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Amino Acid-Dependent Sodium Transport in Plasma Membrane Vesicles from Rat Liver

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Summary. The L-alanine-dependent transport of sodium ions across the plasma membrane of rat-liver parenchymal cells was studied using isolated plasma membrane vesicles. Sodium uptake is stimulated specifically by the L-isomer of alanine and other amino acids, whose transport is sodium-dependent in rat-liver plasma membrane vesicles. The L-alanine-dependent sodium flux across the membrane is inhibited by an excess of Li^+ ions, but not by K^+ or choline ions. Sodium transport is sensitive to -SH reagents and ionophores, and is an electrogenic process: a membrane potential (negative inside) can enhance L-alanine-dependent sodium accumulation. The data presented provide further evidence for a sodium-alanine cotransport mechanism.

Key words: Plasma membrane, rat liver, sodium transport

Studies in several laboratories have demonstrated that accumulative transport of various amino acids in liver parenchymal cells is dependent on the presence of a sodium concentration gradient [2, 7, 10, 12]. This observation is in agreement with the widely accepted gradient hypothesis [1] which implies a cotransport mechanism of amino acids or sugars and sodium ions.

The development of the technique to isolate plasma membrane vesicles has given a great impetus to the transport studies with mammalian cells. Such membrane preparations offer the possibility of precisely defining the role of the cell membrane in the uptake process (for recent reviews *see* [14, 17].

Studies with isolated plasma membrane vesicles from intestinal and kidney epithelial cells provided direct evidence that an experimentally imposed Na^+ gradient, in the absence of both cellular metabolism and an operative Na^+ pump, can stimulate the accumulation of amino acids and sugars in the vesicles.

Recently we developed a procedure for the isolation of plasma membrane vesicles from rat liver [22] and from isolated rat-liver parenchymal cells [19]. These vesicles, capable of Na^+-dependent amino acid transport, enabled us to characterize the plasma-membrane transport of the important glucogenic substrate L-alanine [20].

In order to obtain further insight in the Na^+-dependent amino acid transport in liver parenchymal cells, we decided to perform direct measurements of Na^+ transport in these rat-liver plasma membrane vesicles.

Materials and Methods

Preparation of Membrane Vesicles

Plasma-membrane vesicles from rat liver were isolated from the livers of male Wistar rats (200–350 g), fed ad lib. Tissue homogenization was performed in a homogenization medium, consisting of 250 mM sucrose, 0.2 mM $CaCl_2$ and 10 mM HEPES-KOH, pH 7.5. Dounce homogenization, differential and sucrose density centrifugation were performed as described previously [22]. All operations were performed at 4 °C.

The final membrane preparation was suspended in homogenization medium at a concentration of about 10 mg protein/ml and stored in small aliquots in liquid nitrogen. Before an experiment, membranes were quickly thawed in a waterbath at 40 °C. Membranes were frozen and thawed once only.

Transport Experiments

Unless otherwise stated, plasma membrane vesicles were incubated in homogenization medium, 10 mM $MgSO_4$ and 0.2 mM $^{22}NaSCN$ (sp act 60,000–100,000 cpm/nmol Na^+) for 20 min at 0 °C and subsequently for 10 min at 25 °C. These conditions secured the equilibration of Na^+ ions across the vesicular membrane (data not shown).

Transport experiments were started by the addition of 20-μl aliquots of preincubated membrane vesicles to 80 μl of transport medium, consisting of homogenization medium, 10 mM $MgSO_4$ and 0.2 mM $^{22}NaSCN$. All other additions are given in the legends to the Figures and Tables.

At times indicated, incubations were terminated by dilution of 20- μl aliquots (containing 40–70 μg protein) from the transport

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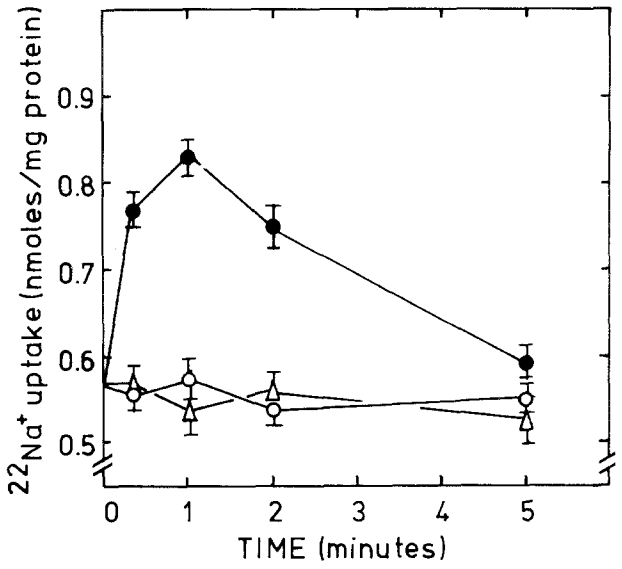

Fig. 1. L-Alanine-dependent $^{22}Na^+$ uptake in rat-liver plasma membrane vesicles. Rat-liver plasma membrane vesicles were preincubated in the presence of 0.2 mM $^{22}NaSCN$ as described in Materials and Methods. At $t=0$, uptake was initiated by dilution in media containing 0.2 mM $^{22}NaSCN$ and either 100 mM L-alanine (●), 100 mM D-alanine (○) or 100 mM L-leucine (△). At times indicated, the uptake was terminated and the intravesicular $^{22}Na^+$ was determined as described

incubation into 1 ml of ice-cold homogenization medium. The dilution was immediately followed by a filtration through Millipore filters (HAWP, 0.45 μm). The filters were washed once with 3 ml of cold homogenization medium and the radioactivity on the filters was determined via liquid scintillation spectrometry.

The results are presented as the mean of triplicate incubations ± SEM. In the course of our studies we observed that the absolute magnitude of the effects varied between various membrane preparations. The relative magnitude of the results shown in this paper was consistent in different membrane batches. Therefore, the results should be 'normalized' to compare between different membrane preparations.

Protein

Protein was measured via the Lowry method [13] as modified by Peterson [15]. Bovine serum albumin (fraction V, Sigma) was used as a standard.

Special Chemicals

$^{22}NaCl$ (222 mCi/mg Na^+) was purchased from The Radiochemical Centre, Amersham, Bucks., Great Britain.

Results

L-*Alanine-Dependent* $^{22}Na^+$ *Uptake in Rat-Liver Plasma Membrane Vesicles*

As reported previously [20], the application of a Na^+ concentration gradient (outside > inside) results in a transient accumulation of L-alanine in rat-liver plasma membrane vesicles.

Fig. 1 shows that a gradient of L-alanine across the vesicular membrane induces a temporary accumulation of Na^+ ions. In the experiment shown, the initial Na^+ concentration was 0.2 mM in both the extra- and the intravesicular compartments. By allowing the $^{22}Na^+$ ions to equilibrate first, the L-alanine-dependent accumulation of free $^{22}Na^+$ can be discriminated optimally. When at $t=0$ the reaction is started by the addition of 100 mM L-alanine, an influx of Na^+ ions is observed which reaches maximal accumulation after about 1 min and is then followed by a net efflux of Na^+ from the vesicles. The efflux is probably caused by the dissipation of the alanine gradient, which is the driving force for the Na^+ influx. In the absence of L-alanine, or upon substitution of L-alanine by its D-isomer, no Na^+ accumulation is observed.

It can thus be concluded from Fig. 1 that L-alanine catalyzes Na^+ transport stereospecifically, which complements previous reports on Na^+-dependent alanine transport. The observed stereospecificity enabled us to correct for "passive" Na^+ transport by subtracting the value of Na^+ uptake in the presence of D-alanine from the Na^+ uptake in the presence of L-alanine.

Stimulation of $^{22}Na^+$ *Uptake by* L-*Alanine Represents Transport*

The question whether the amino acid-dependent association of Na^+ ions with the membrane vesicles represents transport into the vesicular lumen, or alternatively is caused by mere binding, was answered via two different approaches: by studying the effect of medium osmolarity on Na^+ uptake (Fig. 2) and by exchange diffusion experiments (Fig. 3).

Fig. 2 illustrates the effect of increasing osmolarity on the amount of Na^+, which is associated with the vesicles at equilibrium. The variations in osmolarity were brought about by addition of different amounts of cellobiose. It is evident that upon increasing the osmolarity of the surrounding medium, the amount of Na^+ associated with the vesicles decreases. This observation is consistent with the view that the Na^+ ions are, at least partly, located in an osmotically sensitive compartment, *viz.* the intravesicular space.

During our experiments we observed that the final amount of Na^+, associated with the vesicles, could be significantly lowered by the addition of a large excess of K^+ ions. Since this effect could be due to diminished binding of Na^+, the osmotic effect of cellobiose was also tested in the presence of an excess of K^+ ions. Fig. 2 demonstrates that also under these conditions an osmotic sensitivity of the Na^+ ions in the vesicles is seen. However, the excess of K^+

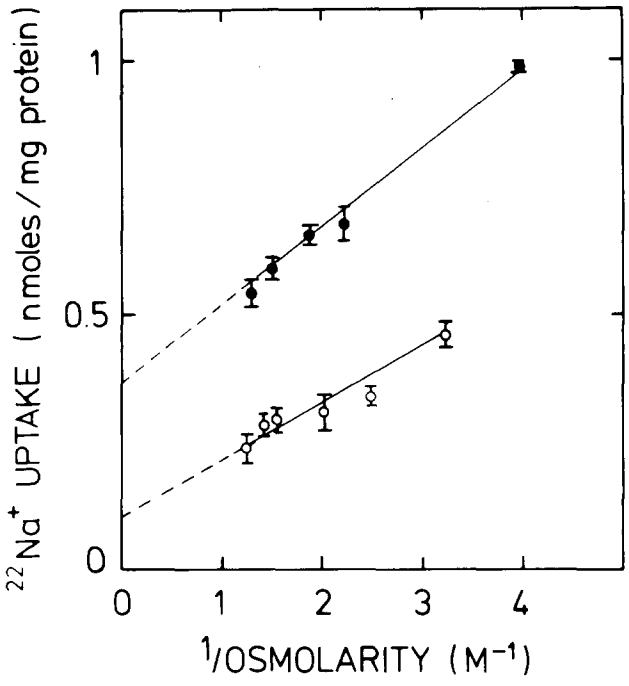

Fig. 2. Osmotic sensitivity of $^{22}Na^+$ uptake. Plasma membrane vesicles were incubated in the presence of 0.2 mM $^{22}NaSCN$ and various amounts of cellobiose for 20 min, either in the absence (●) or in the presence (○) of 50 mM KCl. Intravesicular $^{22}Na^+$ was assayed as described

ions clearly reduces the osmotically insensitive fraction of Na^+ on or in the vesicles.

Further support for transport of Na^+ into the vesicles was derived from exchange diffusion experiments (Fig. 3). Plasma membrane vesicles were preloaded with nonradioactive Na^+ and subsequently diluted in media containing $^{22}Na^+$ and either L-alanine, L-leucine, or D-alanine. As a control, the experiments were repeated, but without preloading the vesicles with unlabeled Na^+. The final concentration of Na^+ in all experiments was 20 mM.

A Na^+ concentration gradient (inside > outside) results in an accelerated influx of labeled Na^+ into the vesicles. The presence of an L-alanine gradient (outside > inside) gives rise to an additional increase in label flux, whereas gradients of L-leucine or D-alanine do not. This amino acid-specific transstimulation of Na^+ transport into the plasma membrane vesicles thus provides support for the contention that the observed accumulation of Na^+ indeed represents carrier-mediated transport, and is not the result of mere binding.

Characterization of L-*Alanine-Dependent* $^{22}Na^+$ *Transport*

Amino Acid Specificity. Studies on amino acid transport in rat-liver plasma membrane vesicles established

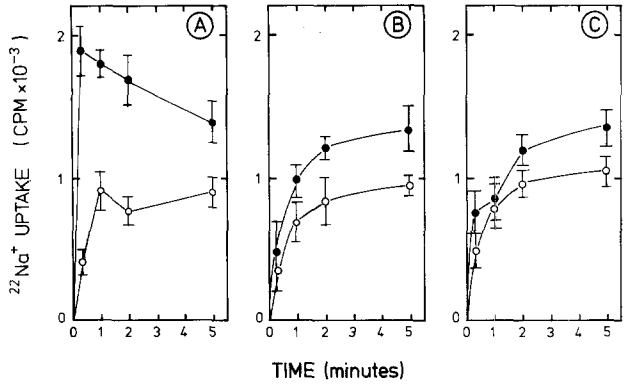

Fig. 3. Transstimulation of L-alanine-dependent $^{22}Na^+$ uptake. Plasma membrane vesicles were preincubated in the presence of 50 mM Na_2SO_4 for 20 min at 0 °C, and 10 min at 25 °C (●). Subsequently, a fivefold dilution was performed in a medium containing $^{22}Na^+$ (carrier-free), and (*A*) 100 mM L-alanine, (*B*) 100 mM L-leucine, or (*C*) 100 mM D-alanine. (○): no preincubation with Na^+ was performed, but Na^+ was added in the incubation to a final concentration of 20 mM

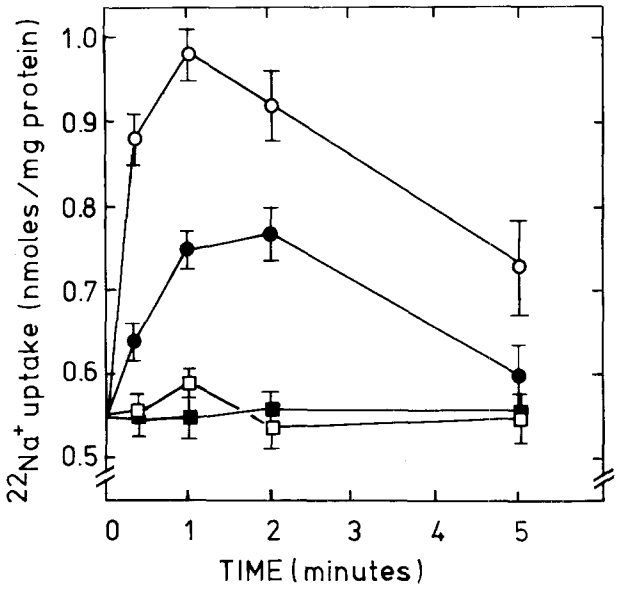

Fig. 4. Amino acid specificity of $^{22}Na^+$ uptake. Plasma membrane vesicles were preincubated in the presence of 0.2 mM $^{22}NaSCN$ and the transport reaction was started via dilution in media containing 0.2 mM $^{22}NaSCN$ and either 100 mM L-glutamine (○), 100 mM L-serine (●), 100 mM L-isoleucine (□), or 100 mM L-leucine (■)

an amino acid specificity for the Na^+ gradient-driven amino acid accumulation [20]. Fig. 4 illustrates that a similar specificity is seen for the amino acid-dependent Na^+ uptake.

Both L-glutamine and L-serine, the transport of which is Na^+ gradient-dependent in isolated liver parenchymal cells [7] as well as in plasma membrane vesicles [19], are capable of inducing the accumulation of Na^+ in the vesicles. On the other hand, L-isoleucine

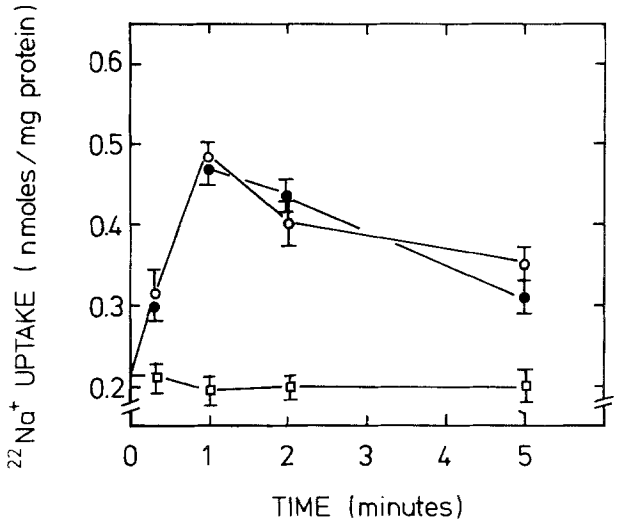

Fig. 5. Effect of monovalent cations on L-alanine-dependent $^{22}Na^+$ uptake. Plasma membrane vesicles were preincubated in the presence of 0.2 mM ^{22}NaSCN and 50 mM KCl. At $t=0$, uptake was initiated via dilution in a medium containing 0.2 mM NaSCN, 100 mM L-alanine and either 50 mM KCl (●), 50 mM choline chloride (○), or 50 mM LiCl (□)

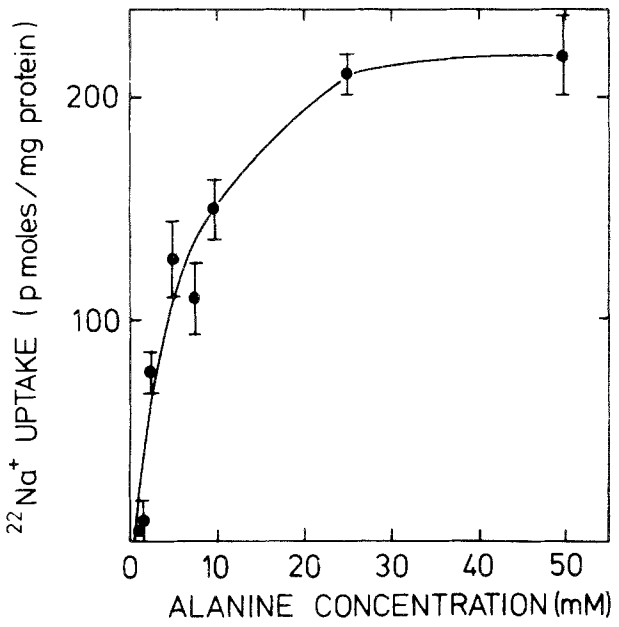

Fig. 6. Kinetics of L-alanine-dependent $^{22}Na^+$ uptake: effect of L-alanine concentration. Plasma membrane vesicles were incubated in the presence of 0.2 mM ^{22}NaSCN. Uptake was started by dilution in media containing L-alanine to final concentrations as indicated in the Figure. Uptake was terminated after 20 sec. In parallel experiments an identical procedure was followed using D-alanine. The difference between uptake in the presence of L-alanine and D-alanine is given

and L-leucine, which are not transported in a Na^+-dependent way, do not influence the distribution of Na^+ across the vesicular membrane.

Ion Specificity. In previous experiments with rat-liver plasma membrane vesicles [20], the specificity of L-alanine transport for Na^+ has been shown: neither K^+, NH_4^+, nor choline gradients (outside > inside) were able to drive amino acid transport. Replacement of Na^+ by Li^+, however, resulted in a marked stimulation of L-alanine transport. A similar specificity was displayed in the alanine-driven Na^+ uptake. The presence of a 250-fold excess of K^+ or choline ions did not inhibit Na^+ uptake, whereas Li^+ almost completely abolished the alanine-dependent overshoot (Fig. 5).

It can thus be concluded that Li^+ can replace Na^+ on the amino acid-ion carrier complex, whereas K^+ or choline are ineffective. It can be noted that in the presence of high salt concentrations the amount of Na^+, which is associated with the vesicles under equilibrium conditions, is much lower than at low salt concentrations, in agreement with Fig. 2.

Kinetics of L-*Alanine-Dependent* $^{22}Na^+$ *Transport*

Figs. 6 and 7 illustrate that the L-alanine-dependent transport of Na^+ in rat-liver plasma membrane vesicles is dependent on the L-alanine and Na^+ concentrations.

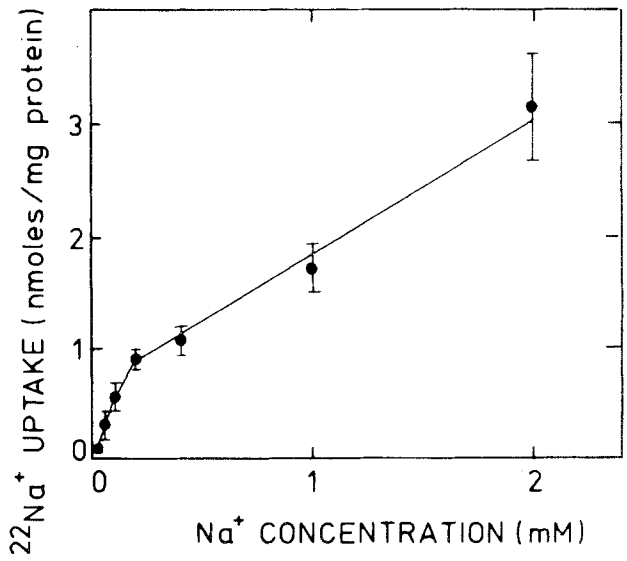

Fig. 7. Kinetics of L-alanine-dependent $^{22}Na^+$ uptake: effect of Na^+ concentration. Plasma membrane vesicles were incubated with ^{22}NaSCN to final concentrations as given in the Figure. At $t=0$, uptake was started by dilution in media containing identical amounts of ^{22}NaSCN, and 100 mM L-alanine or D-alanine. Uptake was stopped after 1 min. The difference between uptake in the presence of L-alanine and D-alanine is given

Table 1. Effect of ionophores and inhibitors on L-alanine-dependent $^{22}Na^+$ uptake[a]

Addition	$^{22}Na^+$ uptake	
	nmol/mg protein	% of control
–	0.26 ± 0.02	100
Monensin (0.10 µg/mg protein)	0.02 ± 0.04	7
FCCP (80 nM)	0.25 ± 0.01	98
Monensin + FCCP	0 ± 0.04	0
Gramicidin (8.5 µg/mg protein)	0 ± 0.02	0
Valinomycin (0.55 µg/mg protein)	0.34 ± 0.02	130
Mersalyl (0.1 mM)	0 ± 0.03	0
NEM (0.7 mM)	0.04 ± 0.02	14
IAA (0.8 mM)	0.23 ± 0.03	88
Ouabain (2.2 mM)	0.24 ± 0.02	93

[a] Plasma membrane vesicles were incubated with 0.2 mM ^{22}NaSCN and ionophores or inhibitors to concentrations as indicated. Uptake after 1 min was determined in the presence of L-alanine or D-alanine (100 mM both) and the values were subtracted.

The Na^+ uptake rate was accelerated with increasing alanine concentrations up to 25–30 mM and complete saturation was observed. Also the concentration of Na^+ ions affected the transport rate, as shown in Fig. 7. Although a deviation from a linear relationship between Na^+ concentration and Na^+ transport was observed, no complete saturation was obtained in the range of Na^+ concentrations tested.

Effects of Ionophores and Inhibitors on L-*Alanine-Dependent* $^{22}Na^+$ *Transport*

Table 1 shows the effects of various ionophores and inhibitors on L-alanine-dependent Na^+ uptake. One should be able to influence the accumulation of Na^+ via interference with the membrane permeability for Na^+ ions, or with the Na^+-alanine carriers.

Addition of monensin, which catalyzes a Na^+-H^+ exchange [16], or gramicidin, which renders the membrane permeable for Na^+ [16], completely inhibits the transient Na^+ uptake. No inhibition was seen, when the membrane was permeabilized for H^+ (by carbonylcyanide *p*-trifluoromethoxyphenylhydrazone), or K^+ (by valinomycin). In the latter case even stimulation was observed.

Reagents reacting with -SH groups, such as mersalyl or N-ethylmaleimide (NEM), were found to inhibit the alanine-driven Na^+ uptake, which is indicative for the presence of -SH groups on the carriers. Hardly any inhibition was seen in the presence of iodacetamide (IAA), which also reacts with -SH groups. Blockage of the (Na^+, K^+)-ATPase by ouabain did not affect the amino acid-induced Na^+ accumulation in the vesicles, nor the subsequent efflux from the vesicles (data not shown): thus apparently Na^+ movement via the ATP-dependent Na^+-K^+ pump is insignificant.

Table 2. Effect of various anions on L-alanine-dependent $^{22}Na^+$ uptake[a]

Salt present	$^{22}Na^+$ uptake	
	nmol/mg protein	% of control
NaSCN	0.56 ± 0.03	100
$NaNO_3$	0.46 ± 0.02	82
Na_2SO_4	0.26 ± 0.03	46
Na_3-citrate	0.17 ± 0.04	30
Na-phosphate	0.06 ± 0.02	11

[a] Plasma membrane vesicles were incubated with ^{22}Na salts to final concentrations of 0.2 mM Na^+. Uptake after 1 min was determined in the presence of L-alanine or D-alanine (both 100 mM), and the values subtracted.

L-*Alanine-Dependent* $^{22}Na^+$ *Transport is an Electrogenic Process*

In accordance with the observations on Na^+ gradient-dependent L-alanine transport [20], the L-alanine-driven transport of Na^+ was also found to be an electrogenic process. The evidence is twofold: Na^+ transport was shown to be stimulated by the presence of mobile charged ions in the incubation mixture and Na^+ transport was enhanced by a K^+ diffusion potential (negative inside).

Both the permeation of positive charges (K^+ ions via valinomycin, Table 1), and the permeation of negative charges influenced the rate of alanine-driven Na^+ uptake, as shown in Table 2. The presence of anions such as citrate, phosphate or sulfate, which are known to be rather impermeant in biological membranes, resulted in a lowered Na^+ transport rate, compared to nitrate or thiocyanate, both examples of very permeant anions. Apparently, the rate of L-alanine-dependent Na^+ transport can be manipulated by means of compensation of charge. Such a compensation can be brought about by positive charges (from the inside of the vesicles to the outside) or by negative charges (from the outside of the vesicles to the inside). The results from Tables 1 and 2 suggest that the operation of the alanine-Na^+-carrier complex leads to charge displacement across the membrane.

Further evidence for the electrogenicity of L-alanine-Na^+ cotransport is shown in Fig. 8. Membrane vesicles were preincubated with K^+ ions and subsequently diluted 10-fold in a medium in which no extra K^+ was added. In the presence of the K^+ ionophore valinomycin the K^+ concentration gradient (inside > outside) creates a diffusion potential (negative inside).

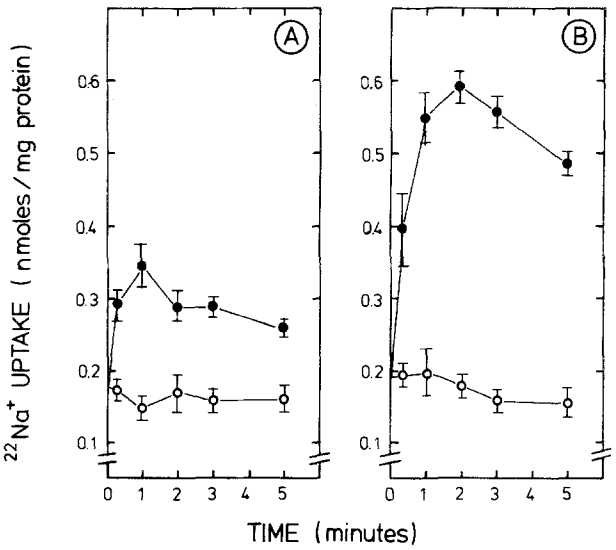

Fig. 8. Effect of a membrane potential (negative inside) on L-alanine-dependent $^{22}Na^+$ uptake. Plasma membrane vesicles were incubated in the presence of 0.1 mM $^{22}Na_2SO_4$ and 50 mM K_2SO_4. At $t=-1$ min, valinomycin was added to the membranes to a concentration of 0.55 μg/mg protein. At $t=0$, the reaction was started by a 10-fold dilution in media, to which no K^+ was added (●), or to which 50 mM K_2SO_4 was added (○). The experiment was carried out in the presence (*B*) or in the absence (*A*) of 100 mM L-alanine

In the control experiments K^+ ions were present in the incubation medium also, so that no concentration gradient and thus no diffusion potential, was generated. It is demonstrated that the presence of a diffusion potential (negative inside) is able to drive L-alanine-dependent Na^+ transport. Even in the absence of alanine the potential stimulates Na^+ flux into the vesicles, but to a much smaller extent: this uptake is probably due to stimulation of passive Na^+ uptake.

Discussion

The results presented in this paper provide direct evidence for the postulate of the gradient hypothesis, *viz.* Na^+ gradient-dependent amino acid transport is a symport of amino acid molecules and Na^+ ions. The characteristics of Na^+ transport described here and those of L-alanine transport as described previously [20] are complementary. From exchange diffusion experiments (Fig. 3) and from osmotic sensitivity (Fig. 2) it can be concluded that the observed accumulation of Na^+ in the vesicles is the result of carrier-mediated transport into the vesicular lumen. The observed exchange diffusion prompted us to stop the transport reactions with a medium in which no Na^+ was included. Otherwise, exchange of intravesicular $^{22}Na^+$ with external unlabeled Na^+ could occur during the stopping procedure and thus lead to an underestimation of the Na^+ uptake.

Figs. 2 and 5 illustrate that the amount of Na^+ in or on the vesicles is strongly diminished in the presence of high cation concentrations. Fig. 2 also shows that at both low and high salt concentrations vesicular Na^+ is osmotically active. So the presence of high cation concentrations does not interfere with free Na^+ in the vesicles, but only with bound Na^+. Upon prolonged incubation of membrane vesicles with Na^+, both binding to the vesicles and transport into the vesicles occur. This binding of Na^+ is not unique for plasma membrane vesicles from liver; it was also observed in studies with isolated intestinal [9] and renal [6, 18] brush-border membrane vesicles. Fig. 2 shows that upon extrapolation to infinite osmolarity a significant amount of Na^+ remains associated with the vesicles (0.1 nmol Na^+/mg protein), even in the presence of high potassium concentrations. It might well be that rat-liver plasma membrane vesicles have a minimal internal volume which is not equal to zero. Similar suggestions have been made previously for these vesicles [20] and also for mitochondria [21]; it is interesting to note, that in experiments on alanine transport in these vesicles the same amount of alanine remained associated with the vesicles when extrapolation to infinite osmolarity was performed and the external alanine concentration was 0.2 mM (i.e. identical to the Na^+ concentration in the experiments described here).

The dependence on the concentrations of both alanine and Na^+ was different for alanine-driven Na^+ transport and for Na^+-driven alanine transport. A possible explanation for this paradox is that the Na^+ transport is measured under completely different experimental conditions (high alanine, low Na^+) from the alanine transport (low alanine, high Na^+). Due to this experimental complication it is impossible to determine the coupling stoichiometry between Na^+ and alanine from the combined data. Comparable problems were encountered in studies on Na^+ transport in membrane vesicles from kidney brush borders [6]. A recent report on alanine transport in intact rat-liver parenchymal cells mentions a coupling ratio between Na^+ and alanine of 1.36:1 [10].

Table 1 shows that alanine-dependent Na^+ transport is sensitive to various -SH reagents. This means that -SH groups are probably involved in the translocation process. However, not all -SH reagents tested proved to be inhibitory: mersalyl and NEM gave almost complete inhibition, whereas IAA had hardly any effect. This difference in sensitivity is possibly caused by the fact that the -SH groups are accessible to some agents, and inaccessible to others. Another explanation for the observations could be that the variable sensitivity towards -SH reagents, which was also observed for alanine transport in these vesicles (H.J. Sips, *unpublished data*), could be the reflection

of different transport systems involved in Na^+-alanine symport. Using various -SH reagents, Young [23] identified different transport systems for alanine in the erythrocyte membrane.

Fig. 8 in this paper illustrates that the alanine-Na^+ symport can be driven by a membrane potential (negative inside). Although Na^+-dependent alanine transport was shown to be an electrogenic process in these vesicles [20] (which was recently confirmed in intact liver parenchymal cells [10]), no alanine uptake was observed with a similar diffusion potential as driving force. It is reasonable to assume that this apparent discrepancy results from the fact that in the alanine uptake experiments a high Na^+ concentration was present, whereas in the Na^+ uptake experiment (Fig. 8) a low Na^+ concentration was used. Thus, the effect of a membrane potential on alanine-Na^+ cotransport depends on the concentration of the solutes involved, of which Na^+ is the most important in this respect. On the basis of theoretical considerations, Heinz [5] has predicted such concentration effects for electrogenic cotransport processes.

It has recently been suggested that Na^+-dependent alanine transport in rat-liver parenchymal cells would coincide with an increased passive permeability of the plasma membrane for K^+ ions [10]. The data presented in this paper do not contradict nor provide support for these observations. It is clear, however, that a certain permeability barrier for K^+ ions remains, since addition of the K^+ ionophore valinomycin stimulates Na^+-alanine cotransport (Table 1, [20]).

It is a well-known fact that Na^+-dependent amino acid transport in liver parenchymal cells is subject to hormonal regulation [3, 8, 11]. Since alterations in amino acid transport activity necessarily result in changes in the Na^+ flux across the plasma membrane, the action of hormones could have implications for the energetics of the cell: increase in Na^+ flux would lead to activation of the plasma membrane-bound (Na^+, K^+)-ATPase. On the other hand, the observed action of glucagon on the ion permeability in liver [4] could well be the result of effects of glucagon on amino acid transport systems, and thus not be an independent hormonal action.

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