J. Membrane Biol. 86, 37–44 (1985)

The Journal of
Membrane Biology

Na^+-Coupled Glycine Transport in Reticulocyte Vesicles of Distinct Sidedness: Stoichiometry and Symmetry

Andrew M. Weigensberg and Rhoda Blostein
Departments of Biochemistry and Medicine, McGill University, Montreal, Quebec, Canada

Summary. Sodium-coupled glycine transport has been studied using membrane vesicles of distinct sidedness, either inside-out or right side-out, prepared from sheep reticulocytes. The activity is chloride dependent and characterized by high and low apparent affinities for glycine ($K'_m \simeq$ 0.5 mM and >10 mM) for both types of vesicles as well as intact cells. Transport is symmetrical with respect to similar apparent affinity constants for glycine, for both the high- and low-affinity systems, and for sodium. Direct measurements of the sodium/glycine coupling indicate a ratio of 2:1, consistent with kinetic data fitted to a Hill-type equation describing glycine flux as a function of sodium concentration.

Key Words Na-glycine cotransport · stoichiometry · symmetry · vesicles

Introduction

One of the major mechanisms for solute entry into polar as well as nonpolar cells involves Na^+-coupled cotransport (for reviews, *see* Schultz & Curran, 1970; Christensen et al., 1973; Kimmich, 1973; Crane, 1977; Johnstone, 1979). The transport efficacy of ion-coupled transport depends not only on the electrochemical driving forces of the transported species, but also on inherent properties of the particular transport system, such as the ion/solute coupling stoichiometry (Aronson, 1981) and the kinetic characteristics of the system.

With intact cells, variable Na^+/solute coupling ratios have been obtained. However, measurements have been relatively few and, in some instances, prone to equivocal interpretation due, among other factors, to effects of the membrane potential on ion permeabilities. Thus, for intestinal glucose transport, as an example, a ratio of 1:1 was obtained for intact tissue (Goldner et al., 1969; Okada, 1979; Hopfer & Groseclose, 1980). In contrast, a value of 2:1 was obtained for isolated intestinal cells with the membrane potential clamped near zero (Kimmich, 1981). Using three independent methods, one a kinetics-independent "Static Head Method," Turner and Moran demonstrated a 1:1 ratio for kidney outer cortex brush-border membranes (Turner & Moran, 1982*a*) and a 2:1 ratio for the outer medullary transporter (Turner & Moran, 1982*b*). Variable stoichiometries have been reported also for Na^+-coupled amino acid transport; for example, values have ranged also from 1:1 (Wheeler et al., 1965) to 2:1 (Vidaver, 1964*a*,*b*) for Na^+-glycine cotransport.

For studies aimed at characterizing the stoichiometry and kinetic properties of Na^+-coupled solute transport, membrane vesicle preparations have the advantage over intact cells since they lack interfering intracellular organelles and metabolic reactants. Nevertheless, the orientation of most vesicle preparations has been difficult to control, so that the kinetic parameters cannot be evaluated with respect to membrane sidedness. Until now, evaluation of kinetic parameters have been carried out with either intact cells as, for example, studies of Na^+-coupled glycine transport in Ehrlich ascites tumor cells (Johnstone, 1978), resealed pigeon red cells (Vidaver & Shepherd, 1968), or membrane vesicles of undefined orientation (for review *see* Lever, 1980). In the former studies with cells, the kinetics of Na^+-dependent glycine transport appeared to be asymmetric with respect to apparent V_{max} and K_m values for glycine entry and exit.

In the present study, we have applied the procedure of preparing inside-out or right-side-out vesicles from human red blood cells (Steck, 1974) to reticulocytes isolated from anemic sheep. These immature cells, in contrast to mature red cells, possess relatively active Na^+-coupled amino acid transport (Benderoff et al., 1978). This has enabled us to evaluate and compare the apparent affinities for sodium (K'_{Na}) and for glycine (K'_{gly}) at both cytoplasmic and extracellular membrane surfaces. In addition, these vesicles have proven to be a particularly advantageous system for evaluating directly

Table 1. Sidedness of vesicle preparations[a]

Prep. No.	Method of prep.	Sheep No.	Retics. (%)	IOV (% of total vesicles)	ROV
24-3-81	IOV	9	15	77	23
6-4-81	IOV	9	70	91	9
2-4-81	IOV	1023	68	100	0
24-2-82	IOV	91	51	97	3
24-3-82	ROV	1023	80	0	100
21-4-82	ROV	364	40	0	100

[a] Vesicles were prepared and assayed as described in Materials and Methods.

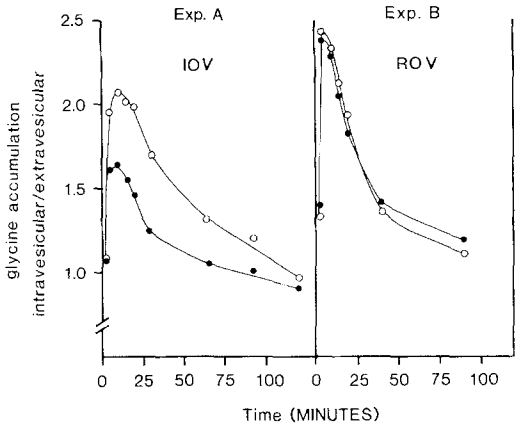

Fig. 1. Effect of ATP on glycine accumulation in IOV and ROV. The data are from separate experiments carried out with IOV (50% IOV; 8% ROV) derived from 58% reticulocytes (sheep #91), and ROV (43% ROV; 0% IOV) derived from 83% reticulocytes (sheep #1023). In experiment *A*, IOV were equilibrated for two days at 0°C with 40 mM KCl, 1 mM $MgCl_2$, 10 mM MOPS-Tris, pH 7.4 and 0.1 mM ^{14}C-glycine (2.5×10^4 cpm nmol) and then concentrated by centrifugation for 10 min at 12,000 × *g*; final protein concentration, 6.56 mg/ml (IOV) and 2.9 mg/ml (ROV). The reaction was started by adding 0.45 ml of prewarmed (37°C) isoosmotic medium containing 0.10 mM ^{14}C-glycine of the same specific activity, 40 mM NaCl, 1 mM $MgCl_2$, 10 mM MOPS-Tris, pH 7.4 and without ATP (open circles) or with 1.0 mM ATP (solid circles) as indicated, to 50 μl of vesicles. Samples were taken at the indicated times and the vesicles were filtered as described in Materials and Methods. In experiment *B*, ROV were equilibrated for 2 hr at 37°C with 40 mM KCl, 1 mM $MgCl_2$, 10 mM MOPS-Tris, pH 7.4 and 1.0 mM ^{14}C-glycine (7.6×10^3 cpm/nmol) and then concentrated to 2.19 mg/ml. The reaction was carried out as in experiment *A* except that the final glycine concentration was 1 mM and ATP, 0.5 mM

the Na^+-glycine coupling ratio. Not only are they free of interfering cellular components, but their high chloride permeability effectively shunts any electrical potential build-up that may ensue from ion-solute cotransport.

Materials and Methods

Reticulocytosis was produced in sheep by phlebotomy. The cells were isolated as described earlier (Weigensberg et al., 1982).

Inside-out vesicles (IOV) and right side-out vesicles (ROV) were prepared from the cells according to the method of Steck et al. (1970) with slight modifications, and their sidedness assessed as described elsewhere (Weigensberg et al., 1982). ATP was obtained from Boehringer Mannheim, and choline chloride from Syntex. ^{22}Na (carrier-free) and [^{14}C]glycine were obtained from New England Nuclear.

Transport assays with intact cells were performed in essentially the same way as described earlier (Weigensberg & Blostein, 1983) except for the modifications indicated in the text and transport assays with IOV and ROV were carried out as described by Weigensberg et al. (1982); velocities are expressed as nmol mg^{-1} min^{-1}.

Results

Table 1 and Fig. 1 summarize results of experiments aimed at assessing vesicle sidedness. Thus, the percentage of IOV and ROV was determined by measuring the extracellular exposed acetylcholinesterase and intracellularly exposed glyceraldehyde-3-phosphate dehydrogenase activities, respectively, in the presence and absence of detergent as described by Steck (1974). Table 1 shows that the percent of sealed vesicles with the desired orientation is usually greater than 80% of the total sealed vesicles.

In an early study (Weigensberg et al., 1982) we showed that IOV are capable of 'reverse' Na^+ gradient-stimulated glycine accumulation. This accumulation showed cardiac glycoside-sensitive inhibition by added ATP due to dissipation of the Na^+ gradient via the Na^+,K^+-pump, providing further evidence that these membranes are, indeed, inside-out. Figure 1*A* is a replot of those results and Fig. 1*B* depicts a similar experiment carrier out with ROV. Similar conditions were used except that the glycine and ATP concentrations were 0.1 and 1 mM, respectively, for IOV and 1 mM and 0.5 mM, respectively, for ROV. For IOV, higher ATP was used to compensate for the higher rate of ATP hydrolysis.

The results show two important characteristics of this glycine transport system, namely (i) the ability of both types of vesicles to accumulate glycine and (ii) the inhibitory effect of ATP added to IOV and lack of affect in ROV confirming the putative sidedness of the two types of vesicles. In the case of ROV, ATP presumably does not have access to the intravesicular ATP site of the sodium pump.

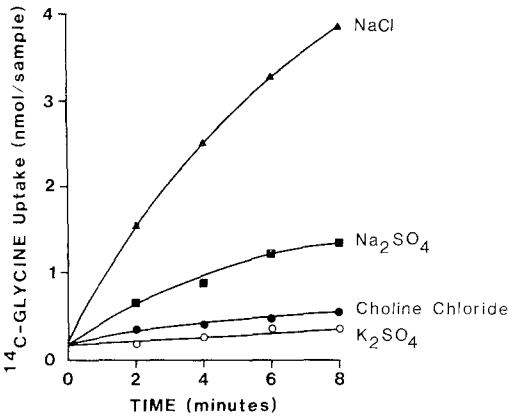

Fig. 2. Effect of anions on Na^+-dependent glycine uptake in intact cells. Reticulocytes (90%) were isolated from an anemic LK sheep (#91) and stored at approximately 5% hematocrit overnight at 4°C in a balanced salt solution (154 mM NaCl, 30 mM sucrose, 6 mM glucose and 5 mM Tris-HCl, pH 7.4, or 103 mM Na_2SO_4, 30 mM sucrose, 6 mM glucose and 5 mM Tris-SO_4, pH 7.4) containing either the chloride or sulfate anion. After the cells were washed twice with isotonic $MgCl_2$ or $MgSO_4$, the glycine uptake was carried out as follows. Reticulocytes (0.08 ml) in isotonic $MgCl_2$ or $MgSO_4$ were added to 0.74 ml of a balanced salt solution containing either the chloride or sulfate anions (5.5 mM potassium phosphate, pH 7.4, 6.6 mM glucose, 1.1 mM $MgCl_2$ or 1.65 $MgSO_4$, 5.5 mM KCl or 3.7 mM K_2SO_4, 11 mM Tris-HCl, pH 7.4, or 11 mM Tris-SO_4, pH 7.4, 165 mM NaCl or KCl or 110 mM Na_2SO_4 or K_2SO_4, with 1.1 mM ouabain). The samples were incubated 5 min at 37°C at which time 0.09 ml of 1 mM ^{14}C-glycine was added (specific activity 550 cpm/nmol). At specified time points, 0.2 ml of the red blood cell suspension was removed and the ^{14}C-glycine uptake was determined as described in Materials and Methods

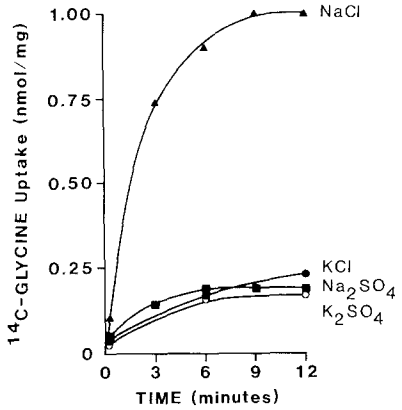

Fig. 3. Effects of chloride and sulfate on Na^+-dependent glycine uptake in IOV. IOV (37%) derived from reticulocytes (30%) were equilibrated with either 50 mM choline chloride, 50 mM KCl, 1 mM MgCl, and 10 mM MOPS-Tris, pH 7.4, or 25 mM K_2SO_4, 63.5 mM $MgSO_4$ and 10 mM MOPS-Tris, pH 7.4, overnight at 4°C and 30 min at 37°C and then concentrated to 2.9 mg protein/ml as described in Fig. 1. The assay was started by adding 0.1 volume of prewarmed (37°C) IOV (either chloride or sulfate-equilibrated) to 0.9 volumes of prewarmed medium (either 50 mM choline chloride, 1 mM $MgCl_2$, 50 mM NaCl, 0.11 mM ^{14}C-glycine, 10 mM MOPS-Tris, pH 7.4, or 63.5 mM $MgSO_4$, 25 mM Na_2SO_4, 0.11 mM ^{14}C-glycine, 10 mM MOPS-Tris, pH 7.4. The specific activity of ^{14}C-glycine was 25 × 10^4 cpm/nmol. The potassium salt was substituted for sodium as indicated. The reaction was terminated as described in Materials and Methods

Anion-Dependence: Effect of Chloride Versus Sulfate

Five glycine transport systems have been operationally defined in the human red blood cell (Ellory et al., 1981). Two are sodium dependent, the differences between them attributed to their anion dependency. Thus, one system appears to be chloride dependent, the other, chloride independent. In the experiments shown in Figs. 2 and 3, intact reticulocytes (Fig. 2) and vesicles (Fig. 3) were equilibrated with either sulfate or chloride after which Na^+-dependent ^{14}C-glycine influx was measured. As shown, the substitution of most of the chloride for sulfate caused a 70 to 80% decrease in Na^+-dependent glycine transport in the cells and a 90% decrease in the vesicles. This is consistent with the conclusion that in sheep reticulocytes the chloride-dependent system is the main, if not only, Na^+-dependent transport system. The residual activity in the sulfate medium may, in fact, be due to small amounts of chloride still present, particularly in the experiment with intact cells (Fig. 2).

Symmetry or Asymmetry of Kinetic Behavior

In order to determine if there is directional asymmetry in the Na^+-dependent glycine transport system with respect to influx and efflux kinetics, the apparent affinities of the system for sodium and for glycine were measured at several glycine and sodium concentrations. In the experiment shown in Fig. 4, IOV and ROV were derived from the same cells; the apparent K'_m for glycine at 90 mM extravesicular Na^+ was determined by following the initial velocity of ^{14}C-glycine influx estimated from triplicate samples taken at 30 sec. This short time interval was used because of the relatively small intravesicular volume and thus short equilibration times. The velocity versus glycine concentration plots in Fig. 4 show that glycine transport may occur by a high-affinity system as well as a low-affinity system although other interpretations for this kinetic complexity are possible (*see*, for example, Glover et al., 1975). Without an *a priori* basis for assuming more than one transport system, approximate values for the apparent kinetic constants were obtained by us-

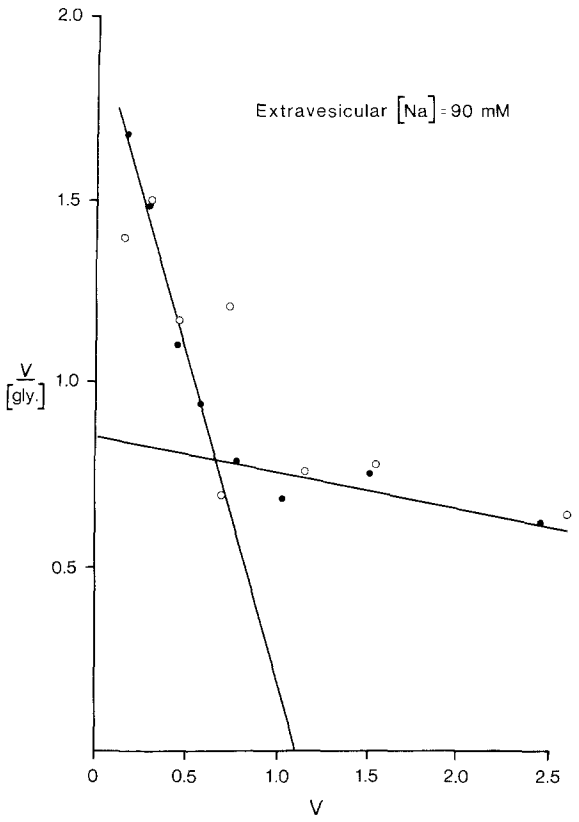

Fig. 4. Eadie-Hofstee plots of Na^+-dependent glycine influx as a function of glycine concentration. IOV (22%) and ROV (18%), prepared concurrently from sheep #8279 (60% reticulocytes) were equilibrated overnight at 0°C, then 15 min at 37°C in 100 mM KCl, 10 mM MOPS-Tris, pH 7.4 and 1 mM $MgCl_2$, and then concentrated by centrifugation to either 5.16 mg/ml (○——○ IOV) or 3.49 mg protein/ml (●——● ROV). The assays were initiated by adding 0.1 volume of prewarmed (37°C) vesicles to 0.9 volumes of prewarmed (37°C) medium containing 10 mM MOPS-Tris, pH 7.4, 1 mM $MgCl_2$, 0.11 to 4.4 mM ^{14}C-glycine (specific activity 1.0×10^4 to 1.1×10^5 cpm/nmol) and 100 mM NaCl, respectively. Values for Na^+-dependent glycine uptake (initial velocities) were obtained after subtraction of the activity observed with NaCl replaced by KCl. The initial velocities were determined using linear regression analysis during the first minute of uptake. From linear regression analyses of the Eadie-Hofstee plots, two values for the apparent K'_{gly} were obtained, 0.72 and 11.4 mM for IOV (r = 0.911 and 0.929, respectively), and 0.52 and 14.1 mM for ROV (r = 0.991 and 0.739, respectively)

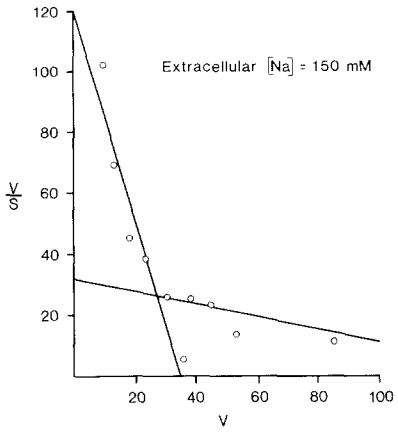

Fig. 5. Eadie-Hofstee plots of Na^+-dependent glycine influx into intact cells as a function of glycine concentration. Reticulocytes (80%, sheep #474) were stored overnight in 150 mM choline chloride, 1 mM $MgCl_2$, 5 mM glucose, 20 mM sucrose, 5 mM potassium phosphate, pH 7.4, 10 mM MOPS-Tris, pH 7.4. One-half of the cells were then washed twice with the above solution, the rest with NaCl replacing choline chloride. The reaction was started by adding 10 μl of ^{14}C-glycine (1 to 80 mM, specific activity 1.19×10^4 to 2.41×10^5 cpm/nmol) to 90 μl cells suspended in either the choline (baseline) or sodium wash solution. After 30 sec the cells were chilled and 1 ml of ice-cold choline chloride solution was added. One ml of this suspension was layered over 0.15 ml of *n*-butyl phthalate and centrifuged for 10 sec in an Eppendorf (Model 3200) micro-centrifuge. The supernatant was removed and the oil surface and tube washed with 1 ml water after which the oil layer was removed and the cells lysed with 0.2 ml water. Following centrifugation (3 min) to remove the stroma, one aliquot of the supernatant was removed for the measurement of radioactivity and another, for hemoglobin determination. Values for Na^+-dependent glycine uptake were obtained after subtraction of the activity observed with NaCl replaced by KCl and the initial velocities were determined using linear regression analysis during the first minute of uptake. Eadie-Hofstee plots were used to determine the apparent K'_{gly} which were 0.23 (r = 0.955) and 3.6 mM (r = 0.886)

ing the data points which distinctly belong to one or the other of the two linear portions of the Eadie-Hofstee plot.[1] As shown, the apparent kinetic parameters, V'_{max} and K'_{gly} of IOV are indistinguishable from those of ROV.

In a similar experiment the K'_{gly} for intact reticulocytes was measured at 135 mM sodium (Fig. 5). The results gave evidence for a change in K'_{gly} expected for the sum of a high- and a low-affinity transport system in intact reticulocytes.

When apparent K'_{Na} values were determined for IOV and ROV similar values were obtained. The velocity versus substrate concentration curves were sigmoidal, consistent with a model whereby two sodium ions are required and possibly cotransported per molecule of glycine. In the experiment shown in Fig. 6, IOV and ROV were derived from

[1] It is not known whether the two-phase kinetics represent two independent saturable transport functions or one system with negative cooperativity as observed by Glover, D'Ambrosio and Jensen (1975), in which case the data were best fitted by the method used in the present study. Nevertheless, an analysis of our data assuming two independent systems as described by Neal (1972) yielded apparent values for K'_{gly} similar to those calculated as described in the text, e.g. 0.34 and 12.6 mM for ROV in Fig. 4 and 0.52 and 14.1 mM for intact cells in Fig. 5.

Table 2. Apparent kinetic constants for Na^+-coupled glycine transport in IOV and ROV[a]

Parameter measured	Prep. No.	Sheep No.	Retics. (%)	Sealed vesicles (%)	Glycine or Sodium conc. (mM)	Value (mM)
K'_{Na} (IOV)	1-12-82	8279	42	32	0.10	37.0
	17-1-84	8279	55	41	0.50	25.0
	21-4-84	1023	60	18	1.00	23.0
	16-3-82	364	37	34	1.67	16.6
K'_{Na} (ROV)	1-12-82	8279	42	63	0.10	66.0
	17-1-84	8279	55	33	0.50	22.0
	21-4-84	364	40	49	1.00	20.4
	16-3-82	364/1023	45	33	1.67	14.0
K'_{gly} (IOV)	21-4-82	364	40	28	36	0.98
	17-1-84	8279	55	41	50	0.31
	9-1-82	8279	60	22	90	0.72, 11.4
K'_{gly} (ROV)	21-4-82	1023	60	13	36	0.68
	17-1-84	8279	55	33	50	0.36
	9-1-84	8279	60	18	90	0.52, 14.1

[a] Vesicles were prepared and assayed as described in Materials and Methods and Figs. 3 and 6.

the same cells (*cf.* Fig. 4). For the determination of kinetic parameters the Michaelis-Menten equation was transformed to the Hill-type equation:

$$v/V_{max} = [Na]^n/(K'_{Na} + [Na]^n).$$

The Eadie-Hofstee plots of $v^{1/n}[Na^+]$ versus $v^{1/n}$ for $n = 2$ approximated a straight line (Fig. 6). The data from Figs. 4 and 6 as well as other similar experiments are summarized in Table 2. As shown the apparent K'_{Na} and K'_{gly} values for IOV and ROV, the one at different concentrations of the other, are generally similar.

Na^+/Glycine Stoichiometry

Table 3 summarizes results of two representative experiments aimed to assess the coupling ratio of sodium to glycine uptake in IOV and ROV. In these experiments, glycine-enhanced uptake of $^{22}Na^+$ and Na^+-enhanced uptake of ^{14}C-glycine were followed concommitantly. As expected, the stoichiometry of the Na^+-dependent glycine transport is the same for IOV and ROV, with two sodium ions being transported for each molecule of glycine transported. This holds true for the experiments carried out at both 1 and 5 mM glycine.

Discussion

In this study, preparations of membrane vesicles of distinct sidedness have been exploited in order to

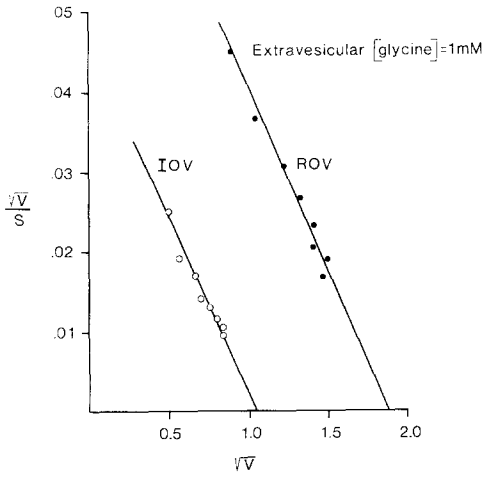

Fig. 6. Eadie-Hofstee plots of sodium-dependent glycine influx as a function of sodium concentration. IOV (41%) and ROV (33%), prepared concurrently from the same reticulocytes (55%, sheep #8279) were equilibrated overnight at 0°C, then 15 at 37°C in 100 mM KCl, 1 mM $MgCl_2$, 10 mM MOPS-Tris, pH 7.4 and then concentrated by centrifugation for 10 min at 13,000 × *g* to either 5.62 mg/ml (open circles), or 4.74 mg/ml (closed circles), respectively. The assays were initiated by adding 0.1 volumes of prewarmed (37°C) IOV or ROV to 0.9 volumes of prewarmed (37°C) medium containing 10 mM MOPS-Tris, pH 7.4, 1 mM $MgCl_2$, 0.56 mM ^{14}C-glycine (specific activity 2.97 × 10^4 cpm/nmol) and 100 mM KCl. In this experiment, the sodium salt was substituted for the potassium one (20 to 90 mM). Values for Na^+-dependent glycine uptake were obtained after substraction of the activity observed with NaCl replaced by KCl. The initial velocities were determined using linear regression analysis during the first minute of uptake. From linear regression analysis of the Eadie-Hofstee plots, the apparent K'_{Na} = 22 mM for IOV (r = 0.980) and K_{Na} = 22 mM for ROV (r = 0.990), based on the Hill-type relationship $v = V[Na]^n/K'^n_{Na} + [Na]^n$ and assuming $n = 2$

Table 3. Na^+/glycine stoichiometry[a]

Uptake measured	Exp. No.	Type of vesicle	Uptake (nmol/mg/min)		
1. Stoichiometry measured at 5 mM ^{14}C-glycine					
			−Na (a)	+Na (b)	Difference (b − a)
Glycine	1	IOV	7.0 ± 0.1	10.1 ± 0.1	3.1 ± 0.1
	2	ROV	6.7 ± 0.2	11.9 ± 0.2	5.2 ± 0.3
			−glycine (c)	+glycine (d)	(d − c)
Sodium	1	IOV	25.5 ± 0.3	31.4 ± 0.2	6.0 ± 0.4
	2	ROV	32.1 ± 0.6	42.2 ± 0.8	10.1 ± 1.0
		Ratio (d − c/b − a): 1.9 ± 0.1 (IOV); 2.0 ± 0.2 (ROV)			
2. Stoichiometry measured at 1 mM ^{14}C-glycine					
			−Na (a)	+Na (b)	(b − a)
Glycine	3	IOV	1.3 ± 0.0	3.8 ± 0.0	2.5 ± 0.1
	4	ROV	1.5 ± 0.0	6.4 ± 0.1	4.9 ± 0.1
			−glycine (c)	+glycine (d)	(d − c)
Sodium	3	IOV	19.0 ± 0.2	24.8 ± 0.1	5.8 ± 0.3
	4	ROV	22.0 ± 0.1	30.5 ± 0.2	8.5 ± 0.3
		Ratio (d − c/b − a); 2.4 ± 0.4 (IOV); 1.7 ± 0.4 (ROV)			

[a] In experiments 1 and 2 vesicles (either IOV or ROV, approximately 0.55 mg protein/ml) derived from reticulocytes (50%) were equilibrated overnight at 0°C, then for 30 min at 37°C with 40 mM KCl, 1 mM $MgCl_2$ and 10 mM MOPS (Tris form), pH 7.4. They were then concentrated by centrifugation to 3.55 and 3.98 mg protein/ml, respectively. The reaction was started by adding 0.09 ml prewarmed (37°C) isoosmotic medium to 0.01 ml prewarmed (37°C) vesicles. For glycine uptake, the media contained 5.56 mM ^{14}C-glycine (1550 cpm/nmol), 30 mM KCl or 22 mM NaCl plus 18 mM KCl, 1 mM $MgCl_2$ and 20 mM MOPS (Tris form), pH 7.4. For sodium uptake the media contained 22 mM ^{22}NaCl (1825 cpm/nmol), 18 mM KCl, 1 mM $MgCl_2$ and 10 mM MOPS (Tris form), pH 7.4 with and without 5.56 mM glycine. The final Na^+ and glycine concentrations were 20 and 5 mM, respectively. The reaction was terminated after 5 min and the vesicles (0.08-ml aliquots) were filtered and washed as described in Materials and Methods using ice-cold solutions of identical ionic composition as the final reaction medium. Experiments 3 and 4 were carried out as described above except that the vesicles were derived from 65% reticulocytes, and equilibrated with 100 mM KCl, 1 mM $MgCl_2$, and 10 mM MOPS (Tris form), pH 7.4. The IOV and ROV were concentrated to 5.59 and 6.74 mg/ml, respectively. For glycine uptake the medium contained 1.11 mM ^{14}C-glycine (1.44×10^4 cpm/nmol), 100 mM KCl or 22 mM NaCl plus 78 mM KCl, 1 mM $MgCl_2$ and 10 mM MOPS (Tris-form), pH 7.4. For sodium uptake, the medium contained 22 mM ^{22}NaCl (2.77×10^3 cpm/nmol), 78 mM KCl, 1 mM $MgCl_2$ and 100 mM MOPS (Tris form), pH 7.4 with and without 1.11 mM glycine. The final glycine concentration was 1 mM. The reaction was terminated after 3 min. Depending on the experiment, 100% of the total sealed membranes were either IOV or ROV. All values are the mean ± SEM of 3 to 5 determinations. The SEM of the ratio was determined according to Calquhoun (1971).

elucidate some basic characteristics of Na^+-dependent glycine transport which are difficult to explore with intact cells. Evidence for the coupled flow of Na^+ and solute in *both* directions has been obtained in experiments with intact cells (Curran et al., 1970; Koser & Christensen, 1971; Kimmich, 1981). However, adequate precision in the measurement of the coupling ratio has not been possible for fluxes occurring from the cytoplasmic to extracellular medium because of interfering substrate (*see*, for example, Koser & Christensen, 1971). Using IOV and ROV derived from reticulocytes it has been possible to examine concurrently the coupled Na^+ and glycine movements in both directions. The results indicate that this ratio is two Na^+ ions per glycine transported in both directions similar to that obtained for Na^+/glycine cotransport in pigeon erythrocytes (Vidaver, 1964*a*,*b*) as well as the rat hepatocyte (Christensen & Handlogten, 1981). It should be mentioned, however, that a 2 : 1 coupling ratio may not be a general phenomenon. Thus, in rabbit reticulocytes a value of 1 : 1 for amino acids other than glycine was obtained (Wheeler et al., 1965).

Na^+-dependent glycine transport in sheep reticulocytes resembles in certain respects that of both avian red cells (Vidaver, 1964*a*,*b*) and rabbit reticulocytes (Winter & Christensen, 1965; Eavenson & Christensen, 1967). With intact cells in 158 mM Na^+, the K'_{gly} for the high-affinity component is generally similar to that obtained for pigeon red cells (Vidaver, 1964*a*). Kinetic complexity apparent as a low affinity component was also apparent with both

intact cells and membrane vesicles, consistent with the high- and low-affinity sites reported by Winter and Christensen (1965) using rabbit reticulocytes. Although the apparent K'_{gly} obtained for the vesicles and intact cells varied to some extent, the preparations we used were not only from different animals, but differed with respect to the state of maturity of the cells from which they were derived. It would be of interest to know whether there is a change in kinetic properties, albeit relatively small, as the cells mature.

From Fig. 6, it is apparent that the data describing ^{14}C-glycine transport as a function of Na^+ concentration fit well to the Eadie-Hofstee plot of the Hill-type equation given above, for $n = 2$. Although such behavior itself is not sufficient evidence for a stoichiometry greater than 1:1, this observation taken together with the Na^+/glycine stoichiometry data argue strongly in favor of a 2:1 ratio. A limitation of the present direct stoichiometry evaluation concerns the questions whether a 2:1 stoichiometry exists under all conditions of substrate concentrations. For technical reasons, it has not been feasible to raise the Na^+ concentration much above 20 mM, to decrease the glycine much below 1 mM or above 5 mM. Nevertheless, with both 1 and 5 mM glycine, a value of 2.0 was obtained. This constant ratio argues against the possibility that the coupling ratio appears greater than unity because of glycine cycling on the Na^+-dependent carrier, in the absence of Na^+, as described for methionine movement across the brush border of the rabbit distal ileum (Paterson et al, 1980).

The efficacy of the high-affinity Na^+-dependent glycine transport system of the sheep reticulocyte resembles that of the pigeon red cell (Vidaver, 1964*a*,*b*; Imler & Vidaver, 1972) in terms of both the Na/glycine stoichiometry and the anion dependence. It is clear that substitution of chloride by sulfate effects close to complete inhibition of Na^+-coupled glycine uptake. The dependence on chloride is probably not absolute, however, since experiments with intact cells (*not shown*) indicate that the system can operate with other anions present, namely thiocyanate, as observed with pigeon cells (Imler & Vidaver, 1972).

Although the system is rheogenic (Benderoff et al., 1978), it is possible that the chloride dependence reflects either a mechanism involving a singly charged complex of transporter, *C*, two sodium ions, glycine and chloride $(Na_2Cl.C.Gly)^+$ as depicted by Vidaver et al. (1976), or that the chloride-dependence reflects only the highly electrogenic character of a complex comprising 1 glycine molecule and 2 Na^+, i.e., $(Na_2.C.Gly)^{2+}$ whereby a highly permeant anion such as chloride rapidly dissipates any (inhibitory) electrical gradient. A consequence of the high chloride conductance, relative to Na^+ or K^+ conductance (for sheep red cells, *see* Tosteson et al., 1972) is that the membrane potential of the vesicles is effectively clamped at a value close to zero. Thus, it may be assumed that the stoichiometry measurements are not complicated by any transmembrane electrical gradients.

The major departure of the results of the present study with membrane vesicles from earlier studies with intact Ehrlich ascites cells (Johnstone, 1978) or hemolyzed and resealed pigeon red cells (Vidaver, 1964*b*) concerns the question of symmetry (or asymmetry). In the present study, the apparent affinities for Na^+ and for glycine, the one at varying concentrations of the other, are similar. In some experiments, even with vesicles derived from the same cells (*see* Fig. 6), a difference in apparent V_{max} was observed. However, the significance of such a difference is questionable since it is quite plausible that inactivation or segregation of the putative transporter in vesicles relative to the residual unsealed membranes is different, and different also in IOV compared to ROV. It is also plausible that the transporter is more labile to inactivation in the one type compared to the other type of vesicle. A comparison of apparent affinities is thus more meaningful.

The similarities in K'_{gly} and K'_{Na} values contrast markedly with the greater K'_{gly} for exit compared to entry in pigeon red cells (fivefold: Vidaver & Shepherd, 1968) and Ehrlich ascites tumor cells (10-fold: Johnstone, 1979). In the latter study $\Delta\mu_{Na}$ was reduced to zero with gramicidin.

Our results raise the question whether the apparent asymmetry noted in the studies cited above is an inherent property of the transport system or secondary to some regulation by cytoplasmic factors. In support of the latter possibility are studies which indicate that both the glucose transport system and the nucleoside transport system of the human red cell change from directional symmetry to asymmetry. In the former case, the change is effected by cytosolic factor(s) (Carruthers & Melchior, 1983), in the latter, by blood storage (Jarvis et al., 1982).

The assistance of Dr. J.L. Neal in the kinetic analysis is gratefully acknowledged. This research was supported by Grant MT 3876 from the Medical Research Council of Canada.

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Received 30 October 1984; revised 18 January 1985