J. Membrane Biol. 38, 333–346 (1978)

Studies on Isolated Subcellular Components of Cat Pancreas

III. Alanine-Sodium Cotransport in Isolated Plasma Membrane Vesicles

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Received 21 March 1977; revised 31 August 1977

Summary. Transport of alanine was studied in isolated plasma membrane vesicles from cat pancreas using a rapid filtration technique. The uptake is osmotically sensitive and the kinetics of L-alanine transport are biphasic showing a saturable and a nonsaturable component. The saturable component is seen only when a sodium gradient directed from the medium to the vesicular space is present. Under this condition an overshooting uptake of L- but not of D-alanine occurs. The Na^+ gradient stimulated uptake of L-alanine is inhibited by L-serine and L-leucine and stimulated when the membrane vesicles had been preloaded with L-alanine, L-serine or L-leucine.

The ionophore monensin inhibits stimulation of uptake caused by a sodium gradient. In the presence of valinomycin or carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (CFCCP), the sodium-dependent transport is augmented in vesicles preloaded with K_2SO_4 or H^+ ions (intravesicular pH 5.5), respectively. In the presence of different anions, the Na^+-dependent transport is stimulated according to increasing anionic penetration through membranes (lipid solubility). We conclude that a sodium dependent electrogenic amino acid transport system is present in pancreatic plasma membranes.

The pancreas is one of the most active protein synthetizing organs and, as might be expected, able to transport and to accumulate a variety of amino acids [1–4, 16, 20]. Since large differences in amino acid concentration between pancreatic cells and medium can be established, on the one hand, and abolished by anaerobiosis, dinitrophenol or ouabain on the other hand [1, 6, 19], the question as to the energy source for the amino acid transport from the medium into the cells arises. Depending on whether the energy for active transport is utilized directly from the coupling to a metabolic chemical reaction such as ATP hydrolysis or to the downhill

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0022-2631/78/0038-0333 $2.80

movement of electrolytes by co- or countertransport, active transport has been classified as primary or secondary [12]. In the last years it has been shown in a variety of tissues that the transport of amino acids is indeed coupled to the inward movement of Na^+ ions and that energy of the Na^+ gradient drives amino acids uphill [7, 10–12, 14, 18, 22–27]. In pancreas slices it was observed that glycine accumulation is depressed if ouabain was added or if either Na^+ or K^+ was omitted from the incubation medium [1, 19]. However, a correlation of glycine uptake with the magnitude of the Na^+ and/or K^+ gradient, or the intracellular level of ATP could not be obtained. Consequently it was concluded that cellular ATP, and not the cation gradients, drives glycine accumulation. The availability of pure cell membrane vesicles which contain an osmotically reactive space made it possible to study amino acid transport independent of metabolic energy and to assess its dependence on Na^+ gradients without the drawbacks of intact cells. The results obtained in this study with plasma membrane vesicles from cat pancreas indicate that these membranes contain a Na^+/L-alanine cotransport system which in the absence of metabolic energy is able to accumulate L-alanine against a concentration gradient at the expense of an inward directed Na^+ gradient. They are, therefore, confirmatory with findings in other animal tissue that amino acid uptake can be driven by a Na^+ gradient.

Materials and Methods

All reagents used were of analytical grade. HEPES (N′-2-hydroxyethylpiperazine-N′-2-ethane sulphonic acid), L- and D-alanine were from Serva International, Heidelberg, L-[^{3}H]alanine and D-[^{14}C]alanine from NEN Chemicals, Dreieichenhain/Frankfurt. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (CFCCP) was purchased from Boehringer, Mannheim, valinomycin from SIGMA, St. Louis, and the filters (SM 113, pore size 0.6 μm) from Sartorius, Goettingen.

Plasma membranes were obtained by centrifugation of cat pancreas homogenate through an exponential ficoll-sucrose gradient in a zonal rotor (Beckmann Ti XIV rotor). The fractionation procedure has been described previously [20].

The lightest fraction F_I recovered from the ficoll-sucrose gradient at a density of 1.07 was diluted 8 times with homogenization buffer and spun down for 1 hr at 100,000 × *g*. This step produced a membrane pellet enriched in marker enzymes for plasma membranes such as secretin-stimulated adenylate cyclase, pancreozymin-stimulated adenylate cyclase and Na^+-, K^+-ATPase by a factor of 30, 30 and 23 as compared to the total homogenate. This fraction was virtually free of rough endoplasmic reticulum, mitochondria and zymogen granules, as judged from the determination of ribonucleic acid (RNA), succinic dehydrogenase, and trypsin.

The membrane pellet was resuspended by homogenization with a Teflon glass homogenizer (10 strokes, 1200 rpm) in 15 ml of a solution containing 100 mM mannitol, 10 mM Tris/HEPES buffer (pH = 7.4), 0.5 mM $MgCl_2$ and 0.5 mM KCl and centrifuged for

15 min at 50,000 × g at 4 °C. The resulting pellet was incubated in the same buffer with L-[^{3}H]alanine or D-[^{14}C]alanine as indicated, 100 mM of Na^+ or K^+ salts and 0.16–1.60 mg of membrane protein/ml. Uptake experiments were carried out in 1-ml Eppendorf tubes according to Hopfer et al. [17]. After different incubation times 20 µl of incubation mixture was withdrawn and added to 1 ml of ice-cold "stop"-solution: 200 mM mannitol, 10 mM Tris/HEPES, 1 mM $MgCl_2$, 1 mM KCl and 150 mM NaCl. The resultant suspensions were rapidly filtered through Sartorius filters and washed with 5 ml of the same solution. The filters were dried, dissolved in Bray's solution and counted in a Packard Tricarb liquid scintillation counter. The amount of medium radioactivity bound to the filters in the absence of membranes was always lower than 2.5% of the equilibrium value in the presence of membranes.

Results

1) Time Course of L-*alanine Uptake*

Fig. 1 shows the time course of L-alanine uptake in the presence of a 100-mM NaSCN gradient directed from the medium to the inside of the vesicles. At low (0.1 mM) L-alanine concentration the vesicular L-alanine content

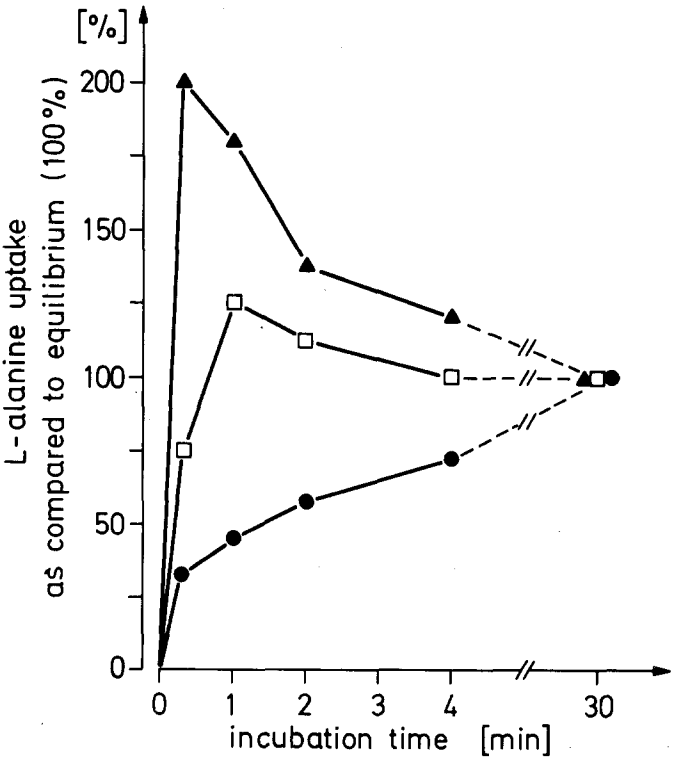

Fig. 1. Time course of L-alanine uptake; dependence on the L-alanine concentration in the medium. The reaction was started by addition of membranes at 0 time to a medium containing 100 mM NaSCN and 0.1 mM (▲), 1.0 mM (□), or 10 mM (●) of L-alanine, respectively

transiently reached values higher than at equilibrium. In some experiments at 1–2 min of incubation a content as much as sevenfold higher than at equilibrium was observed. This "overshoot" was present only if the concentration of L-alanine in the incubation medium was sufficiently low. With higher L-alanine concentrations in the incubation medium (10 mM) the uptake approached the equilibrium in a hyperbolic fashion, whereas a curve between both extremes was obtained at 1 mM L-alanine. At all three L-alanine concentrations studied, the vesicular volume at equilibrium (evaluated as the ratio of L-alanine taken up in the vesicles to the medium concentration) did not change significantly.

In order to decide whether the uptake of L-alanine represents adsorption to membranes, or transport into an intravesicular space, the dependence of L-alanine uptake on the size of the intravesicular space was tested. As shown

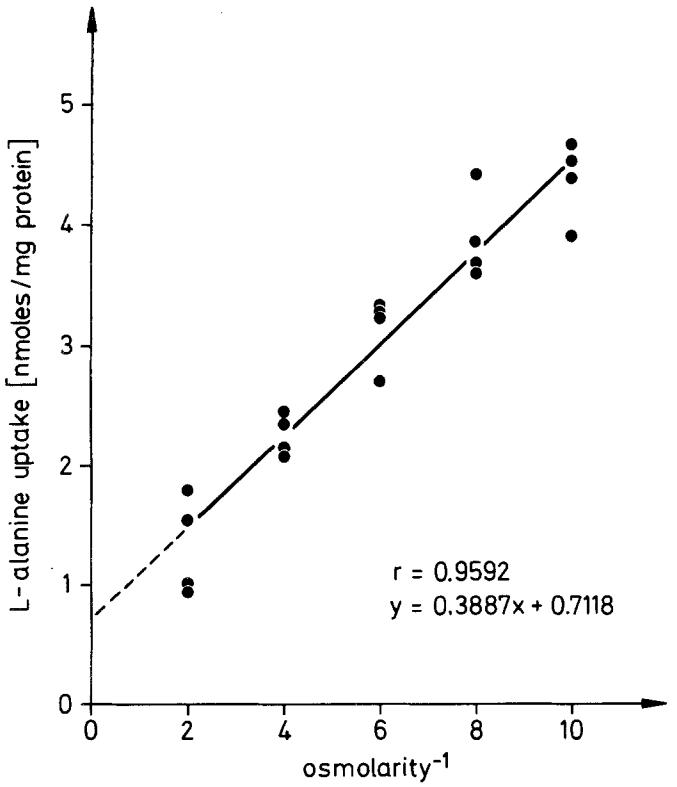

Fig. 2. Effect of increasing medium osmolarity on L-alanine uptake at equilibrium. Membranes were prepared in a medium containing 100 mM raffinose, 10 mM NaSCN and 1 mM L-alanine. Uptake after 30 min of incubation was determined in the same medium with raffinose added to obtain osmolarities as indicated

in Fig. 2 the amount of L-alanine taken up decreased when osmolarity of the incubation medium was increased by the addition of raffinose. When uptake was extrapolated to infinitely high osmolarities, at which the intravesicular space should approach 0, the intercept on the *Y*-axis should represent binding of L-alanine to plasma membranes or slow penetration of raffinose into an osmotic active space at relatively long incubation periods. Additional evidence that L-alanine uptake was not due to significant binding, was obtained from experiments in which distilled water was used instead of the hypertonic "stop" solution to rupture the vesicles. Under these conditions "uptake" was always less than 10% of that into vesicles at equilibrium. Similar conclusions could also be drawn from the measurements of L-alanine uptake values at 30 min, which were linearly related to the medium concentration at all L-alanine concentrations tested (i.e., up to 100 mM).

2) Concentration Dependence of L-*Alanine Uptake*

Fig. 3A shows the 20 sec, i.e., initial rate, of L-alanine uptake in dependence on rising L-alanine concentrations in the incubation medium. In the absence of a sodium gradient, the L-alanine uptake increases linearly with the L-alanine concentration in the incubation medium within the whole concentration range studied, no matter whether the vesicles were preincubated with Na^+ or whether a K^+ gradient or only mannitol was present (the latter curves are not shown). This indicates a nonsaturable, probably simple, diffusion process. If, however, a Na^+ gradient was present at low L-alanine concentration in the medium, a saturation-type uptake component was superimposed on the linear uptake component (Fig. 3*B*); the total uptake could be expressed by the equation $v=\frac{c \cdot V_{max}}{c+K_m}+P \cdot c$. In the example shown in Fig. 3*A*; $V_{max}=0.45$ nmoles $\cdot$ mg protein$^{-1} \cdot 10\,sec^{-1}$; $K_m=0.2$ mM and the proportionality factor for the linear component $P=1.7\,\mu l \cdot$ mg protein$^{-1} \cdot 20\,sec^{-1}$. As can be seen from Fig. 4, the initial uptake rate for L-alanine increased with increasing Na^+ gradient showing a saturation type uptake. A replot of the curve of Fig. 4 reveals a K_m for Na^+ of 10 mM and a V_{max} for L-alanine of 0.53 nmoles $\cdot$ mg protein$^{-1} \cdot 20\,sec^{-1}$ at a concentration of 0.05 mM L-alanine in the incubation medium (the values at zero Na^+ being subtracted). For comparison the values obtained after 30 min of incubation corresponding to equilibrium concentrations are given.

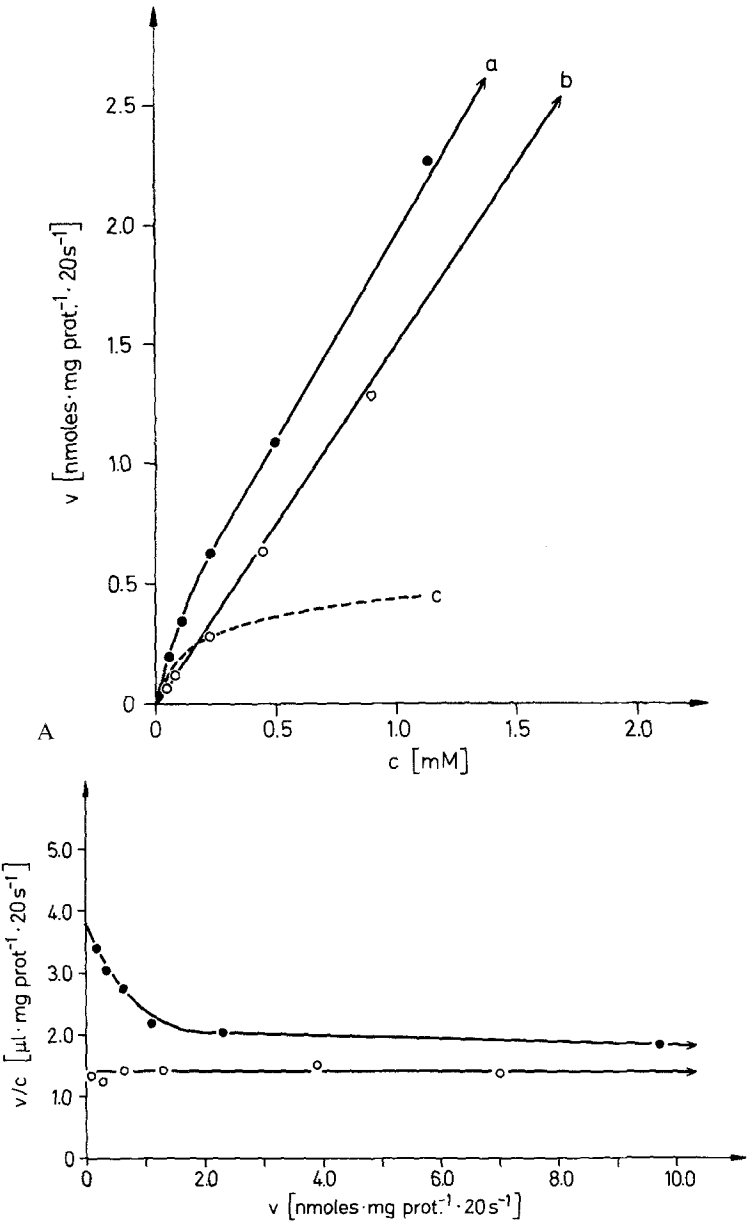

Fig. 3. (*A*): The concentration dependence of the initial rate of L-alanine uptake. Membranes were added to a medium containing L-[^{3}H]alanine in a concentration as indicated and 100 mM NaSCN (●), or after the membranes were preincubated for 10 min in a medium containing 100 mM NaSCN (○); the reaction was started by addition of L-[^{3}H]alanine. Each point represents the average of two measurements. The arrow means that points up to 8 mM are on the same line. Line *c* is obtained by subtracting the ordinate values of line *b* from those of line *a*. (*B*): The same data plotted according to Eadie [9] and Hofstee [15]: $V=\frac{c \cdot V_{max}}{c+K_m} + P \cdot c$; $V_{max}=0.45$ nmoles · mg prot^{-1} · 20 s^{-1}; $K_m=0.2$ mM; $P=1.7$ µl · mg prot^{-1} · 20 s^{-1}

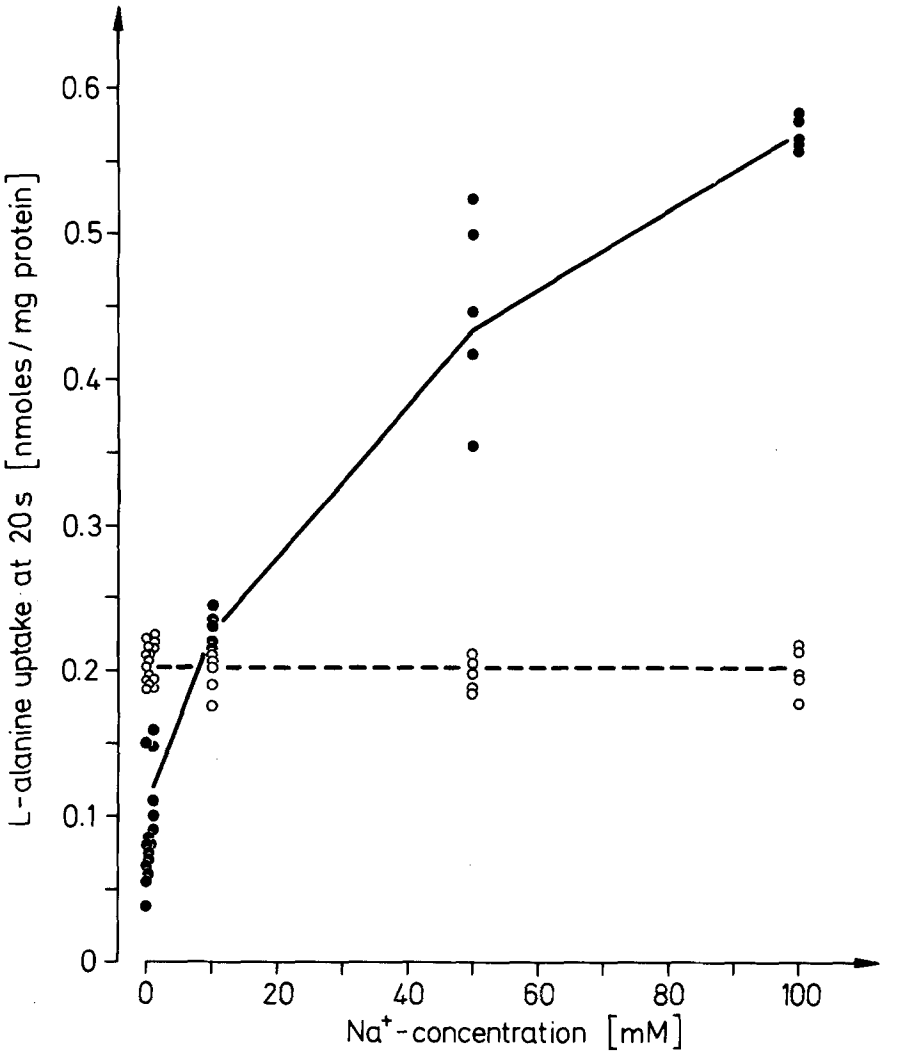

Fig. 4. The effect of different sodium gradients (medium [Na] > vesicular space [Na]) on the initial rate and the equilibrium value of L-alanine uptake by plasma membrane vesicles. The uptake was started by the addition of membranes to the medium containing NaSCN in concentrations as indicated. Ionic strength and osmolarity of all solutions were maintained constant by the addition of KSCN so that the sum of both cation concentrations was constant (100 mM). L-alanine concentration in the incubation medium was 0.05 mM. ●—●, L-alanine uptake after 20 sec (initial rate); o------o, after 30 min (equilibrium value)

3) Stereospecificity and Mutual Interaction of Amino Acid Transport

Fig. 5 shows the time course of L- and D-alanine uptake in the presence and absence of a Na^+ gradient. L-alanine uptake resulted in an overshoot, while D-alanine uptake was slightly increased only when a sodium gradient

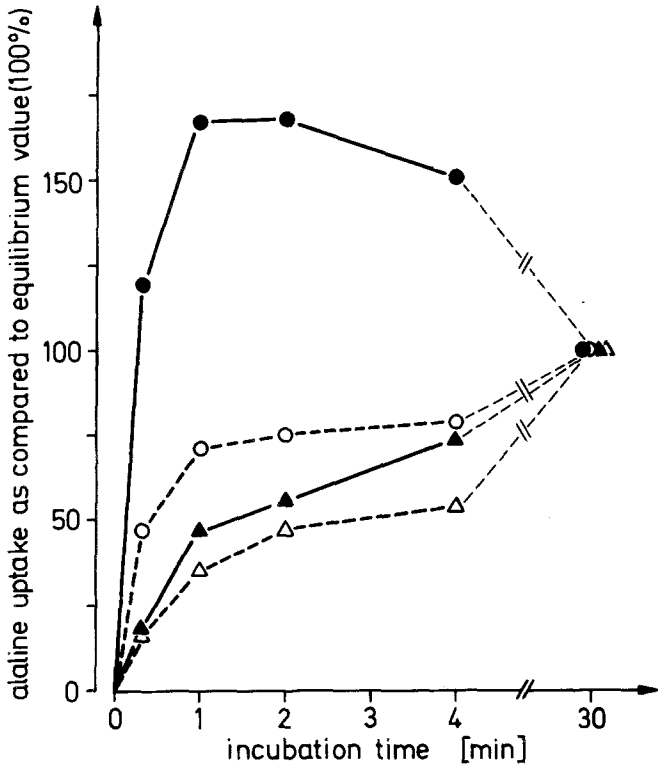

Fig. 5. Stereospecificity of an alanine transport system in isolated plasma membranes. The membranes were preincubated for 10 min with 100 mM NaSCN, and the reaction was started by the addition of 0.05 mM L-[^{3}H]alanine (○—○) or D-[^{14}C]alanine (△—△). The reaction was started by adding membranes to a medium containing 0.05 mM L-[^{3}H]alanine (●—●) or D-[^{14}C]alanine (▲—▲) and NaSCN (100 mM)

Table 1. Cis- and trans-effects of L-alanine transport in isolated plasma membranes from pancreas (results of two experiments, *I* and *II*)

Experiment	Control	Cis-effects		Trans-effects		
		L-serine	L-leucine	L-alanine	L-serine	L-leucine
I	0.132	0.057	0.085	0.183	0.216	0.144
II	0.162	0.118	0.115	0.208	0.226	0.225

Values give the initial rates of uptake in nmoles per mg protein at 20 sec. Cis-effects were observed by preincubating membranes in a medium containing 1 mM of L-serine or L-leucine, respectively. For trans-effects membranes were preincubated in a medium containing 1 mM of L-alanine, L-serine or L-leucine, respectively. Uptake was started by transferring the membranes to a medium containing 0.05 mM of L-[^{3}H]alanine.

was present. Addition of L-serine or L-leucine in concentrations 20 times higher than for L-alanine to the incubation medium caused an inhibition of the inital rate of L-alanine uptake to almost half of the control value (Table 1, cis-effect). An outward directed gradient of these amino acids, however, augmented the initial transport rate of L-alanine by 35% for all applied amino acids (Table 1, trans-effect).

4) Effect of Different Cat- and Anions on L-Alanine Transport

When membranes were added to a medium containing 0.05 mM of L-alanine and 100 mM of either NaSCN, LiSCN or KSCN, only the Na^+ gradient produced the overshoot phenomenon (data not shown). Li^+ stimulated the initial L-alanine uptake by 40–50% compared to K^+, but without producing an overshoot.

In Table 2 the effect of different anions on the initial L-alanine uptake with and without Na^+ gradients are given. In the presence of the readily permeating thiocyanate ions Na^+-stimulated L-alanine uptake was highest and decreased when SCN^- was replaced by less permeable Cl^- ions. In the presence of practically nonpermeating SO_4^{2-} ions L-alanine uptake was even smaller.

5) Effect of Ionophores on L-Alanine Transport

To evaluate the influence of an electrical gradient on sodium-stimulated L-alanine uptake, membranes were preloaded with either K^+ or H^+ ions to create a K^+ or H^+ gradient from the inside to the outside of the vesicles, thus creating a diffusion potential. In the presence of a K^+ gradient and valinomycin, Na^+-dependent L-alanine uptake was stimulated by 23%, whereas in the presence of CFCCP and a H^+ gradient, L-alanine uptake was

Table 2. Effect of different anions on L-alanine uptake by isolated plasma membranes (results of two experiments, *I* and *II*)

Experiment	Sulfate		Chloride		Thiocyanate	
	Na_2SO_4	K_2SO_4	NaCl	KCl	NaSCN	KSCN
I	5.04	2.20	5.70	2.10	7.00	1.80
II	2.90	2.40	3.80	2.20	4.80	2.10

The initial rate of L-alanine uptake (1 mM L-alanine being present in the incubation medium) is shown in nmoles per mg protein at 20 sec.

stimulated by 11 % (Table 3). However, if the K^+ gradient (50 mM K_2SO_4) was directed from the outside to the inside of the vesicles the overshoot phenomenon mediated by an inwards directed Na^+ gradient (50 mM NaSCN) was abolished in the presence of valinomycin, the initial uptake at 20 sec being inhibited by 40 %. Preincubation of membranes with the ionophore monensin (0.1 µg/µg protein) which mediates electroneutral Na^+-H^+ exchange completely abolished the Na^+-gradient dependent transport of L-alanine, lowering the initial rate of uptake to 30 % to 50 % in three different experiments (data not shown). Similarly preincubation of membranes with carbamylcholine (10^{-4} M) inhibited the initial L-alanine uptake in the presence of a 100-mM Na^+ gradient by 20 % (data not shown).

6) Temperature Dependence of the L-Alanine Transport

Only Na^+-gradient dependent L-alanine transport is temperature-dependent. There is no influence of lowering of temperature on the Na^+-independent transport system (data not shown), but lowering the temperature from 30 °C to 4 °C, completely inhibited the Na^+-gradient dependent L-alanine uptake.

Discussion

1) Use of Closed Osmotically Reactive, Plasma Membrane Vesicles for Transport Studies

As studies on amino acid uptake into the pancreas so far have been done only in the intact organ of mouse or rat [1–6, 19], or in slices of pigeon

Table 3. Effect of K^+ and H^+ gradients (medium concentration ≦ intravesicular concentration) in the presence or absence of valinomycin and CFCCP, respectively, on initial rates of L-alanine uptake (results of three experiments, *I*, *II* and *III*)

Experiment	K^+ gradient		H^+ gradient	
	+valinomycin	−valinomycin	+CFCCP	−CFCCP
I	0.115	0.097	0.114	0.092
II	0.170	0.135	0.111	0.106
III	0.147	0.121	0.112	0.104

The initial rate of uptake is given in nmoles per mg protein at 20 sec. The membranes were prepared in solutions containing 50 mM Na_2SO_4 and 50 mM K_2SO_4 with or without valinomycin (0.01 µg per µg protein) or in a solution containing 50 mM Na_2SO_4 and 20 mM MES/Tris buffer, pH 5.5, with or without CFCCP (10 µM). The uptake was started by transferring membranes to a medium containing 50 mM Na_2SO_4 and 0.05 mM L-alanine.

pancreas [16], the question as to the energy source for amino acid transport has not been unequivocally established. Use of isolated membrane vesicles has the advantage of allowing investigation of the driving forces for membranal transport independent of cellular metabolism. The following observations support the view that uptake of the amino acid represents transport into a vesicular space and not membrane binding: Increasing medium osmolarity decreased uptake of L-alanine indicating that L-alanine uptake represents transport into an osmotically reactive space (Fig. 2). The cis- and trans-effects of alanine transport should be due to a carrier-mediated process rather than to binding (Table 1). The transient nature of "cumulative" uptake of L-alanine in the presence of a Na^+ gradient should be observed only in the presence of closed membrane vesicles.

2) Transport Components of L-*Alanine Uptake*

Our data on L-alanine movement across the vesicular membrane showed two components: in the absence of a Na^+ gradient or, as not shown in the figure, when a K^+ gradient or only mannitol was present, the initial L-alanine uptake rose linearly with the L-alanine concentration in the medium (Fig. 3) which probably represents diffusion. The small temperature dependence of this component supports this interpretation.

If, however, a sodium gradient was present, a superimposed transport component of L-alanine became apparent. The Na^+-dependent uptake processes showed not only saturability with regard to L-alanine but also with regard to Na^+ (Fig. 4). It is stereospecific since D-alanine uptake was not stimulated by a Na^+ gradient. It showed tracer interaction, i.e., cis-inhibition and trans-stimulation (Table 1). Na^+ stimulation was abolished at $+4\,°C$ showing a high temperature coefficient. Therefore, we can conclude that the Na^+-dependent L-alanine transport is carrier-mediated.

The stereospecificity of the carrier as well as transstimulation and cis-inhibition of alanine transport (Table 1) indicate that the carrier is specific for L-forms of neutral amino acids and is possibly comparable to the *A*-system described by Christensen [7].

3) What does the Overshoot Phenomenon Mean?

The overshoot phenomenon of L-alanine in the presence of a Na^+ gradient in principle could be due to several factors: (i) an initial but transient swelling of the vesicles; (ii) a transvesicular electrical potential

difference; (iii) Na^+-L-alanine cotransport. The first possibility is not very likely, since transfer of membranes to a hypertonic medium should cause initial shrinking rather than initial swelling. Concerning the second possibility, a negatively charged L-alanine-carrier complex could produce an overshoot of L-alanine influx into vesicles if the permeability for Na^+ would be much larger than for the accompanying anion, thus creating a transient, vesicle lumen positive, diffusion potential difference. Our data, however, show that the overshoot is abolished if a possible membrane potential is short-circuited in the presence of valinomycin and K^+. In the presence of monensin or of carbamylcholine which both increase the membrane permeability for Na^+ [13, 21], the transport system can be uncoupled. These findings contradict the second but favor the third possibility of a Na^+-L-alanine cotransport system.

4) Is the Na^+ Amino Acid Cotransport Electrogenic or Not?

We have tested the nature of coupling by three types of experiments which support the electrogenicity of L-alanine transport. (i) As shown in Table 2 in the presence of a Na^+ gradient the lipophilic anion SCN^- was the most effective accompanying anion, whereas with the less permeable SO_4^{2-} ion the L-alanine uptake was smallest. (ii) The creation of a negative diffusion potential in the presence of valinomycin and a K^+ gradient or of CFCCP and a H^+ gradient increased L-alanine uptake to a small but significant extent (Table 3). (iii) If the electrochemical Na^+ gradient was counteracted by an inward-directed K^+ diffusion potential the overshoot phenomenon was abolished. Consequently the mechanism of L-alanine uptake into membrane vesicles can be considered as electrogenic cotransport of the neutral amino acid with a positively charged sodium ion.

The findings described show that in the pancreas a Na^+ cotransport mechanism for neutral L-amino acid uptake is present which confirms the findings in other tissues [7, 8, 10, 12, 14, 18, 22, 24, 27]. Since active amino acid transport is energized by the electrochemical Na^+ gradient probably generated in vivo by the Na^+-K^+-ATPase pump, it is not necessary to invoke an energy source other than the electrochemical potential gradient of Na^+, although an ATP-driven uptake of other amino acids into the acinar cells of the pancreas cannot be excluded.

The authors wish to thank Professor K.J. Ullrich, Professor R. Kinne, Dr. P. Geck, and Dr. H. Murer for valuable discussions.

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