right)\frac{1}{G} + \frac{K_2}{Na^+} + 1$$

and V_{max}, as determined, should vary with Na^+. While this does not appear to be so, if K_2 were small the variation would escape detection and the two equations would be indistinguishable. There may be a suggestion of curvature in Figure 2 and a possible difference in the V_{max} values in Figure 1b, though neither is large enough to be called significant. More and more accurate data would be required to establish a first-power term in Na^+ or to lower the upper limit of its value. Since small systematic errors could cause such marginal effects, the experiments have not been attempted.

A number of simplifying assumptions were made to obtain the above equations; among them, that there is a fixed total of E in all its forms at the outside of the cell (as opposed to, e.g., supposing a carrier shuttling between inside and outside), that there is a single strict order of combination of Na^+ and glycine with E, that no third "cosubstrate," e.g., Cl^-, enters the complex, and that glycine enters the cell only from or in the form of ENa_2G (e.g. not as or from ENaG). Any or all of these may be dropped. The resulting equations, which gain terms as each assumption is dropped, still give linear double-reciprocal plots of glycine entry vs. glycine concentration. If suitable values are chosen for the appropriate K values, the equations also can be fitted to the data of Figures 1–3. Since so many equations can be fitted to the data, few conclusions can be drawn regarding mechanism. The chief conclusions from Figures 1–3 are simply that there is a major rate-limiting complex containing two sodium ions and one glycine, and that Na^+ probably governs the binding of glycine to the transport mechanism rather than the transfer of glycine across the membrane.

There is the question of whether glycine accumulation is due to an active (energy-requiring) process or merely binding by sites on or within the cell. The data of Figure 4 are taken to indicate that internal glycine is not bound, since if it were the exit rate should be less than the entry rate at the same apparent concentration. It is possible that the true Na^+-independent exit is less than entry but hidden by an outpumping of glycine. The method of plotting used in Figure 4A obscures the fact that exit is somewhat greater than entry at low internal glycine concentrations. This fact is revealed in the plot of the same data as "fraction of load" in Figure 4B (fraction of load = cpm leaving or entering cells during incubation ÷ average cpm in the cells or in an equivalent volume of medium). With rising glycine concentration, the exit and entry curves in Figure 4B approach each other. These properties are similar to those observed by Helmreich and Kipnis (1962) for α-aminoisobutyric acid in lymph node cells. The difference seen in Figure 4B between entry from Na^+-free medium and exit from cells is most simply accounted for as a result of a small remnant of Na_i^+. The following calculation indicates that only a small amount might be needed. The difference between exit and entry at 2 mM $glycine_o$ is here *ca.* 0.1 μmole per ml cell H_2O per 25 minutes, or *ca.* 0.067 μmole per ml pellet H_2O per 15 minutes. If the same equation describes Na^+-dependent exit and entry and the average V_{max} for entry, 0.83 μmole per ml pellet H_2O per 15 minutes, is used for the exit V_{max}, the calculated value for K_m would be 22.8 mM and the corresponding Na^+ concentration 6 mM. It may also be noted that glycine accumulation arises from a Na_o^+-augmented glycine uptake which is difficult to explain by binding of glycine by the cell.

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