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Efflux and exchange of glycine by plasma membrane vesicles isolated from glioblastoma cells

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The efflux and exchange of glycine were studied in plasma membrane vesicles isolated from cultured glioblastoma cells. The mechanism of glycine translocation has been probed by comparing the ion dependence of net efflux to that of exchange. Dilution-induced efflux requires the simultaneous presence of internal sodium and chloride, while influx is dependent on the presence of these two ions on the outside (Zafra, F. and Giménez, C. (1986) Brain Res. 397, 108–116). Glycine efflux from the membrane vesicles is stimulated by external glycine, this exchange being dependent on external sodium, but not on external chloride. The parallelism observed in influx and efflux processes suggests that glycine is translocated in both directions across the membrane, probably by interacting with the carrier. To account for all the observed effects of external ions, glycine concentrations and membrane potential on glycine influx and efflux, a kinetic model of the Na^+/Cl^-/glycine cotransport system is discussed.

Introduction

High-affinity, Na^+-dependent uptake of neurotransmitter amino acids by neural tissue is throught to represent reuptake by nerve endings and to be a major means of termination of their action [1]. On the other hand, several lines of evidence suggest that glial cells of the central nervous system play an important role in the modulation of neural excitability via control of the levels of neuroactive substances in the extracellular millieu of neurons [2–4]. Moreover, transport systems similar to those found in presynaptic terminals for several amino acids have been identified in glial cells [5–10].

Glycine, besides its important role in numerous metabolic functions [11], has been postulated to be an inhibitory neurotransmitter in the mammalian central nervous system [12], mainly in the spinal cord [13] and probably in some localized areas of the brain, such as the substantia nigra [14].

On the other hand, membrane vesicles isolated from various bacterial and mammalian cells, including neurons [15–17], and specifically from the glial plasma membrane [18], have been shown to be extremely useful for studying the amino-acid transport mechanisms, entailing a well-defined ion environment and energy sources and avoiding metabolic and compartmentation interference.

We have previously reported [18] that the transport of glycine in membrane vesicles derived from glial cells is an electrogenic process strictly depen-

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Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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dent on the presence of Na^+ in the medium. In addition, the influx process of glycine is absolutely dependent on the simultaneous presence of Cl^- [19].

The measurement of efflux, in addition to that of influx, can provide better knowledge of the roles of these ions in the transport process. Moreover, the ability of a solute to trans-stimulate its efflux through the membrane shows that the same transport system can move the solute in both directions reversibly, and that the direction of movement across the membrane may be related to the bioenergetics and mechanisms of uptake.

In this article we report the effects of membrane potential, Na^+ and Cl^-, and homoexchange across the membrane on the efflux of glycine from plasma membrane vesicles isolated from glial cells.

Materials and Methods

Materials

[U-^{14}C]Glycine was obtained from Amersham International, U.K., Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum were obtained from Gibco. Dishes for tissue culture were purchased from Costar, The Netherlands. Valinomycin, furosemide, *N*-ethylmaleimide, diethyl pyrocarbonate and phenylglyoxal, were purchased from Sigma, U.S.A. Diazotized sulfanilate was obtained from Calbiochem. Ficoll was provided by Pharmacia Sweden and was exhaustively dialysed against water before use. All other reagents used were of the highest purity available.

Cell culture

The cell line was obtained from the American Type Culture Collection. Cultures were grown at 37 ° C in a humidified atmosphere of 5% CO_2 and 95% air. The growth medium was changed at 3-day intervals, the medium containing 10% fetal calf serum. Only cell cultures at the stationary growth phase were used for cell membrane isolation.

Membrane vesicle preparation

Membrane vesicles were prepared essentially as described previously [18]. Cells from about 20 confluent roller bottles were used for each membrane preparation. Bottles were rinsed twice with 0.3 M mannitol/1 mM EDTA/10 mM Tris-HCl (pH 7.4) (solution 1) and then cells were harvested by scraping with a rubber blade. Subsequent steps were carried out at 4 ° C. Cells were centrifuged at $3000 \times g$ for 10 min, resuspended in 60 ml of solution 1 and broken down with ten strokes of an all-glass Potter-Elvehjem with a tight-fitting pestle. The homogenate was centrifuged for 10 min at $3000 \times g$ in a Sorvall SS34 rotor and the pellet was washed by resuspension in 30 ml of solution 1 and centrifugation at $3000 \times g$ for 10 min. Both supernatants were combined (S1) and centrifuged for 20 min at $27000 \times g$ to obtain a crude mitochondrial pellet (PII). PII was resuspended in about 3 ml of solution 1 and layered over two discontinuous gradients, each consisting of five layers, of 20, 16, 12, 8 and 2% Ficoll in solution 1, respectively. The gradients were then centrifuged in a Beckman SW28 rotor at $75000 \times g$ for 90 min. The 2–8% and 8–12% interfaces (F_1 and F_2, respectively) were collected, combined and diluted with 4 vols. of solution 1. F_1 and F_2 were centrifuged at $27000 \times g$ for 20 min. The pellet was subjected to osmotic disruption in 20 ml of 5 mM Tris-HCl/1 mM EDTA (pH 7.4). After stirring for 45 min, the suspension was centrifuged again (20 min at $27000 \times g$) and the pellet resuspended in 10 ml of a 300 mosM medium (pH 7.4) with ionic composition depending on each particular experiment. Finally, the suspension was centrifuged in the former medium to a protein concentration of 5 mg/ml. Portions were frozen in liquid nitrogen, and stored at −70 ° C. Under these conditions, membrane vesicles were functional for at least 1 month.

Efflux from actively loaded vesicles

Portions of 10 μl (about 45 μg protein, unless otherwise indicated) were preincubated for 2 min at 25 ° C. Uptake was started by adding 40 μl of a solution containing [U-^{14}C]glycine (20 μM final concentration) in 120 mM NaCl/22 mM sodium phosphate/1 mM $MgSO_4$ (pH 7.4) (influx solution). After the indicated times, the incubation mixtures were diluted 20-fold with the specified efflux solutions. The experiment was terminated by dilution with 5 ml ice-cold 0.8 M NaCl, and immediate filtration through a moistened Millipore filter RAWP 02500 (1.2 μm pore size) attached to a vacuum assembly. The filters were rinsed twice with the ice-cold medium. The dilution, filtration and washing procedures were performed in less than 15 s. The filters were dried at 60 ° C, placed in microvials and their radioactivity was measured by liquid scintillation spectrometry. The zero-time value was obtained by adding the cold stop solution before the efflux solution. In order to avoid possible bacterial contamination, all solutions used in the experiments were prepared with distilled-deionized water and filtered through Millipore filters (0.45 μm). The osmolarity of all solutions was kept constant during the experiments. All incubations were carried out in triplicate. Each experiment was repeated at least three times with different membrane preparations.

Membrane pretreatments

Membranes were preincubated with reagents in the presence or absence of 5 mM glycine before osmotic disruption. Preincubations were performed during 15 min at 37 ° C (pH 7.4) for all reagents except for diethylpyrocarbonate (0 ° C, pH 6.6), and were terminated by dilution with 20 vols. hypotonic buffer (5 mM Tris-HCl/1 mM EDTA (pH 7.4)). After centrifugation at 27 000 × *g* for 20 min, membranes were resuspended in the final internal medium. The centrifugation-resuspension process was repeated three times.

Protein determination

Membrane proteins were determined according to the method of Resch et al. [20].

Results

Fig. 1 shows that the glycine efflux from plasma membrane vesicles actively preloaded with [^{14}C]glycine is highly dependent on temperature, being inhibited at 4 ° C. This fact rules out a process of simple diffusion of glycine through the membrane as the only way to explain the efflux of the substrate induced by dilution of the membrane vesicles.

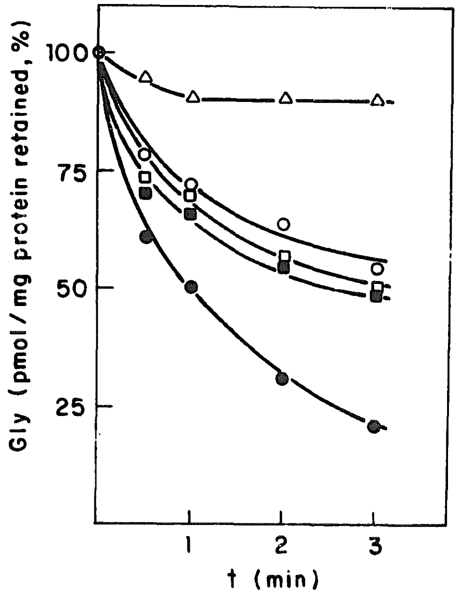

Fig. 1. Influence of temperature and internal Na$^+$ ions on glycine efflux. Membrane vesicles from C6 cells, preloaded with 150 mM potassium gluconate/5 mM Hepes-Tris/1 mM $MgSO_4$ (pH 7.4) were actively loaded during 2 min with the NaCl medium (150 mM NaCl/5 mM Hepes-Tris/1 mM $MgSO_4$ (pH 7.4)) containing 20 μM [^{14}C]glycine. The efflux was initiated by adding NaCl medium at 4 ° C (△) or 25 ° C (○, ●) or LiCl medium (150 mM LiCl/5 mM Hepes-Tris/1 mM $MgSO_4$ (pH 7.4)) at 25 ° C (□, ■). Dilution was made in the absence (empty symbols) or in the presence of 5 μM nigericin (full symbols).

The dependence of glycine efflux on internal sodium is shown in the experiment described in Fig. 1. It uses nigericin, which is able to exchange Na$^+$/K$^+$ or Na$^+$/H$^+$ electroneutrally across the membrane. In vesicles preloaded with potassium gluconate, the addition of nigericin to the Na$^+$-containing efflux medium produces an increase in the internal sodium concentration which results in an enhanced glycine efflux. No efflux stimulation by nigericin is observed when Na$^+$ is replaced by Li$^+$ in the efflux medium.

The experiment in Fig. 2 shows the effect of the internal chloride on glycine efflux. The vesicles containing either KCl or potassium gluconate were actively preloaded with radioactive glycine in NaCl medium. The time of influx was adjusted, both in order to limit the entrance of chloride, which was necessary at this stage, and to obtain similar glycine levels in both kinds of vesicle at the time when efflux is initiated. Glycine efflux from vesicles loaded with potassium chloride ($[Cl^-]_{in} > [Cl^-]_{out}$) is more active than the efflux from vesicles containing potassium gluconate. The efflux medium included nigericin and CCCP (proton ionophore) to avoid the limitation of Na$^+$ in the internal medium and to prevent the build-up of membrane potential, respectively.

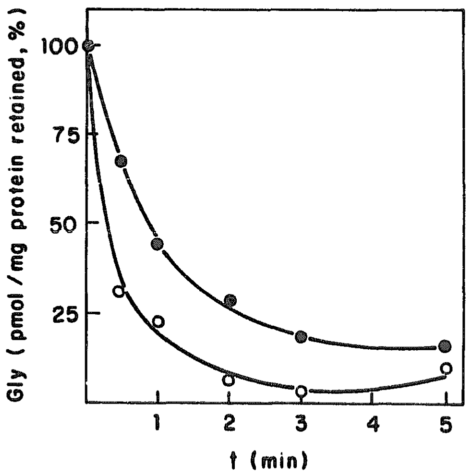

Fig. 2. Effect of internal chloride on glycine efflux. Membrane vesicles from C6 cells, preloaded with potassium gluconate medium (●) or KCl medium (150 mM KCl/5 mM Hepes-Tris/1 mM $MgSO_4$ (pH 7.4)) (○) were actively loaded with the influx solution during 45 s (●) or 60 s (○) in a NaCl medium containing 20 μM [^{14}C]glycine. Efflux was initiated by adding sodium gluconate medium containing 5 μM nigericin and 5 μM CCCP. The compositions of sodium chloride, sodium gluconate and potassium gluconate media are described in the legend to Fig. 1.

Fig. 3 indicates that the presence of unlabeled glycine in the external medium stimulates the efflux of radioactive glycine by a concentration-dependent homoexchange process. This exchange process occurs via the transporter. Because this process is very rapid, an accurate estimation of the initial rate was difficult to make. Although this imposes an important restriction on the determination of the K_m for external glycine in its ability to promote exchange, its half-maximal effect was obtained with 50–100 μM glycine concentration, a value which is in agreement with the K_m for glycine influx [19].

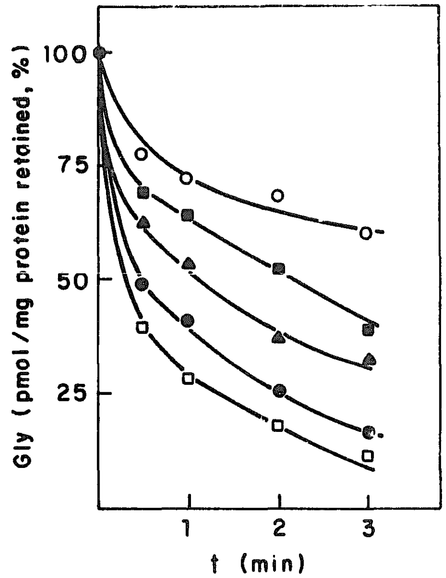

Fig. 3. Influence of external glycine concentrations on glycine efflux. Membrane vesicles were preloaded with potassium gluconate medium. After 2 min of influx, the efflux was induced by dilution with NaCl medium and the following additions: none (○), 50 μM (■), 100 μM (▲), 200 μM (●) or 400 μM (□) glycine.

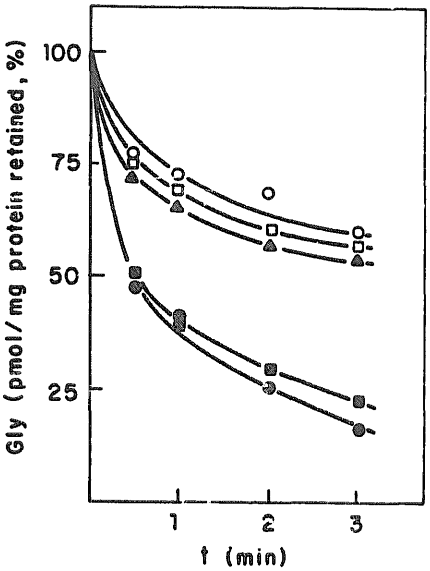

Fig. 4. Effect of external ions on the glycine exchange. Membrane vesicles from C6 cells preloaded with potassium gluconate medium were actively loaded during 2 min in a NaCl medium containing 20 μM [^{14}C]glycine. Efflux was initiated by adding 20 vols. of each of the following media: NaCl medium (○), NaCl medium + 400 μM glycine (●), sodium gluconate medium (□), sodium gluconate medium + 400 μM glycine (■) or LiCl medium + 400 μM glycine (▲).

Fig. 4 shows the effect of external Na^+ and Cl^- on the exchange of glycine. If Cl^- is replaced by gluconate, the efflux is also stimulated by external glycine. On the other hand, when the Na^+ ion is omitted, the ability of external glycine to stimulate efflux is lost.

These results indicate that the interaction of external glycine with the transporter under exchange conditions is different from that during net influx. The later requires both Na^+ and Cl^-, the former only Na^+. Interestingly, when the isotopic exchange of glycine was analyzed as a function of the Na^+ concentration under chemical-equilibrium conditions [21], a biphasic curve was ob-

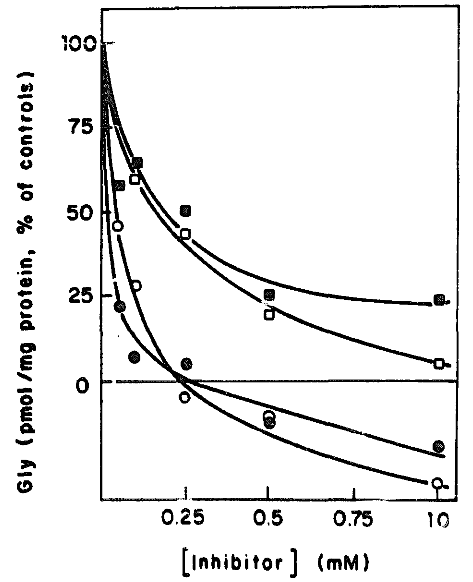

Fig. 5. Effect of group reagents on the glycine transport. Membrane vesicles preloaded with potassium gluconate were pretreated as detailed in Materials and Methods in the presence of the indicated concentrations of *N*-ethylmaleimide (●), diazotized sulfanilate (○), diethyl pyrocarbonate (□), or phenylglyoxal (■). After exhaustive washing, transport activity was determined for 10 min in a NaCl medium containing 20 μM [^{14}C]glycine, 0% corresponds to the transport observed in the absence of ionic gradients.

tained, with a maximum of stimulation of glycine exchange at 200 mM Na$^+$ and an inhibition at higher (600 mM) Na$^+$ concentrations (data not shown).

Additional data about the binding order, independent of the topographic information, were obtained from the following experiment, using reagents of functional group of proteins in the presence or absence of glycine in different ionic conditions.

The experiment depicted in Fig. 5 shows the inhibitory effect of several group reagents on the glycine transport. Diazotized sulfanilate, which is known to react with histidine and tyrosine residues of proteins, was the most effective reagent tested (IC$_{50}$ = 25 μM) followed by *N*-ethylmaleimide (selective for sulfhydryl group) with IC$_{50}$ = 40 μM, diethylpyrocarbonate (reagent of histidine) (IC$_{50}$ = 150 μM) and phenylglyoxal (selective for guanidino groups) with IC$_{50}$ = 200 μM. When the preincubation of the membrane vesicles was carried out in the simultaneous presence of glycine and a protein-modifying reagent, protection of the inhibitor effect of diazotized sulfanilate was observed, but this protection was not seen when the three other reagents were used (Table I). The

TABLE I

PROTECTION BY GLYCINE FROM THE INACTIVATING EFFECT OF PROTEIN REAGENTS ON GLYCINE TRANSPORT

Membrane vesicles were pretreated as described in Materials and Methods with the indicated additions. After exhaustive washing, transport assay was carried out for 10 min in NaCl medium containing 20 μM [^{14}C]glycine. Results are the mean ± S.E. of triplicate determinations in a representative experiment.

Additions during preincubation	Glycine uptake (pmol/mg protein per 10 min)
None	205 ± 13
5 mM glycine	199 ± 10
20 μM *N*-ethylmaleimide	131 ± 17
20 μM *N*-ethylmaleimide + 5 mM Gly	143 ± 11
60 μM diazotized sulfanilate	52 ± 12
60 μM diazotized sulfanilate + 5 mM Gly	150 ± 13
150 μM phenylglyoxal	139 ± 15
150 μM phenylglyoxal + 5 mM Gly	131 ± 10

TABLE II

IONIC DEPENDENCE OF PROTECTION BY GLYCINE FROM THE EFFECT OF DIAZOTIZED SULFANILATE

Protection from the inactivating effect of 75 μM diazotized sulfanilate was carried out preincubating membrane vesicles as indicated in the legend to Fig. 5, with the indicated ionic media in the presence of 5 mM glycine. After exhaustive washing, transport assay was carried out for 10 min in NaCl medium containing 20 μM [^{14}C]glycine. Results are the mean ± S.E. of triplicate determinations in a representative experiment and represent the recuperated activity in the presence of 5 mM glycine as the percentage of controls obtained without diazotized pretreatment. 100% corresponds to 174 pmol/mg protein per 10 min.

Ionic medium	Additions	% of recuperated activity
Sodium chloride	none	100 ± 9
Sodium chloride	75 μM diazotized sulfanilate	61 ± 10
Potassium chloride	75 μM diazotized sulfanilate	63 ± 15
Sodium gluconate	75 μM diazotized sulfanilate	68 ± 5
Potassium gluconate	75 μM diazotized sulfanilate	74 ± 8

experiment in the Table II indicates that the protecting effect of glycine on the diazotized sulfanilate inactivating effect was independent of the presence or absence of Na$^+$ or Cl$^-$ during the preincubation of membrane vesicles with the reagent. This experiment suggests that glycine binds to the transporter before or independently of either ion.

Discussion

Considering the results reported herein, it is possible to establish that the Na$^+$-coupled transport system for glycine, in membrane vesicles derived from C6 glioma cells, catalyzes glycine transport in either the inward or outward direction with vectorial translocation determined by the specific ionic gradients. The glycine efflux appears to require internal Na$^+$ and Cl$^-$, just as the glycine uptake was shown to be strictly dependent on the simultaneous presence of both ions in the external medium [19].

The exchange of internal radioactive glycine by external glycine is another mode of exit of glycine from vesicles. This process appears to be independent of external Cl$^-$ and dependent on external sodium. Evidence in support of the fact that external glycine exerts its effect via the carrier system is provided by the effect of the external glycine concentration on the stimulation of efflux. The half-maximal effect coincides with the K_m value proposed for glycine influx [18].

In Fig. 6, we present a kinetic model of the Na$^+$/Cl$^-$/glycine transport system that appears to be consistent with the present experimental observations and those reported in previous studies [19]. The fact that the process is electrogenic [19] imposes restrictions on the possible values for the stoichiometry of the process. Assuming that glycine is transported in its predominant form at neutral pH, the zwitterion, a relationship for the carrier like 2 Na$^+$: 1 Cl$^-$: 1 glycine suggested from our results (unpublished data), is adequate to explain the electrogeneity of the process. Similar models have been proposed for L-glutamic acid [22], β-alanine [17] or glycine in presynaptic preparations [23]. The model implies that only a fully loaded carrier (2 Na$^+$: 1 Cl$^-$: 1 Gly) can be translocated, and suggests that binding and debinding sequences occur through an ordered mechanism for Na$^+$ and glycine and a random mechanism for Cl$^-$ binding. Net influx occurs by step 1 to 7, net efflux occurs in the opposite direction (clockwise), while exchange may occur as a result of shuttling back and forth through steps 2 to 5. External Na$^+$ is needed for exchange to occur, thus at least one Na$^+$ ion should be released on the outside (step 1) before glycine (step 2). Several facts are consistent with this possibility, but other models can not be ruled out. Firstly, protection by glycine of the inactivating effect of diazotized sulfanilate on the transport does not require the presence of Na$^+$. Moreover, the biphasic curve obtained for glycine exchange, as a function of the Na$^+$ concentration in equilibrium conditions (data not shown), is consistent, according to Segel [24] or Hopffer and Groseclose [21], with an ordered mechanism for Na$^+$ and glycine binding or debinding. And, finally, the fact that both K_m and V_{max} are affected by a reduction in Na$^+$ concentration on the external face of the membrane [22] is consistent with a binding order, in which at least one Na$^+$ binds after glycine during the influx process [25]. For the sake of simplicity, it is assumed that both Na$^+$ ions are associating or dissociating together, but in fact we have no information on the binding of the second Na$^+$.

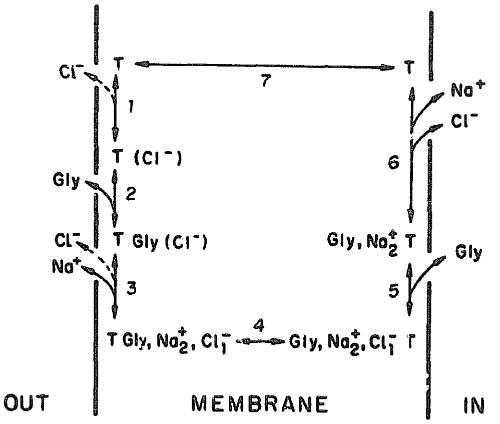

Fig. 6. Proposed model for the mechanism of the glycine translocation cycle. As discussed in the text, the binding and debinding orders of Na$^+$, Cl$^-$ and glycine, indicated here for the inside and the outside of the membrane, fit the experimental data; however, other models can not be ruled out.

For Cl$^-$, the situation is different. The fact that efflux is not dependent on the presence of Cl$^-$ on the external face is compatible with one of the two following possibilities: (a) Cl^- binds before glycine, thus, after radioactive glycine is released to the outside, unlabeled glycine can rebind to the transporter before Cl^- is released (step 1); or (b) the binding of glycine is independent of the binding of Cl^-. This second possibility is supported by the fact that the carrier is protected by glycine from the effect of diazotized sulfanilate in the absence of Cl^-, so glycine can bind to the carrier in the absence of Cl^-.

We have less information about the order of the process in the internal face of vesicles, but the fact that exchange occurs very rapidly when vesicles are diluted (when the concentrations of Na^+ and Cl^- are supposed to be low in the inner part of vesicles) suggest that after the release of one molecule of unlabeled glycine to the inside, a molecule of labeled glycine can bind before the Na^+ and Cl^- are lost (step 6) and so, the radioactive glycine can exit (step 5 to 1).

Acknowledgement

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