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THE BASIC ASYMMETRY OF Na^+-DEPENDENT GLYCINE TRANSPORT IN EHRLICH CELLS

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Summary

Influx and efflux of glycine have been examined as a function of external and internal Na^+ concentrations, respectively, when $\Delta\tilde{\mu}_{Na} = 0$. With $\Delta\tilde{\mu}_{Na} = 0$ it was found that at comparable external and cellular Na^+ levels, the K_m for efflux was larger by an order of magnitude than the value for influx and the V for efflux was several times greater than the V for influx. For both fluxes the major effect of Na^+ was to decrease the K_m value. The observations are consistent with the conclusion that the Na^+-dependent transport system is asymmetric per se. Influx and efflux of glycine were increased in a near linear manner by increasing the Na^+ concentration from 13 to 100 mM, the half-time for glycine equilibration being a function of the Na^+ concentration in absence of an electrochemical potential difference for Na^+. In Na^+-free media ($[Na^+] <$ 5 mM) equilibration of glycine between cells and medium was not achieved after 60 min at 25°C. With $\Delta\tilde{\mu}_{Na} = 0$, efflux (or uptake) of glycine was not affected by internal (or external) K^+ between 20 and 120 mM suggesting that K^+ plays no direct role in Na^+-dependent transport of glycine in Ehrlich cells.

Introduction

The mechanism by which Na^+ enhances the transport of organic solutes in mammalian systems has been intensively studied since the report by Crane [1] over 15 years ago. Earlier observations [2—4] had suggested a possible role for Na^+ in transport of organic solutes. However, the experiments of Crane and his colleagues clearly defined that role and showed that the kinetic parameters for solute transport were altered by Na^+. The first reports [1] suggested that only the K_m for translocation was affected. However, it became apparent as more

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid.

experimental systems were examined, that either the K_m or the V (or both) for organic solute were altered by the presence of Na^+ [5,6]. In the Ehrlich cell Na^+ affects the K_m value to a greater extent than the V value with a number of amino acids [7—9].

Studies of the effects of Na^+ on organic solute transport have usually been executed under conditions where an electrochemical potential difference for Na^+ has existed across the plasma membrane. It now appears clear that in many animal systems, the driving force for the accumulation of organic solutes, particularly sugars and amino acids across the plasma membranes, is the electrochemical gradient for Na^+ [5,6,10—16]. Generally, it is not feasible to change the external Na^+ concentration without altering the Na^+ driving force. The question therefore arises whether the original reports on the K_m and V alterations described the effects of the Na^+ per se, or mixed effects of the driving force and Na^+ concentration on these parameters.

Furthermore, although most models for amino acid transport assume that the basic system is symmetrical (for example see ref. 6) and the asymmetric behaviour of the system is imposed by the cation distributions, there is in fact little direct evidence for symmetry (or lack of symmetry) of the system. Where the system has been tested specifically, in absence of ion gradients, it does not appear to behave symmetrically [17]. Even in equilibrating transport systems, such as the glucose transport system in red blood cells [18] or thymocytes [19], the kinetic constants for the system are different at the internal and external membrane surfaces.

To study the effect of Na^+ per se on the coupled transport of organic solutes, it is necessary to have a system where the Na^+ concentration can be varied in absence of a Na^+ electrochemical gradient (where $\triangle\tilde{\mu}_{Na} = 0$). Under such conditions the action of Na^+ per se can be examined on the kinetic parameters for organic solute influx and efflux and the basic asymmetry (or symmetry) of the system can be assessed. This is the subject of the present communication.

It is known that gramicidin [20] rapidly abolishes monovalent cation electrochemical potential gradients across synthetic and natural membranes. A previous report [21] showed that with Ehrlich cells, gramicidin caused a rapid equilibration of Na^+ (and depolarization) across the plasma membrane. This agent can therefore be used to examine kinetic effects of Na^+, as opposed to the thermodynamic effects of a Na^+ gradient, on solute fluxes in either direction. In a previous communication it was shown that with gramicidin, a rapid Na^+-dependent efflux of amino acids occurred in Ehrlich cells [21]. The apparent first-order rate constant (k_e) for glycine efflux in a sodium medium (0.04 min^{-1} at 37°C) was enhanced by more than an order of magnitude in presence of gramicidin. A similar increase in efflux may account for the observations by Terry and Vidaver [23] on the reduction of amino acid uptake in avian red cells. The sodium-dependent amino acid efflux in Ehrlich cells behaved as if it were catalyzed by the Na^+-dependent amino acid transport system (System A of Christensen) operating in the reverse direction, that is, from in to out, although no movement of the amino acid against its electrochemical potential was observed [21]. In this report the kinetic constants for influx and efflux are compared when $\triangle\tilde{\mu}_{Na} = 0$.

The question of the direct action of K^+ on amino acid efflux has not been

assessed. Evidence is presented in this communication that cellular K^+ has little effect on the Na^+-dependent amino acid efflux in gramicidin-treated cells.

Materials and Methods

Ehrlich cells were maintained in Swiss white mice and prepared for experiments as described in an earlier communication [21]. [1-^{14}C]Glycine was purchased from New England Nuclear Corp., Boston, Mass. Gramicidin D was obtained from Sigma Biochemicals Corp., St. Louis, Mo., and kept in 95% ethanol at a concentration of 0.7 mg/ml. Other reagents were obtained from local suppliers, primarily Fisher Scientific Co.

The basic medium used for incubation contained 150 mM NaCl, 12 mM K^+, 1.5 mM $MgCl_2$ and 2.0 mM $NaKHPO_4$. The buffer was 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (Hepes) at pH 7.4. Replacements for Na^+ or K^+ are indicated in the text. Samples for [1-^{14}C]glycine uptake or efflux were diluted with 10 times their volume of cold isotonic choline chloride in tared centrifuge tubes and centrifuged rapidly. The weighed cell pellet was extracted with 95% ethanol and the samples were counted in a Packard Liquid Scintillation Counter. The procedure for measuring uptake (or efflux) has been described in detail elsewhere [21].

Samples for cation determinations were washed with 10-fold excess of cold isotonic choline chloride. Ions were measured by flame photometry using an internal Li^+ standard.

Fluorescence measurements were made using a fluorescent dye (3',3'-dipropylthiadicarbocyanine iodide, referred to as the dye) which was obtained from Dr. A. Waggoner of Amherst College. The procedure and concentration of the dye used were identical to those described by Laris et al. [22].

All incubations were carried out at 25° C in air. Cells were usually prepared at room temperature and incubated at 25° for 10 min before initiating measurement of glycine uptake. In experiments with gramicidin, the latter was present for at least 2 min with the cells (to dissipate ion gradients, etc.) before initiating uptake or efflux measurements.

The cell water/medium water ratio for uptake measurements was approx. 1 to 100 (20 mg fresh weight of tissue per ml of cell suspension). For efflux studies it was 1/250—1/300 (approx. 8 mg fresh weight per ml of cell suspension). In our hands, 20 mg fresh weight of tissue contained 11 μl cell water and 3 mg dry weight.

Results

Action of Na^+ on influx

The first question to which we addressed ourselves was whether the kinetic constants for glycine influx and efflux were the same when $\Delta\tilde{\mu}_{Na}$ was near zero. The latter was achieved by incubating cells with gramicidin before initiating flux measurements. All experiments were done at 25° C because the rate of glycine loss at 37° C with gramicidin at high Na^+ (>50 mM) is too rapid for precise measurements.

The results in Fig. 1 show that a fixed external glycine concentration

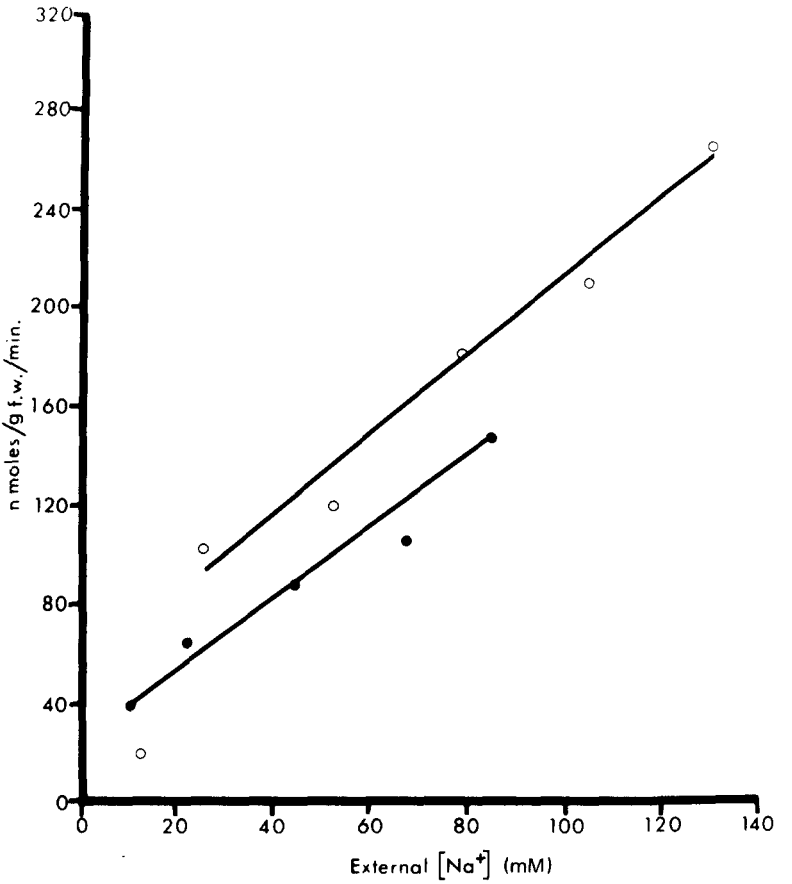

Fig. 1. Effect of extracellular Na^+ on the initial rate of [1-^{14}C]glycine uptake when $\Delta\tilde{\mu}_{Na} = 0$. Cells were incubated in media where Na^+ was varied. LiCl was used as a Na^+ substitute. 10 mM K^+/Hepes at pH 7.4 was used as buffer. After 5—10 min equilibration at 25°C, gramicidin was added to a concentration of 7 μg/ml and incubation continued for a further 2 min. [1-^{14}C]Glycine was added to a final concentration of 1 mM (specific activity 500 cpm/nmol). The given rates are based on values of uptake after 30 s incubation. The uptake of [^{14}C]glycine in choline chloride has been subtracted from all the values given. Results from two representative experiments are shown.

(1 mM), glycine influx increased as the Na^+ concentration increased. It appears that the relationship between Na^+ concentration and influx of glycine (at 1 mM) is linear or nearly linear between 13 and 130 mM Na^+. The relationship between Na^+ concentration and glycine influx may be subject to some (perhaps appreciable) error at low Na^+ concentrations, making it difficult to detect deviations from linearity at low Na^+. The reasons for this difficulty are: (1) The uptake at 1 min in a choline medium represents about 50% of the uptake after 1 min incubation at the lowest Na^+ concentration (10 mM Na^+, 140 mM Li^+). Therefore the absolute value of the Na^+-dependent amino acid uptake at low Na^+ levels is subject to large errors. All the data in Fig. 1 were corrected for uptake in a medium where choline chloride replaced NaCl. (2) Li^+ was used as a substitute for Na^+. This substitution may also introduce complications since Li^+ may, to some extent, duplicate the effect of Na^+. The latter, however, is prob-

ably minimal. The data in Table I compare [1-^{14}C]glycine uptake and loss in normal (Na^+-containing) Ringer, Li^+-substituted solutions and choline-substituted solutions in presence of gramicidin. The data for uptake in Table I have been corrected for the residual uptake found at 0°C at zero time in an all choline medium. This level is not reduced by the presence of 10 mM methionine. It is greater however than the contamination expected from adherence of the diluted medium (nearly double). A similar problem of a "residual" uptake was noted earlier [24]. It is evident that uptake and loss in Li^+ are small compared to those in Na^+ but they are nonetheless greater than those in choline chloride. It should be noted in these experiments that there was no apparent change in fresh weight when cells were incubated in NaCl, LiCl or choline chloride indicating that the cells do not have variable volumes with these NaCl replacements. That glycine loss is enhanced by Li^+ compared to choline was shown earlier [21]. Eddy and Hogg [25] also reported that amino acid uptake is greater in Li^+ than choline. Since the objective here was to estimate glycine fluxes at variable Na^+ in absence of a potential difference across the membrane, it was necessary to use a monovalent cation replacement for Na^+ whose permeability would be increased by gramicidin. The choice of Li^+ over K^+ as replacement was made to permit us to study the effects of K^+ itself on transport of amino acids. The weak Na^+-like action shown by Li^+ is unlikely to distort the relative effects of Na^+ on uptake and efflux.

It is known that external Na^+ is required to obtain accumulation of organic solutes against their concentration gradients in many mammalian systems (for reviews see refs. 1, 5, 6 and 26). The data presented in Fig. 2 show that the rate of equilibration of glycine (i.e. the time required to attain a cellular [^{14}C]-glycine concentration equivalent to that of the medium) is a function of the Na^+ concentration in absence of an electrochemical difference for Na^+. It is

TABLE I

COMPARATIVE DATA ON GLYCINE INFLUX AND EFFLUX IN Na^+, Li^+ AND CHOLINE MEDIA IN PRESENCE OF GRAMICIDIN

For measurements of glycine uptake or loss, cells were incubated at 25°C in a medium composed of 150 mM NaCl (or Li^+ or choline chloride), 1.5 mM $MgCl_2$, 1.5 mM phosphate (K^+ salt) and 10 mM K^+/Hepes buffer, pH 7.4). The final K^+ concentration was 12 mM. Influx measurements: 2 min before addition of [1-^{14}C]glycine (final concentration 1 mM, specific activity 860 cpm/nmol), gramicidin to a final concentration of 7 μg/ml was added as a solution in 95% ethanol. The concentration of ethanol did not exceed 1%. Efflux: the cells were preincubated with [^{14}C]glycine at 37°C for 30 min in a normal Ringer medium. The initial cellular [^{14}C]glycine concentration was 0.6 mM. The cells were centrifuged, washed once with cold isotonic choline chloride, suspended in isotonic choline chloride and injected into an amino acid-free medium containing 7 μg/ml gramicidin. Samples were washed with ice-cold, isotonic choline chloride, 10-fold excess (for uptake) or 5.5 excess (for efflux). Influx measurements are based on uptakes after 15 s incubation. Values for uptake have been corrected for uptake at 0°C at 0 time in a all choline medium. The latter is twice that expected of the medium contamination. It is not reduced by the presence of excess methionine. A representative experiment is shown. Results are expressed in nmol/min per g (fresh weight).

Major cation of the incubation medium	Influx	Efflux
Na^+	315.0	235.0
Li^+	75.0	27.0
Choline	32.0	13.5

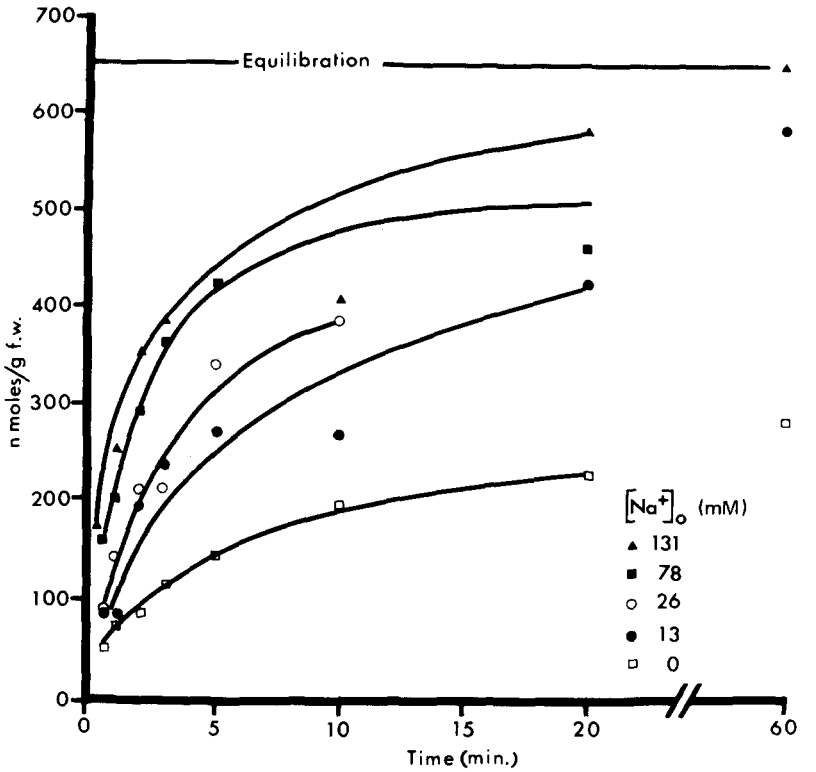

Fig. 2. Effect of external Na^+ concentration on equilibration time for glycine. Conditions were identical to those described in Fig. 1 except that the incubation was continued for 60 min. The data in this figure can also be expressed as the effect of Na^+ on the $t_{1/2}$ for equilibration. These values (in min) are 1.5; 2.5; 5.5; 10.5; and >60 min for 131 mM Na^+; 78 mM Na^+; 13 mM Na^+ and 0 Na^+, respectively. Assuming that the approach to equilibration is a first-order process, the apparent rate constant (per min) for these values would be 0.46 and 0.07 at 131 and 13 mM Na^+, respectively.

clear from this data, that equilibration does not occur within 60 min of incubation in the absence of external Na^+. It is also apparent that without a Na^+-driving force, even at high Na^+ (>100 mM Na_o^+), equilibration requires more than 20 min at 25°C. It is unlikely that the reduced rates of glycine uptake are due to an inhibitory effect of Li^+ rather than a reduction of Na^+, since the results show (Table I and Fig. 2) that uptake in choline chloride is even less than in LiCl. Additional experiments (not shown here) with other cation replacements including sucrose show rates of uptake comparable to those with choline chloride. It was reported earlier that in ATP-depleted cells, and in Na^+-free medium, the cellular [^{14}C]glycine (or [^{14}C]methionine) concentration was less than that of the medium after 60 min incubation [27]. These data show that, in addition to any effect of the Na^+ gradient on amino acid uptake in the Ehrlich cells, Na^+ has an effect on the apparent permeability of the membrane to glycine and that despite the presence of a transmembrane concentration difference for the amino acid, the rate of penetration is very slow in absence of Na^+.

To determine whether Na^+ (in absence of a Na^+ gradient) influenced the K_m

or V, the uptake of glycine at difference glycine and Na^+ concentrations was plotted according to the procedure of Lineweaver and Burk [28]. The data from a typical experiment are shown in Fig. 3. The major effect of Na^+ is on K_m which is decreased 3-fold when Na^+ is raised from 24 to 115 mM. It cannot be concluded that V is not altered but the effect on the V is small compared to

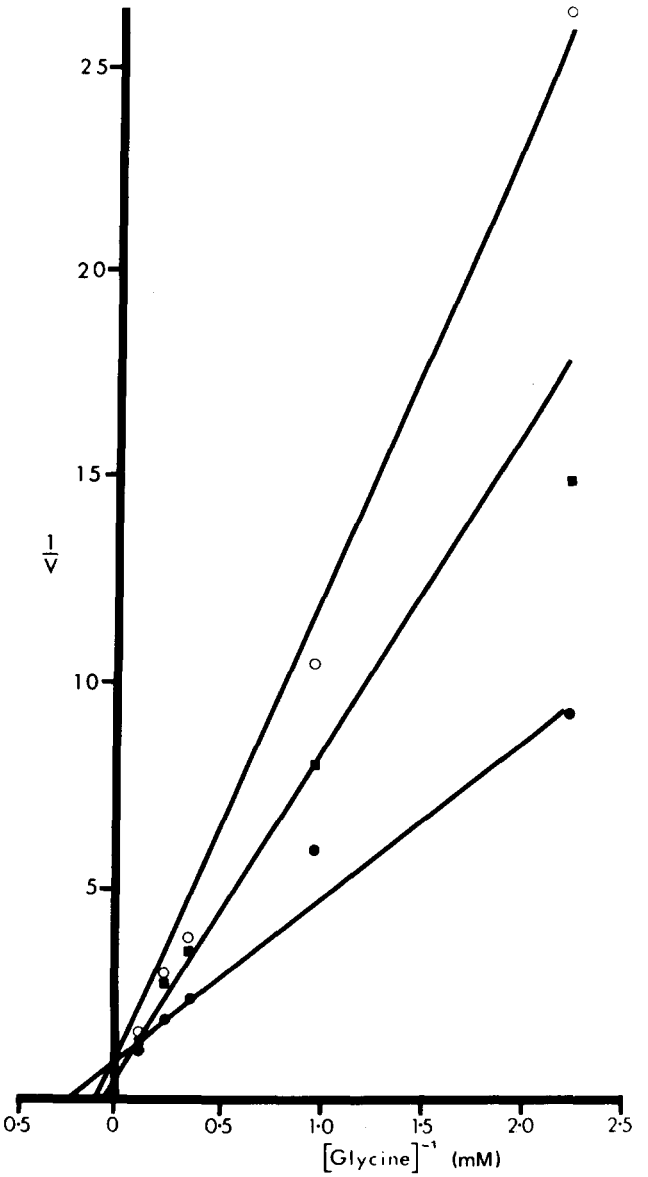

Fig. 3. Effect of Na^+ concentration on the kinetic constants for glycine uptake when $^{\Delta}\tilde{\mu}_{Na} = 0$. Ehrlich cells were incubated as described for Fig. 1. [1-^{14}C]Glycine was added 2 min after gramicidin and samples taken at 30-s intervals for 2 min. The data are expressed per min per g fresh weight of tissue. All uptakes are corrected for uptake in choline medium. The Na^+ concentrations used were 115 mM (●——●), 45 mM (■——■) and 24 mM (○——○).

that on the K_m value (see also Table II). Inui and Christensen [7] have reported that in the Ehrlich cells, the K_m for α-aminoisobutyrate uptake is altered more than the V by varying the external Na^+ concentration.

Effect of Na^+ on efflux

As with Na^+-dependent glycine uptake, the efflux of glycine responds in a linear (or near linear) manner to cellular Na^+ concentration (Fig. 4). To assess the effect of Na^+ on efflux, the loss of [^{14}C]glycine from cells preincubated with [^{14}C]glycine was determined at different cellular Na^+ levels with Li^+ as the replacement cation for Na^+. The apparent first-order rate coefficient for efflux is shown to increase in a linear (or near linear) fashion with increasing internal Na^+ concentration up to 100 mM Na_i^+. As with influx, Na^+ increases the permeability of the cell to glycine. In absence of Na^+, despite the presence of a very steep glycine gradient, (initially 40—80 mM inside, <0.1 mM outside) the cell loses little glycine, the $t_{1/2}$ for glycine loss being between 45 and 60 min at 25° C. It should be stressed that the movement of glycine in these experiments is down its own concentration gradient.

Effect of cellular amino acid concentration on the rate of exodus

In an earlier communication, it was reported that amino acid efflux behaved like a first-order process over a 10-fold range of cellular glycine concentrations [24]. These experiments were done with cells where the initial [^{14}C]glycine concentration was not in excess of 20 mM. When the cellular glycine concentration was raised above 25 mM (25—80 mM) it was found that efflux deviated from first order, i.e. $t^{-1}\ \ln[(a - x)/a]$ (where a is the original amino acid concentration and $(a - x)$ is the remaining concentration at time t) decreased as the internal glycine concentration increased. In a typical experiment with gly-

TABLE II

K_m AND V VALUES FOR GLYCINE INFLUX AND EFFLUX AT VARIABLE Na^+

V values are expressed per g fresh weight. To express these values per ml cell water or per g dry weight, the values given should be multiplied by 1.9 or 6.7, respectively. Experimental conditions were as described in Table I. For uptake, the range of glycine concentrations used was 0.45—10 mM, for efflux, 4—70 mM. Samples were taken at 30-s intervals for 2 min for uptake and at 2-min intervals for efflux. The fluxes were measured at 25°C. Preloading of the cell with [^{14}C]glycine prior to efflux was done at 37°C.

Expt. No.	Influx				Expt. No.	Efflux			
	Na_0 (mM)	K_m (mM)	V (μmol · min^{-1} · g^{-1})	$\frac{V}{K_m} \times 10^3$		Na_i (mM)	K_m (mM)	V (μmol · min^{-1} · g^{-1})	$\frac{V}{K_m} \times 10^3$
1	22	6.5	2.0	0.31	4	30	97	9.2	0.09
	112	1.5	1.3	0.83		90	55	10.0	0.18
2	24	13.5	3.1	0.23	5	27	103	11.6	0.11
	45	5.0	1.9	0.38		82	38	8.1	0.21
	115	4.1	2.5	0.61	6	30	58	—	—
3	11	20.0	2.0	0.10		60	46	5.5	0.12
	112	4.0	1.1	0.27		90	22	4.0	0.18
					7	90	54	5.5	0.10

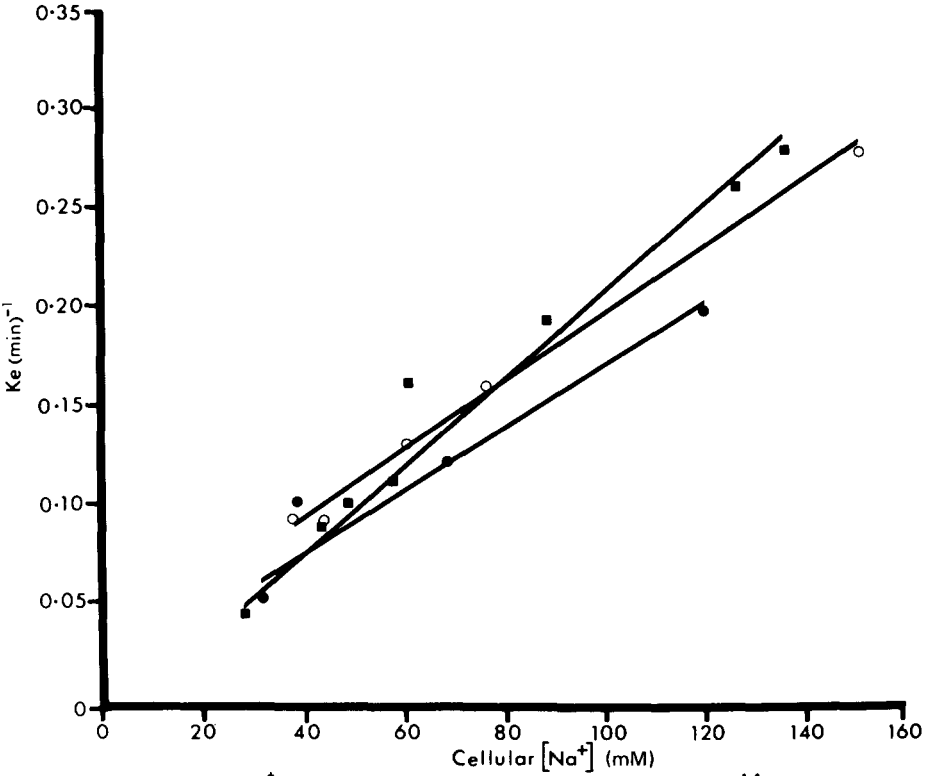

Fig. 4. Effect of cellular Na^+ on pseudo first-order rate coefficient (k_e) for [1-^{14}C]glycine efflux. Ehrlich cells were preincubated with [1-^{14}C]glycine for 30 min at 37°C. After a quick wash with cold isotonic choline chloride, the cells, suspended in isotonic choline chloride, were brought to the temperature of the bath (25°C) in presence of 7 μg/ml gramicidin for 2 min. Then the cells were injected into amino acid-free medium containing K^+/Hepes buffer, variable NaCl and 7 μg/ml gramicidin. Isotonicity was maintained with LiCl. Initial [1-^{14}C]glycine concentrations were 2 mM (○———○), 8 mM (■———■), and 50 mM (●———●). Three representative experiments are shown.

cine at 25°C and 150 mM Na_i^+ the value of $t^{-1}\ln[(a-x)/a]$ falls from 0.28 min^{-1} at an initial cellular concentration of 15 mM to 0.14 min^{-1} at 70—80 mM cellular glycine. Starting with various internal glycine concentrations, a series of pseudo first-order rate coefficients is obtained where the apparent coefficient decreases as the cellular glycine level increases. However, $\ln[(a-x)/a]$ versus time is approximately linear for 5—7 min of incubation with initial glycine concentrations as high as 80 mM. This interval results in a decrease of 35—65% in the initial intracellular amino acid level. A typical experiment is shown in Fig. 5 with 87 mM Na_i^+ and initial internal [^{14}C]glycine concentrations from 8 to 78 mM. In the figure each initial glycine level is normalized to 100%. These observations suggested that efflux tends to become saturated at elevated internal glycine levels and provides an approach to estimate the K_m and V and the effects of Na^+ on these parameters using the Lineweaver-Burk [28] transformation of the Michaelis-Menten relationship.

The fact that cellular glycine is rapidly lost, (and therefore the substrate concentration is changing significantly during the course of measuring exodus)

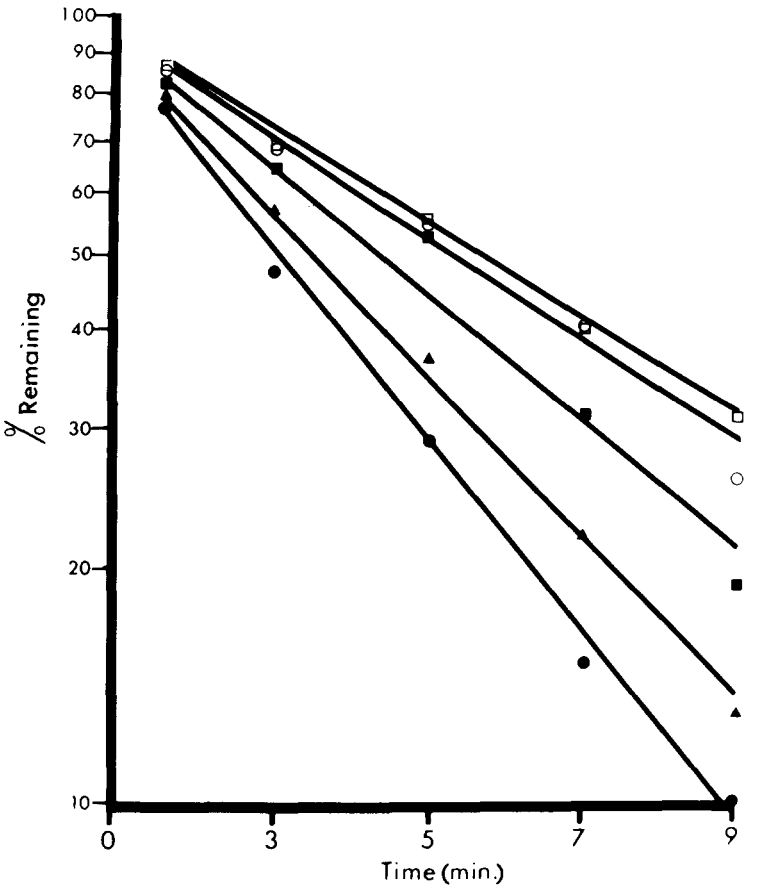

Fig. 5. Effect of cellular glycine concentration on the pseudo first-order rate of loss of cellular glycine. The experimental conditions were described in Fig. 4. The cellular Na^+ concentration was maintained at 87 mM throughout. Initial cellular [1-^{14}C]glycine concentrations were 8 mM (●———●), 25 mM (▲———▲), 60 mM (■———■), 70 mM (○———○) and 78 mM (□———□).

introduces difficulties in obtaining initial velocities except at very early time (less than a minute). Technically, measurement of a small percentage loss in experiments of this type is subject to large errors. However, the initial velocity can be obtained from the data in Fig. 5, since the initial velocity is the product of the initial cellular glycine concentration, a, at $t = 0$ (obtained by extrapolation) and the slope of the line describing $\ln[(a - x)/a]$ versus t. As the initial cellular level of glycine is raised the slope of the line decreases. Using such data from a number of experiments at different initial glycine and Na^+ concentrations, the values for K_m and V were calculated from the Lineweaver-Burk plots namely, $1/v_{obs} = 1/V + K_m/V \times 1/S$ where S is the initial substrate concentration at $t = 0$ and v_{obs} is the product of the initial glycine concentration and the slope of the line describing the loss of glycine at various initial glycine concentrations. The best fit for the Lineweaver-Burk expression was obtained using a linear regression analysis. The correlation coefficients were better than 0.9 in all cases. The values obtained for K_m and V for efflux from a number of

experiments at different Na^+ levels are shown in Table II. It is clear that the major effect of Na^+ is on the K_m value for efflux. As with uptake, the V value may also change at different Na^+ concentrations although to a lesser extent. The high K_m for glycine efflux in these experiments (around 50 mM at 90 mM Na^+) is in contrast to values reported by Christensen and collaborators [29,30] and Schafer [31] for efflux of α-aminoisobutyrate. During this investigation their observations [29,31] that efflux of α-aminoisobutyrate is saturated at lower levels than glycine were confirmed. A K_m of 12 ± 2 mM was obtained for α-aminoisobutyrate efflux at 90 mM Na_i^+ (results not shown). Earlier work by Oxender and Christensen [30] had in fact suggested that the K_m for glycine efflux was larger than that for α-aminoisobutyrate. Morville et al. [32] have reported a K_m of 25 mM for methionine efflux with tumor cells at high Na^+.

Despite the fact that there is appreciable variation of the values for K_m and V obtained with different batches of cells in these experiments, it is equally clear that when $\Delta\tilde{\mu}_{Na} = 0$ both K_m and V values for glycine efflux are greater than those for uptake. At high Na^+, the K_m values for influx and efflux may differ by an order of magnitude (Table II).

Effect of K^+ on the rate coefficient for amino acid efflux

In Crane's [1] original and insightful proposal of the Na^+ gradient hypothesis, a competitive role was assigned to K^+ opposite to that of Na^+. Internal K^+ was postulated to decrease the binding of Na^+ to the transport system and increase the K_m for the organic solute at the cytoplasmic surface. Thus K^+ was

TABLE III

THE EFFECT OF CELLULAR K^+ ON THE PSEUDO FIRST-ORDER COEFFICIENT (k_e) FOR GLYCINE EFFLUX

Ehrlich cells were preincubated with 1 mM [1-^{14}C]glycine, specific activity = 300 cpm/nmol, for 30—40 min at 37°C. The cells were centrifuged and quickly washed in cold isotonic choline chloride and suspended in isotonic choline chloride. The incubated cells were added to flasks containing various mixtures of Na^+, K^+ and LiCl, final concentration of the monovalent cation being 150 mM. The buffer solution was 10 mM Na^+/Hepes at pH 7.4. After 5 min equilibration at 27° C, gramicidin was added to a final level of 7 μg/ml and samples were taken at intervals, washed with 5.5-fold excess of cold isotonic choline chloride and centrifuged. The pellets were weighed and extracted with 95% ethanol for amino acid measurements or nitric acid for ion determinations. The rate coefficients were determined from the slope of the relationship between cellular glycine concentration versus time. To vary intracellular K^+, extracellular Na^+ was maintained constant and the proportions of K^+ and Li^+ were varied. The temperature of incubation was 25° C. In the values given below no corrections have been applied for efflux in absence of Na^+. The apparent first-order rate constants for the latter were 0.01, 0.05 and 0.01 in experiments 1, 2 and 3, respectively, in all choline media. The three experiments reported were done with different batches of cells.

Expt. No.	$[Na^+]_0$	$[K^+]_i$ (mM)	$[Na^+]_i$	k_e (min^{-1})
1	50	22	63	0.13
	50	90	71	0.14
2	28	25	42	0.15
	28	73	40	0.15
	28	97	40	0.15
	28	120	42	0.15
3	30	23	53	0.12
	30	130	43	0.09

TABLE IV

RELATIVE CHANGE IN FLUORESCENCE IN MIXTURES OF Na^+, Li^+ AND K^+ SALTS

Changes in fluorescence were measured as described [22]. Cells, at a cytocrit of 0.3%, were incubated in isotonic Ringer solutions with the cation composition given below. The other components of the medium were given in Table I. After equilibration with the dye (about 5 min) to reach a constant base line, gramicidin to a level of 2 μg/ml was injected and the change in fluorescence measured. The percentage change in fluorescence from the base line is given.

	Cation composition of medium (mM)	Percent change in fluorescence with gramicidin
I	Na^+, 50; K^+, 75; Li^+, 25	22
	Na^+, 50; K^+, 100; Li^+, 0	20
	Na^+, 50; Li^+, 100	22
	Na^+, 50; K_i^+, 50; Li^+, 50	24
II	Na^+, 30; Li^+, 120; K^+, 4	22
	Na^+, 60; Li^+, 90; K^+, 4	22
	Na^+, 90; Li^+, 60; K^+, 4	23
	Na^+, 120; Li^+, 30; K^+, 4	21

ascribed to have a direct role in Na^+-coupled transport. Using Na^+-dependent efflux with gramicidin as a test of the Na^+-dependent system, we were in a position to examine directly whether K^+ affects efflux and thereby deduce whether K^+ has a direct action on the Na^+-dependent transport system. The results in Table III show clearly that over a wide range of cellular K^+ concentrations there is little effect of K^+ on the rate coefficient for glycine efflux. Each of the experiments reported is with a different batch of cells. Since there is some variation of k_e for efflux from day to day, the values of k_e should be compared only within each experiment. These data are not consistent with a direct participitation of cellular K^+ on Na^+-dependent amino acid transport in Ehrlich cells.

Membrane potential changes with gramicidin with various cations

The results have shown that influx and efflux are directly proportional (or nearly so) to the extra- and intracellular Na^+ concentrations, respectively (Fig. 4). To ascertain that with the various cations used as replacement, the cell was comparably depolarized in the presence of gramicidin, the membrane potential was determined using a fluorescent probe [22,32]. When gramicidin is added to Ehrlich cells in presence of monovalent cations, there is extensive depolarization (increase in fluorescence). The data in Table IV show that with mixtures of Li^+ and Na^+, the extent of depolarization is equivalent at high and low Na^+ concentrations (the increase in fluorescence is the same). Moreover, when various mixtures of Li^+ and K^+ are used at constant Na^+ the extent of depolarization is also comparable. In each experiment, the base line before gramicidin was the same. These data substantiate the conclusion that under conditions used to vary internal Na^+, the resulting membrane potentials in presence of gramicidin are equivalent.

Discussion

Many investigators studying Na^+-coupled transport of organic solutes support the idea that the electrochemical gradient for Na^+ is a major factor, if not the

only factor, involved in the energization of the Na^+-coupled transport of organic solutes in mammalian systems [5,6,10—16,25]. We have examined the action of Na^+ itself on amino acid uptake in the absence of a Na^+-driving force. The results show that the influx and efflux of glycine may be increased by an order of magnitude in presence of 150 mM Na^+ as compared to the respective fluxes in Na^+-free media. Early reports by Vidaver [34], Eddy and Hogg [25] and by Bihler et al. [35] as well as previous work from this laboratory [27] have shown that in cells depleted of ATP, the presence of Na^+ increased the rate of passage of the organic solute across the membrane down its own electrochemical gradient. The present studies show that in absence of Na^+, equilibration of glycine at 25° C is an extremely slow process.

The present studies also show that the constants governing amino acid fluxes originating from the two surfaces of the membrane are not the same even in absence of an electrochemical or concentration difference for Na^+. The K_m for glycine efflux at 100 (±10) mM Na^+ is about an order of magnitude greater than corresponding value for influx (i.e. 50 mM for efflux compared to 4.0 mM for influx). The ratios V/K_m, however are more similar in magnitude in either direction. Since the fluxes in both directions should be equal when $\triangle\tilde{\mu}_{Na} = 0$ and $\triangle\tilde{\mu}_{glycine} = 0$, the difference in K_m may be compensated by a difference in V. Attempts to measure flux ratios at equivalent intra- and extracellular amino acid levels when $\triangle\tilde{\mu}_{Na} = 0$, were only modestly successful because of the inability to balance precisely the intracellular and extracellular solute concentrations so that the net flux of amino acid was never zero during the experimental period. However, the net movement was small and the ratio of the two fluxes was close to 1 when measured under these conditions (results not shown). The lack of symmetry for the kinetic constants for solute fluxes originating at the two membrane surfaces has been shown in several systems including the glucose transport systems in red cells [18] and thymocytes [19] and the glycine transport system in avian erythrocytes under conditions where $\triangle\tilde{\mu}_{Na} = 0$ [17]. Other reports have also appeared concerning the asymmetry of the amino acid transport system(s) in Ehrlich cells [29,30,32]. Christensen and Handlogten [29] reported that under normal conditions (that is with a normal Na^+ gradient) the K_m for efflux about 20 times greater than that for influx with several amino acids tested. Morville et al. [32] reported a 20-fold greater K_m for methionine efflux than influx in ATP-depleted cells. In this communication with $\triangle\tilde{\mu}_{Na} = 0$, the K_m for glycine efflux is larger than for influx by an order of magnitude at several Na^+ concentrations.

In contrast to the studies of Christensen and Handlogten [29] which showed that with a normal Na^+ gradient, V for influx and efflux are nearly equal, the present findings indicate that at several Na^+ concentrations the V for efflux is several-fold greater than that for influx in presence of gramicidin. V values for glycine influx reported here vary from 2.1 to 5.9 μmol/ml cell water per min and are within the ranges reported for a number of amino acids (including glycine) by Oxender and Christensen [30] namely, between 1.5 and 4.6 μmol/ml cell water per min.

Schafer [31] reported that metabolic inhibition (which normally results in an elevation of cellular Na^+) increased the maximal velocity for efflux. This observation is consistent with the present work which shows that gramicidin treat-

ment and a decreased $\Delta\tilde{\mu}_{Na}$ have a similar effect (see below).

The role of K^+ in ion-coupled amino acid transport has been investigated for many years. Riggs et al. [3] originally suggested that the K^+ gradient was responsible for energization of amino acid transport in Ehrlich cells. Subsequently, less prominent roles have been postulated for K^+ including the proposal that cellular K^+ competed for Na^+ and increased dissociation of the Na^+ bound to the amino acid carrier [1]. Whether or not K^+ has a direct role in Na^+-coupled solute transport has not been resolved. Some investigators favour a direct participation of K^+ suggesting a competition between Na^+ and K^+ [1,6,25,36]. Others have reported little effect of K^+ [16,37] on Na^+-coupled transport. With isolated plasma membrane vesicles from Ehrlich cells, K^+ was not required for Na^+-coupled transport [12]. The present study shows that variation of intracellular K^+ from 20 to 130 mM does not affect amino acid efflux when $\Delta\tilde{\mu}_{Na} = 0$. Similarly no effect of K^+ between 0 and 50 mM was detected on glycine influx with gramicidin when Na^+ (inside and out) was about 100 mM (results not shown). The known requirement [38] of low levels of extracellular K^+ for amino acid transport in Ehrlich cells is probably associated with the requirement to maintain Na^+ pump activity and a Na^+ gradient [39,40].

When gramicidin is used to elevate cell Na^+, cellular K^+ is lost [21]. Since variable cell K^+ at constant cell Na^+ has no apparent effect on efflux of amino acids, the high rates of amino acid loss with gramicidin are unlikely the result of altered cellular K^+ levels (Table III). Therefore the higher rates of amino acid loss at equivalent cellular Na^+ seen with gramicidin-treated cells compared to other agents [21] which elevate cell Na^+ cannot be due to differences in cell K^+. Of a variety of agents examined it has been observed that depolarization (increased fluorescence of the cyanine dye) is greater with gramicidin in a medium containing monovalent cations than with other agents (unpublished). Therefore it is suggested that a decrease in membrane potential contributes to the increased efflux with gramicidin.

In several systems, particularly bacterial systems, investigators have addressed the question whether a Na^+ (or H^+) chemical gradient is as efficacious as an electral gradient for energization of organic solute transport [41—43]. In some reports differences have been found [41,42] between the electrical and chemical gradients suggesting that transport is better with one or the other gradient. Other investigators [43] have reported that a pH difference can compensate for an electrical difference to obtain equivalent sugar accumulation in *Escherichia coli*. While on purely energetic grounds, they may be equivalent, the present observations suggest they are not equivalent in Na^+-coupled transport systems, and that the rate of transport at variable Na^+ may not be compensated by an energetically equivalent electrical potential difference.

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