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Na^+-LINKED COTRANSPORT OF GLYCINE IN VESICLES OF EHRLICH CELLS

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Summary

Vesicles have been prepared from Ehrlich cells by using a method based on the early experiments of Forte et al. (Forte, J.G., Forte, T.M. and Heinz, E. (1973) Biochim. Biophys. Acta 298, 827—841) with some minor modifications, using the filter technique. From electron micrographs and from the sensitivity of these vesicles towards osmotic pressure changes in the medium induced by (nonpermeant) sucrose, it is concluded that the vesicles are closed. The counterflow phenomenon with glycine and Na^+-linked cotransport of glycine appears to indicate that these vesicles are still functioning. The observation of the overshoot phenomenon interpreted in terms of theoretical predictions confirms that the active accumulation of glycine is energized by the Na^+ electrochemical potential gradient. In particular, the contribution of the electrical components of these gradients is evidenced by the effects of the anion of the added sodium salt or of the addition of valinomycin. In contrast to observations by others, we found that ouabain does not directly affect Na^+-linked cotransport of glycine whereas $HgCl_2$ does so. Nor could any significant overshoot be demonstrated in the absence of an Na gradient. Since these vesicles were not metabolically active, these experiments do not exclude the possibility that in intact cells glycine is in addition transported primarily or partially actively.

Introduction

The gradient hypothesis postulates that the active transport of neutral amino acids is driven by the electrochemical potential gradient of Na^+. There is still

debate, however, as to whether the electrochemical Na gradient is energetically adequate for this purpose. So this gradient, if estimated on the basis of extra- and overall intracellular Na^+ concentrations and of the electrical potential as measured by microelectrodes of Cl^- distribution, would be grossly insufficient [1]. On the other hand, taking into account nuclear sequestration of Na and deriving the potential difference from the distribution of lipophilic cations or from the quenching of fluorescent dyes, one may arrive at energetically adequate Na gradients. It appears that the final solution of this dilemma can hardly be obtained with intact cells where the crucial information, namely the effective electrochemical potential gradient of Na, appears to be obscured by cellular subcompartments, in particular the nucleus and mitochondria.

Vesicles prepared from pure cell membranes, to the extent that they are closed and have retained the transport system under investigation, appear to be preferable for this purpose as they are devoid of cellular organelles and subcompartments. One should keep in mind, though, that owing to the lack of continuous metabolism the permeability of the vesicular membrane, to the extent that it depends on energy supply (phosphorylation), may be different from that of the intact cell. For the same reason, an Na gradient imposed to induce amino acid accumulation, not being stabilized by a pump, is bound to dissipate quickly. Hence, the active accumulation, whenever it occurs, should be transient manifesting itself often only by the 'overshoot' phenomenon.

Whereas numerous studies have been reported on vesicles of various tissues and cells, only a few have been carried out with vesicles from Ehrlich cells. The most important ones are those reported by Colombini and Johnstone [2,3], which, however, are not yet conclusive with respect to Na^+-linked cotransport. Whereas some of their observations support the gradient hypothesis, others are not easily compatible with it; for instance, that some accumulation of amino acids occurs in the absence of an electrochemical Na gradient and that the Na-stimulated amino acid uptake could be inhibited by ouabain, as would suggest the involvement of a mechanism different from cotransport. We therefore repeated and extended the above studies, using glycine as the test solute, with the main objective to test whether the electrochemical potential difference of Na^+ specifically is necessary and sufficient for producing a true cotransport, and furthermore whether the latter bears a positive relationship to the driving force. The overshoot phenomenon, provided that other causes could be excluded, was taken to be the most reliable indication of cotransport, whereas any residual accumulation of the amino acid, i.e., after the dissipation of the gradient, was considered questionable. Due to the extremely high surface : volume ratio of these preparations ($1—6 \cdot 10^{13}$ μm/ml), even minute adsorption of amino acid to the outer and inner vesicle surfaces may easily simulate accumulation where there is none. As quantitative evaluation of the complete overshoot curve is difficult, the initial rate and amplitude of the overshoot, i.e., the height of its peak, served as a crude basis to relate the effectiveness of the cotransport to the magnitude of the electrochemical Na gradient. In contrast to Colombini and Johnstone, we varied this gradient by changing the anion of the added sodium salt, rather than by using only ionophores. It will be shown further below that at equal initial chemical potential difference of Na^+, the electrical potential difference should predictably vary in a wide range with the relative permeancy of the anion.

Vesicles were prepared by a modified method based on previous studies of Forte et al. in this laboratory [4]. Various tests to be described in detail below appeared to indicate that the vesicles were closed against the suspending medium by a reasonably tight permselective barrier and that the transport system for glycine was still functioning. The present studies with these vesicles fully agree with the existence of Na-specific secondary active transport of glycine which, in contrast to previous studies by others [2,3], does require the presence of an Na gradient and is not directly inhibitable by ouabain.

Some theoretical considerations on overshoot

The overshoot phenomenon is usually interpreted as indicating an active, though temporary, accumulation of the test solute. Its subsequent decline toward equilibrium distribution between intra- and extravesicular space is attributed to a decay of the electrochemical potential gradient of Na^+ or H^+, respectively, owing to leakage.

There may be alternative causes of overshoot which have to be excluded before cotransport can be postulated such as:

(a) A transient membrane diffusion potential, due to different ionic mobilities of the added salt. This might cause accumulation of certain highly permeant ionic test solutes, but not neutral test solutes, and is not likely to be specific for Na^+.

(b) Transient swelling of vesicles due to osmotic water shift as may occur if the added salt enters the vesicles faster than intravesicular solutes leave it. Also this added effect is not likely to be specific for Na^+.

(c) Transient alkalinization of the intravesicular space, owing to Na^+-H^+ antiport. This may cause accumulation of weak acids which penetrate faster in the non-ionic than in the ionized form, but should not affect neutral solutes or electrolytes which are fully ionized at the outside pH.

The overshoot as a result of true Na^+-cotransport should be specific for Na^+ and apply to ionized and neutral test solutes as well. It should, however, depend on the relative mobility of the anion of the sodium salt, in a way predictably related to the ionic nature of the test solute. The principles of these relationships can easily be derived if the following simplifying assumptions are made, which appear to come close to reality in the system under investigation:

(1) The concentration of the test solute, A, is very small as compared to that of the sodium salt so that it will not appreciably affect the electrical potential difference nor the equivalence between Na^+ and X^-.

(2) The test solute (A) can penetrate the membrane rapidly by cotransport with Na^+, but barely so in the free form.

(3) The concentrations of permeant ions other than of the sodium salt are negligible.

The distribution of the test solute (A) can now be treated as a monitor of the effective electrochemical activity ratio of Na^+ between the intra- and extravesicular compartments. The slope of the ascending limb of the curve should give the rate at which the distribution of A tends to adjust to the (declining) electrochemical activity ratio of Na^+. When the distribution ratio of A meets the latter, the entry of the substrate becomes zero, and its distribution ratio passes its 'peak' value. The subsequent decline of this ratio, i.e., the descending

limb of the distribution curve, presumably monitors closely the declining electrochemical activity ratio of Na^+, and is therefore of little informative value for the present purpose; the amplitude of the overshoot, i.e., the height of the peak in cotransport, should be related to the electrochemical activity ratio of Na^+ as may be demonstrated on the basis of the following considerations:

At the peak of the overshoot, the net uptake of test solute has vanished. Hence, its electrochemical activity ratio must have attained the reciprocal electrochemical activity ratio of Na^+: *

$$\frac{a''}{a'} \cdot \xi^{z_a} = \frac{c_s'}{c_s''} \cdot \xi^{-z_{Na}}$$

where $\xi = \exp(-F\Delta\psi/RT)$; z_a and z_{Na} are the electrical valences of A and Na^+, respectively; a and c_s stand for the concentrations of the test solute and the salt, respectively. The superscripts, $'$ and $''$, refer to outside and inside the vesicle, respectively. The accumulation at the peak of the overshoot is therefore:

$$\frac{a''}{a'} = \frac{c_s'}{c_s''} \cdot \xi^{-(z_a + z_{Na})}$$

As for a neutral test solute, like glycine, $z = 0$ and for Na^+, $z = 1$. The overshoot peak is:

$$\left(\frac{a''}{a'}\right)_{gly} = \frac{c_s'}{c_s''} \cdot \xi^{-1}$$

i.e., equal to the inverse electrochemical activity ratio of Na^+ at the peak of the overshoot. If the test solutes were a monovalent anion ($z = -1$) or a divalent anion ($z = -2$) the inverse of the corresponding electrochemical activity ratios of Na^+ would be c_s'/c_s'' and $c_s'/c_s'' \cdot \xi$, respectively. ξ for a given concentration ratio of a binary sodium salt depends on the relative mobility of the anion as follows:

$$F\Delta\psi = \frac{r-1}{r+1} \cdot RT \cdot \ln(c'/c'')$$

$$\xi = \left(\frac{c'}{c''}\right)^{(1-r/1+r)}$$

where r indicates the relative mobilities (u) of cation and anion ($u+/u-$). z_a and z_{Na} are the electrical valencies of test solute (A) and Na^+, respectively. Hence, the maximum accumulation ratio of the test solute at the peak is:

$$\left(\frac{a''}{a'}\right)_{peak} = \left(\frac{c_s'}{c_s''}\right)^{2/(1+r)}$$

It is clearly seen that at a given value of (c_s'/c_s'') the peak value of (a''/a') increases with decreasing r, with the limits $(c_s'/c_s'')^2$, i.e., if the permeancy of the

* In keeping with the assumptions, leak pathways for the test solute have been ignored. Taking such pathways into account, the maximum attainable electrochemical activity ratio of the test solute is less than the reciprocal electrochemical activity ratio of Na^+. Even so, the main conclusion of this analysis will not be affected.

anion greatly exceeds that of Na^+. As for ionic test solutes, it follows that at $z_a = -1$, the electrical potential difference has no influence on the accumulation in the system whereas at $z_a = -2$ the peak accumulation should be:

$$\left(\frac{a''}{a'}\right)_{peak} = \left(\frac{c_s'}{c_s''}\right)^{2r/(1+r)}$$

i.e., the dependence on the relative mobilities of Na^+ and its anion should be reversed. Hence, one would predict that the peak of the overshoot of glycine cotransport would be the higher the more the permeancy of the salt anion exceeds that of Na^+. This relationship should become most pronounced if the peak is reached very soon and if the time of the peak is fairly similar for all sodium salts tested, as is approximately true in our experiments. On the other hand, the rate of decay of the concentration gradient should also increase with increasing relative mobility of the anion, an effect that somewhat weakens but does not abolish the above relationship. It may, therefore, be useful to test also the initial rate of the overshoot curve which should rise with necessary mobility of the salt anion for a neutral test solute, as follows from the following considerations.

The rate of entry of A via cotransport should be a function of the difference between the electrochemical activities of the ternary complex between carrier, solute and Na^+ at the two sides of the barrier. Assuming that the penetration of the barrier is by jumps rather than by diffusion through a homogeneous membrane, we can write for this rate:

$$J_a = P_a^*(a'c_s' \, \xi^{1/2} - a''c_s'' \, \xi^{-1/2})$$

To obtain the initial rate we set a'' and c_s'' equal to zero so that this rate is

$$J_a^0 = P_a^* \, a'c_s' \, \xi^{1/2}$$

At constant values of a' and c_s', J_a^0 depends mainly on $\xi^{1/2}$ which, according to the Goldman-Hodgkin equation, is for a binary salt, NaX:

$$\xi = \frac{P_X}{P_{Na}}$$

It is seen that the initial rate should rise with the permeancy of the anion relative to that of Na^+, if $z_a = 0$; if $z_a = -1$ or -2, the corresponding values would be 1 and P_{Na}/P_x, respectively.

Materials and Methods

Isolation of plasma membranes. Ehrlich ascites tumor cells were propagated as described by Pietrzyk and Heinz [1]. Plasma membranes of these cells were isolated according to the procedure outlined by Forte et al. [4], except for some changes.

The most significant modification concerned the composition of the discontinuous sucrose gradient which in our studies had the following composition (w/v): 60.5%, 5 ml; 47.1%, 15 ml; 40.4%, 15 ml; 26.95%, 15 ml. All sucrose solutions contained 25 mM NaCl, 20 mM Tris-HCl (pH 8), 0.5 mM $MgCl_2 \cdot 6\,H_2O$, 0.5 mM $CaCl_2$. A crude membrane preparation in the same buffer

without sucrose was overlayed. Following centrifugation at 103 000 × *g* for 90 min, a pellet and bands of material at each of the interfaces resulted. Enzymatic tests and amino acid transport activity assay appeared to indicate a bimodal distribution of plasma membranes which were located at two interfaces: 26.95—40.4% (band II) and 40.4—47.1% (band III). Except for density, most likely reflecting differing lipid composition, no major difference between these two fractions was observed, either enzymatically or electron microscopically. This is in contrast to the observations reported by Forte et al. [4]. After washing the membranes free of sucrose, they were resuspended in buffer using a syringe and a 25 gauge needle.

Enzymatic and chemical assays. Ouabain-inhibitable ($Na^+ + K^+$)-ATPase and K^+-stimulated phosphatase [3] were assayed as markers for plasma membrane. Endoplasmic reticulum was monitored by NADH oxidoreductase activity [5] with the addition of KCN [6]. Acid phosphatase was employed as an indicator of lysosomes [7] using *p*-nitrophenylphosphate as substrate at pH 4.5 after pretreating the samples at 0°C with 0.15% (v/v) Triton X-100. Succinate:cytochrome *c* oxidoreductase [8] was employed as a marker for inner mitochondrial membranes. DNA was assayed by monitoring mithramycin fluorescence [9] while protein was determined by using the method of Lowry et al. [10]. Inorganic phosphate was measured according to the method of Gerlach and Hiby [11] with slight modifications.

Electron microscopy. Samples of membrane preparations and washed cells were fixed with 2.5% glutaraldehyde, buffered with sodium cacodylate/HCl at pH 7.2.

For thin sectioning, samples were additionally fixed with buffered 1% OsO_4, then dehydrated and embedded in Spurr's resin [12].

For freeze-fracturing, glutaraldehyde-fixed material was stepwise infiltrated with glycerol up to about 25% and then frozen in liquid nitrogen. Fracturing was performed with an EPA 100 freeze-fracture machine (Leybold-Heracus, Cologne, F.R.G.) at —90°C. Platinum-carbon replicas, thin sections and membrane samples were negatively stained with ammonium molybdate and subsequently examined with a Philips 300 electron microscope.

Transport experiments. Most of the transport experiments were performed at 25°C with freshly prepared membranes. In a few cases, membranes stored at —16°C in buffer containing 20% glycerol were used. The latter membranes were thoroughly washed free of the storage buffer prior to uptake experiments. The standard buffer usually employed for membrane preparation and uptake studies was 100 mM mannitol/5 mM Hepes-Tris (pH 7.4)/0.1 mM $MgSO_4$ supplemented with salts and radioactive tracers. Uptake of labelled substances by isolation of plasma membrane vesicles was determined by a rapid filtration technique similar to that described by Murer et al. [13]. At distinct times after initiation of a transport experiment, a 20 μl aliquot of the reaction mixture was diluted into 1 ml of ice-cold quenching buffer, followed by rapid filtration through a Sartorius cellulose nitrate membrane filter (pore diameter 0.6 μm). The filter membrane was immediately washed with 4 ml of ice-cold quenching buffer and was ready for liquid scintillation spectroscopy after 15—20 s. The composition of the quenching buffer is described in each figure legend.

For some experiments the vesicles were preloaded with various salts, amino

acids or radioactive tracers by a 60-min preincubation at 25°C in buffer containing the substance.

Uptake of radioactively labelled substances is reported as a relative uptake or as a clearance, these terms being interchangeable. They are calculated as the amount of substance taken up/unit membrane protein per unit extravesicular concentration, giving dimensions of volume per unit membrane protein. Uptake can also be quantified as a relative content, which is the amount of radioactively labelled substance per unit membrane protein relative to that of a reference state. In this instance the reference state is specified in the figure legend.

Materials. All chemicals were reagent grade. Valinomycin and mithramycin were purchased from Serva Feinbiochemica. Radioactively labelled substances, [2-^{3}H]glycine, K^{35}SCN and ^{22}NaCl (carrier-free) were obtained from New England Nuclear.

Results

Characteristics of isolated membranes

Examination of freeze-fracture electron micrographs reveals that the final preparation contains vesicles of diameter 0.5 μm or less. These studies, however, do not indicate the type of membrane or the relative impermeability of the vesicles.

As shown in Table I, the protein yield of the combined membrane fraction,

TABLE I

CHEMICAL AND ENZYMATIC CHARACTERIZATION OF SUBCELLULAR FRACTIONS

Units of enzyme specific activities: NADH diaphorase and succinate:cytochrome *c* reductase, μmol substrate transformed/min per mg protein; all others, μmol substrate transformed/h per mg protein. DNA is expressed as μg/mg protein and protein is expressed as a percentage of homogenate value. Data are expressed as mean ± S.E., *n* is the number of preparations (in parentheses). Percent yield is shown in parentheses. Mitochondrial fraction is the 100 000 × *g* · min pellet obtained from the supernatant of the crude membrane pellet.

	Homogenate	Mitochondrial	Combined membrane fraction
Protein	100%	13.9% ± 1.3 (2)	0.88% ± 0.037 (34)
DNA	75 ± 12 (2) (100%)	29 (1) (5.8%)	12.4 ± 2.3 (2) (0.15%)
K^+-stimulated phosphatase	0.20 ± 0.0085 (90) (100%)	—	2.82 ± 0.14 (39) (16.6%)
($Na^+ + K^+$)-ATPase	1.31 ± 0.10 (3) (100%)	2.56 ± 0.26 (2) (23%)	21.5 ± 2.2 (7) (20%)
NADH oxidoreductase	0.247 ± 0.031 (3) (100%)	1.06 ± 0.085 (2) (61%)	0.166 ± 0.033 (4) (0.55%)
Acid phosphatase	1.61 ± 0.047 (3) (100%)	1.35 ± 0.049 (3) (11.4%)	1.83 ± 0.13 (7) (1.28%)
Succinate:cytochrome *c* reductase	20.5 ± 1.4 (2) (100%)	88.1 ± 13 (2) (60%)	22.8 ± 6.0 (2) (0.95%)

bands II and III, is about 0.9% of the initial amount. K^+-stimulated phosphatase and ($Na^+ + K^+$)-ATPase, enzyme markers for plasma membrane, are enriched in the membrane fraction about 14- and 19-fold, respectively. Their yields are approx. 17%. The corresponding yield of DNA is 0.15%, of NADH diaphorase 0.55%, of acid phosphatase 1.28% and of succinate:cytochrome *c* reductase 0.95%. These results indicate a significant enrichment of plasma membrane with respect to other membrane systems and organelles.

Test of vesicular structure

In order to find out whether the vesicles are closed compartments surrounded by a fairly tight, permselective membrane comparable to that of intact cells, the equilibrium uptake of labelled glycine was studied as a function of osmolarity which was varied with sucrose. Fig. 1 shows that the glycine uptake decreases considerably with increasing osmolarity, tending towards zero as the osmolarity of the medium approaches infinity. The course of the curve, however, deviates from what would be expected for ideal osmometers of which the volume and, consequently, the equilibrium glycine uptake should rise linearly

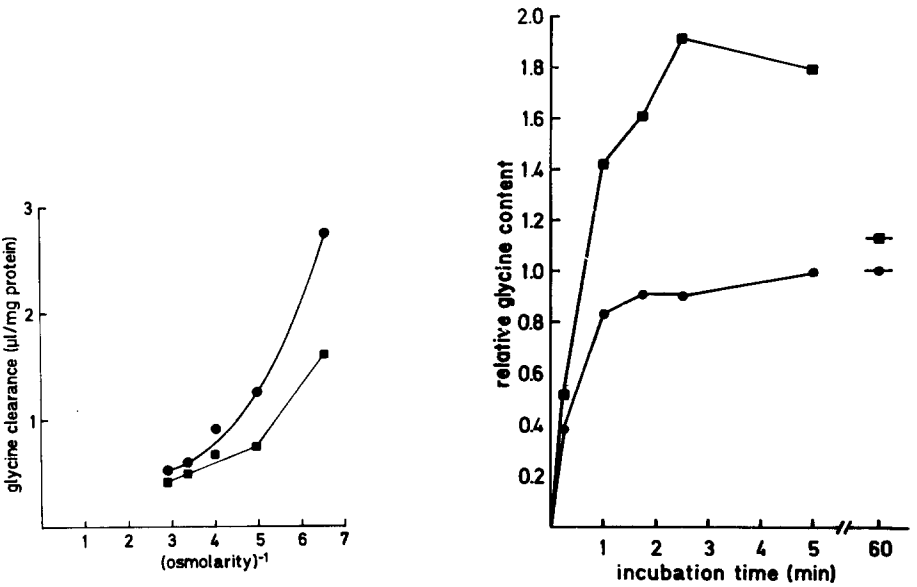

Fig. 1. Effect of osmolarity on equilibrium level of glycine uptake by isolated membrane vesicles from Ehrlich ascites cells. The standard buffer was supplemented with 25 mM NaCl and the incubation media additionally contained 0.83 mM [^{3}H]glycine and varying sucrose concentrations. Incubation time was 11—16 min before termination by the rapid-filtration technique. In the quenching buffer, choline chloride was substituted for NaCl.

Fig. 2. Counterflow of glycine uptake by glycine-loaded, isolated membrane vesicles. Fresh membranes were prepared in 100 mM mannitol/5 mM Hepes-Tris (pH 7.4)/0.5 mM $MgCl_2$/0.5 mM $CaCl_2$. Vesicles were preloaded with either D-sorbitol (•) or glycine (▪), each at 27.3 mM final concentration. Uptake was initiated by an 11-fold dilution of preloaded vesicles into a medium containing, at final concentrations, 90.9 mM mannitol/82.6 mM NaCl/2.48 mM [^{3}H]glycine/2.48 mM D-sorbitol/0.45 mM Hepes-Tris (pH 7.4)/0.45 mM $MgCl_2$/0.45 mM $CaCl_2$. Quenching buffer consisted of 100 mM mannitol/100 mM choline chloride/5 mM Hepes-Tris (pH 7.4)/0.5 mM $MgCl_2$/0.5 mM $CaCl_2$. Uptake is relative to that of control vesicles at 60 min.

with the reciprocal osmolarity of the medium. Instead, the glycine uptake appears to rise more strongly than expected. No explanation of this behavior can be given at the present time, though leakiness of the vesicles to sucrose would cause a bend of the curve in the opposite direction.

Transport function of vesicles

To test whether the glycine transport system is still functioning in the vesicles, the exchange of glycine by counterflow (homeo exchange) was studied. It is seen (Fig. 2) that after preloading vesicles with unlabelled glycine, the influx of labelled glycine added to the outside is greatly enhanced. Further evidence of a functioning transport system follows from the observation of Na^+-linked transport treated in more detail below.

Unspecific binding of glycine

As the extrapolated uptake curve in Fig. 1 tends towards the origin as the osmolarity approaches infinity, binding of glycine to vesicular material appears to be small. However, since the glycine uptake at such high osmolarities is difficult to measure, another test for glycine binding has been provided. This was based on the expectation that the number of binding sites is limited, leading to a saturation phenomenon at higher glycine concentrations. Vesicles were equilibrated at constant osmolarity with increasing concentration of glycine in the presence of Na^+. The relative uptake of glycine should be independent of the glycine concentration if all vesicular space were filled with glycine, but should decrease with increasing glycine concentration to the extent that saturable binding is involved. It was found that the relative uptake of glycine for any given preparation appears to be independent of the outside glycine concentration, over the range 0.23—23 mM. Unless the adsorption to binding sites is still very far away from saturation, which appears very unlikely, these observations point against significant adsorption of glycine to vesicular material.

Cation specificity of overshoot

Fig. 3 shows that the overshoot phenomenon is obtained only in the presence of a gradient of Na^+, i.e., if no Na^+ is initially present inside. Similar results are obtained if the vesicles are preloaded with choline chloride instead of mannitol. If vesicles are pre-equilibrated with the Na^+-containing medium, no overshoot is observed. In contrast to NaCl, KCl does not produce any over-

TABLE II

CATION SPECIFICITY OF GLYCINE UPTAKE BY ISOLATED MEMBRANE VESICLES

Vesicles were pre-equilibrated in a medium of final concentration 8.33 mM mannitol/4.2 mM Hepes-Tris (pH 7.4)/0.42 mM $MgCl_2$/0.42 mM $CaCl_2$/83.3 mM salt. The initial 15-s uptake of glycine in the absence of a salt gradient was determined and expressed as a percentage of uptake in the presence of NaCl. Quenching buffer was 100 mM mannitol/100 mM choline chloride/5 mM Hepes-Tris (pH 7.4)/0.5 mM $MgCl_2$/0.5 mM $CaCl_2$. Values are expressed as percentages.

Fraction	NaCl	LiCl	KCl	Choline chloride
BII	100	37.8	55.4	34.7
BIII	100	46.3	52.2	43.6

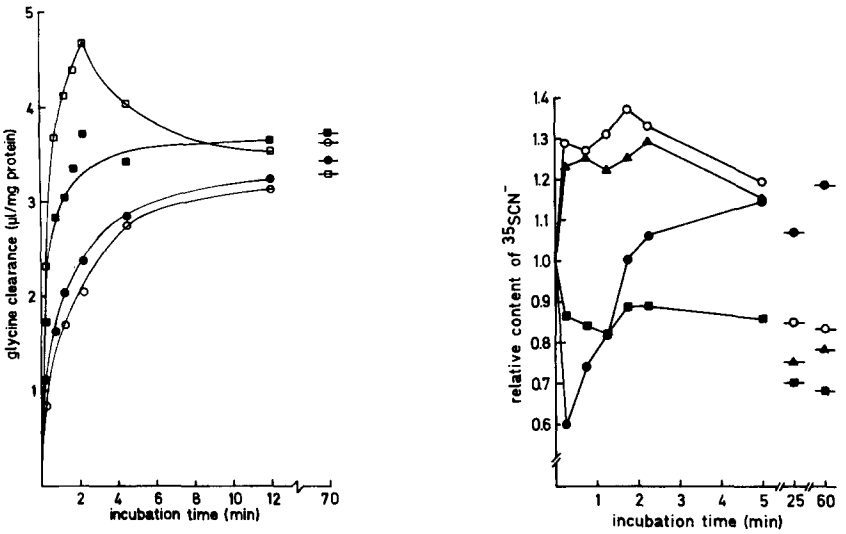

Fig. 3. Na^+-gradient-stimulated glycine uptake by isolated plasma membrane vesicles. Incubation media were of the same composition as the standard buffer but supplemented with 0.2 mM [^{3}H]glycine and either 100 mM NaCl (□, ■) or 100 mM KCl (○, ●). Glycine uptake was obtained at 25°C under conditions of an initial gradient (outside > inside) of NaCl (□) or KCl (○) or in the absence of gradients using vesicles pre-equilibrated with either 100 mM NaCl (■) or 100 mM KCl (●). Choline chloride was substituted for NaCl and KCl in the quenching buffer.

Fig. 4. Redistribution of SCN^- across vesicular membranes by diffusion potentials of various sodium salts. Vesicles were prepared in the standard buffer and pre-equilibrated with 0.52 mM $K^{35}SCN$. Time course of vesicular content of $^{35}SCN^-$ was followed in the presence of 100 mM NaCl (●), 100 mM sodium gluconate (▲), 100 mM sodium cyclamate (■) or 50 mM Na_2SO_4 (○). Initial vesicular content of $^{35}SCN^-$ was determined without the 1.1-fold dilution step. Quenching buffer: 150 mM mannitol/50 mM Na_2SO_4/5 mM Hepes-Tris (pH 7.4)/0.1 mM $MgSO_4$. Uptake is relative to vesicular content of $^{35}SCN^-$ prior to addition of salt.

shoot phenomenon whether there is a K^+ gradient or not. Even without a gradient, the equilibration of glycine in the presence of Na^+ is significantly faster than in the presence of K^+.

Table II shows the initial rate of glycine entry into vesicles that were pre-equilibrated with the chlorides of various alkali ions. Apparently, the rates obtained with NaCl exceed by 2- to 3-fold those obtained with any of the other salts tested.

Effect of anions on overshoot

According to our theoretical considerations for a neutral test solute, both the initial uptake rate and the peak value of overshoot should rise with the relative permeability of the vesicle membrane to the anion of the sodium salt. Before testing the present system in this respect, the relative permeability coefficients (P) of the anions of various sodium salts were estimated from their perturbing effect on the distribution of SCN^-. For this purpose, vesicles pre-equilibrated with KSCN were exposed to various sodium salts added to the medium, producing only a 10% dilution of the SCN^-. The SCN^- distribution should be transiently altered due to a diffusion potential caused by the added salt. In Fig. 4, it is seen that NaCl initially produces a rapid exit of SCN^- indi-

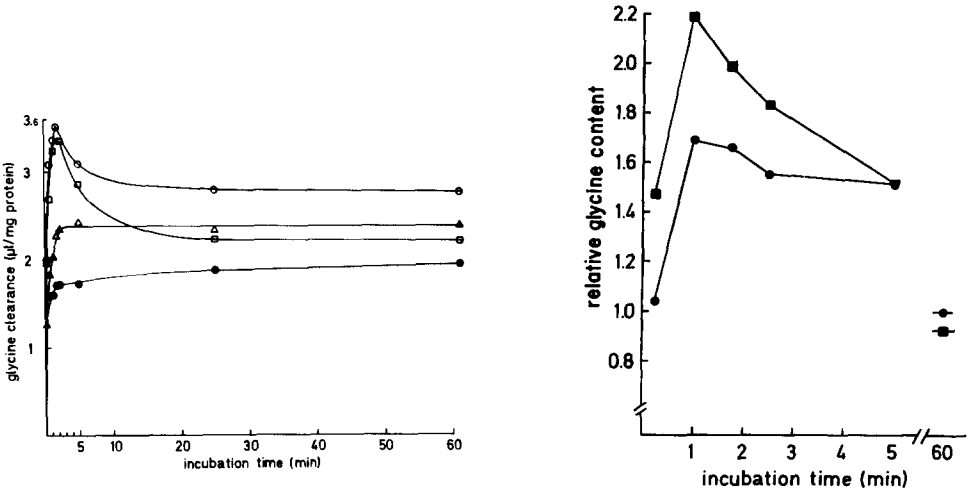

Fig. 5. Effect of anions on Na^+-gradient-stimulated glycine uptake by isolated membrane vesicles. Vesicles were prepared in the standard buffer. Uptake medium was composed of the standard buffer supplemented with 0.2 mM [^{3}H]glycine and 100 mM NaCl (○) or 100 mM NaSCN (□) or 100 mM sodium cyclamate (△) or 50 mM Na_2SO_4 and 50 mM mannitol (●). Quenching buffer was the standard buffer with 100 mM choline chloride.

Fig. 6. Effect of valinomycin-induced K^+-diffusion potential on Na^+-gradient-stimulated glycine uptake by isolated membrane. Vesicles were prepared in 100 mM KCl/20 mM Tris-HCl (pH 7.4)/0.5 mM $MgCl_2$/0.5 mM $CaCl_2$. At zero time, membranes were diluted 11-fold into an incubation medium of the same composition as above with an isosomotic replacement of KCl by NaCl and additions of 0.18 mM [^{3}H]-glycine, 0.9% (v/v) absolute ethanol with (■) or without (●) valinomycin (4.6 µg/mg protein). the composition of stop and wash solutions was 100 mM mannitol/100 mM choline chloride/20 mM Tris-HCl (pH 7.4)/0.5 mM $MgCl_2$/0.5 mM $CaCl_2$. Uptake is relative to that of control vesicles at 60 min.

cating a transient electric potential difference, negative inside the vesicle, suggesting that Cl^- is more permeant than Na^+. The opposite occurs with SO_4^{2-} and gluconate, presumably because both of these anions are much less permeant than Na^+. Cyclamate affects SCN^- distribution only slightly, presumably because the permeability of the vesicle membrane to this anion is not much different from that of Na^+.

From these findings we would expect that the initial rates and the overshoot peaks of glycine uptake should be highest for NaCl and lowest for Na_2SO_4. Fig. 5 shows that both these values are maximal in the presence of either NaCl or NaSCN, whereas in the presence of cyclamate or of SO_4^{2-} no overshoot is observed. It would appear that these anions, in contrast to Cl^- and SCN^-, are so slow that before glycine exceeds the equilibrium distribution the electrochemical potential gradient of Na^+ has already been dissipated. From both initial rates and overshoot peaks one would expect that Cl^- and SCN^- enter the cell equally rapidly. The descending limbs of the respective curves, however, seem to suggest that the dissipation of the Na gradient is faster with SCN^- than with Cl^-. As in the presence of fast anions, the rate of dissipation of this gradient should primarily be determined by the mobility of Na^+; a fully satisfactory explanation for this different behavior cannot be given at the present time.

Effect of valinomycin on overshoot

The significance of an electrical potential difference for the Na^+-linked

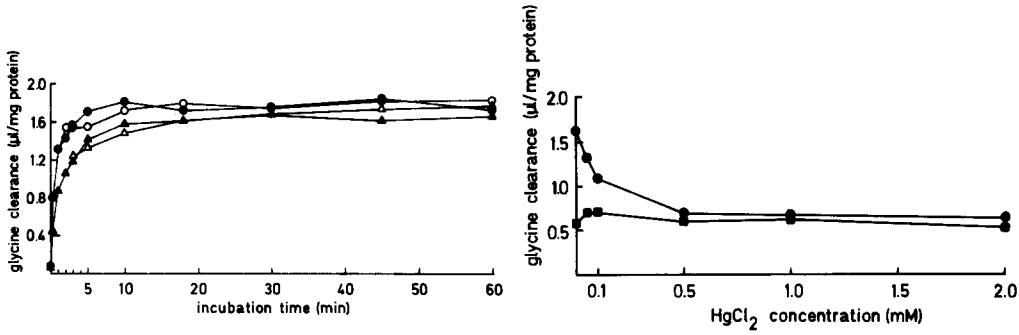

Fig. 7. Effect of ouabain on glycine uptake by isolated membrane vesicles. Vesicles were prepared in the standard buffer and pre-equilibrated with 100 mM NaCl (○, ●) or 100 mM KCl (△, ▲). Uptake of glycine from a medium containing 0.2 mM [^{3}H]glycine with (●, ▲) or without (○, △) 1 mM ouabain was studied in the absence of a salt gradient. Quenching buffer was the standard buffer supplemented with 100 mM choline chloride.

Fig. 8. Effect of $HgCl_2$ on glycine uptake by isolated membrane vesicles. Vesicles were prepared in the standard buffer and pre-equilibrated with either NaCl (●) or KCl (■), each at 100 mM. Uptake of glycine was initiated by dilution into medium of the same composition supplemented with 0.2 mM [^{3}H]glycine and $HgCl_2$ at the indicated concentration. Transport activity was terminated after 15 s. Quenching buffer was the standard buffer with 100 mM choline chloride.

cotransport of a neutral solute into the vesicles is confirmed by experiments with opposite K^+ gradients in the presence of valinomycin. Fig. 6 shows that the height of the peak, and most likely also the initial rate of uptake, is strongly enhanced by the addition of valinomycin to vesicles preloaded with KCl and suspended in an NaCl medium. Apparently, valinomycin raises the electrical potential difference by increasing the membrane permeability to K^+.

Effect of inhibitors on glycine uptake

It has been claimed by Colombini and Johnstone [3] that ouabain affects the uptake of glycine by vesicles of Ehrlich cells either directly or, at least, not merely by inhibiting ($Na^+ + K^+$)-ATPase. So they reported that ouabain decreases the rate of aminoisobutyric acid uptake in the presence of an NaCl gradient. If ouabain inhibits cotransport of glycine and Na^+ directly, it should do so also in the absence of an Na gradient, i.e., under conditions in which Na^+ functions only by catalytically accelerating the glycine equilibration. In order to test this, vesicles were equilibrated with NaCl and subsequently studied for the uptake of glycine in the presence and absence of ouabain (Fig. 7). Clearly, ouabain added at the same time as glycine has no effect on either Na^+-dependent or Na^+-independent glycine uptake. Therefore, these results point against a direct effect of ouabain on Na^+-dependent processes in general.

In contrast to ouabain, $HgCl_2$ does have a specific inhibitory effect on Na^+-linked glycine inflow in the absence of an Na gradient. As seen in Fig. 8, 0.5 mM $HgCl_2$ reduces the glycine influx in the presence of Na^+ to the influx obtained if NaCl is replaced by KCl, while it does not affect the latter appreciably.

Discussion

Vesicles were prepared from Ehrlich cells by an extended method based on that of Forte et al. [4]. Enzymatic tests and electron microscopic examinations

indicated that the preparation contained mainly membraneous material of plasma membrane origin. While the present method yielded only half the amount reported by Colombini and Johnstone [14] it appeared to have the advantage that zinc-fixation is avoided, that intravesicular volumes relative to protein are higher (5—10-fold) and that a potassium swelling effect [2], reminiscent of whole cells, is absent.

Since the uptake of glycine relative to the protein content gradually decreased as the osmolarity of the suspending fluid was increased by sucrose, the vesicle preparation appears to consist of closed compartments. The vesicular structure of the preparation was also confirmed electron microscopically. Since the glycine uptake extrapolated to infinite osmolarity tended towards zero, binding to membrane material appears to be small. This conclusion agrees with the observation that at constant osmolarity the equilibrium uptake of glycine did not show saturation phenomena. As the phenomena of exchange as well as of Na^+-linked cotransport could be demonstrated, it was assumed that the transport mechanism for glycine was still functioning. The orientation of the vesicles, i.e., whether inside-out or right side-out, was not tested because, as Na^+-linked cotransport of amino acids should in principle work in either direction, greatly different behavior between inside-out and right side-out vesicles with respect to the Na^+-linked amino acid transport was not expected.

In the presence of NaCl, the rate of glycine uptake into these vesicles is specifically enhanced with and without an NaCl gradient. In the presence of an NaCl gradient, i.e., if NaCl was added to NaCl-free vesicles, the uptake of glycine showed the overshoot phenomenon usually considered typical for Na^+-linked cotransport. Besides flux coupling, alternative causes of overshoot such as (1) membrane diffusion potentials due to unequal ion mobilities of the added sodium salt, (2) pH gradients due to Na^+-H^+ antiport, or (3) transient water shifts due to osmotic effects, can be dismissed in the present case since none of these is likely to be both Na^+-specific and effective with an electroneutral solute such as glycine. On the other hand, that the observed overshoot was indeed due to Na^+-linked cotransport was confirmed by its responses to changes in electrical potential difference, which were as predicted on the basis of our theoretical considerations for a neutral test solute, glycine, leading to a positive charge transfer in cotransport with Na^+ ($e = +1$). Accordingly, the peak was the higher the more negative the intravesicular electrical potential, whether this potential was due to the greater mobility of the salt anion or whether it had been induced by the addition of valinomycin to vesicles preloaded with K^+. Also the initial rate of glycine entry rose with the relative permeability of the salt anions, in agreement with the prediction.

In contrast to Colombini and Johnstone [2,3], no accumulation without an Na^+-gradient was found, nor could transport be inhibited by ouabain directly. That the Na^+-linked influx of glycine in these vesicles is specifically inhibitable under these conditions is shown by the experiments with $HgCl_2$, which does not affect glycine uptake in the presence of KCl but inhibits the increment in uptake rate caused by the presence of Na^+.

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* A membrane protein yield of 1.8% was actually obtained by Colombini and Johnstone [14] (Johnstone, R., personal communication).

Some observations remain unexplained and need further investigation. First, the uptake of glycine does not rise linearly with the reciprocal osmolarity of the medium. This cannot be attributed to leakiness of the vesicles to the osmolar agent, sucrose, since in this case the deviation from linearity should be in a direction opposite to that observed. Second, whereas the initial rates and height of the peaks appear to be almost equal for NaCl and for NaSCN, it is surprising that the decay of the gradient is slower with Cl^- than with SCN^-, although the rate of decay should be primarily determined by the permeability of the membrane to the cation, which is the same in both cases.

In spite of these uncertainties, the present experiments appear to show that at least part of the glycine uptake is driven by the electrochemical potential gradient of Na. Due to the absence of energy metabolism, these experiments cannot exclude the possibility that in intact cells the glycine transport is to some extent primary, i.e., directly coupled to a metabolic reaction. It may, however, be recalled that such direct coupling has been rejected for active transport of aminoisobutyrate in the cells on the basis of coupling experiments [15].

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