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Electrogenic Properties of the Sodium-Alanine Cotransporter in Pancreatic Acinar Cells: I. Tight-Seal Whole-Cell Recordings

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Summary. Electrical currents associated with sodium-coupled alanine transport in mouse pancreatic acinar cells were studied using the method of whole-cell recording with patch pipettes. Single cells or small clusters of (electrically coupled) cells were isolated by collagenase treatment. The composition of the intracellular solution could be controlled by internal perfusion of the patch pipette. In this way both inward and outward currents could be measured under "zero-*trans*" conditions, i.e., with finite concentrations of sodium and L-alanine on one side and zero concentrations on the other. Inward and outward currents for equal but opposite concentration gradients were found to be of similar magnitude, meaning that the cotransporter is functionally nearly symmetric. The dependence of current on the concentrations of sodium and L-alanine exhibited a Michaelis-Menten behavior. From the sodium-concentration dependence of current as well as from the reversal potential of the current in the presence of an alanine-concentration gradient, a sodium/alanine stoichiometric ratio of 1 : 1 can be inferred. The finding that N-methylated amino acids may substitute for L-alanine, as well as the observed pH dependence of currents indicate that the pancreatic alanine transport system is similar to (or identical with) the "A-system" which is widespread in animal cells. The transport system is tightly coupled with respect to Na^+; alanine-coupled inward flow of Na^+ is at least 30 times higher than uncoupled Na^+ flow mediated by the cotransporter. The current-voltage characteristic of the cotransporter could be (approximately) determined from the difference of transmembrane current in the presence and in the absence of L-alanine. The sodium-concentration dependence of the current-voltage characteristic indicates that a Na^+ ion approaching the binding site from the extracellular medium has to cross part of the transmembrane electric field.

Key words cotransport · electrogenic transport · sodium-coupled amino-acid transport · pancreatic acinar cells · whole-cell recording

Introduction

Cotransport systems which utilize an electrochemical sodium gradient as a driving force for the accumulation of amino acids are known to exist in many animal cells (Johnstone, 1979; Christensen, 1984; Stevens, Kaunitz & Wright, 1984; Kinne, 1985). Most sodium, amino-acid cotransporters are electrogenic, i.e., they translocate electric charge across the cell membrane (Heinz, Geck & Pietrzyk, 1975; Philo & Eddy, 1978). Accordingly, electrophysiological techniques may be applied to the investigation of Na^+-coupled amino-acid transport (Hoshi, Sudo & Suzuki, 1976; Kehoe, 1976; Okada, Tsuchiya, Irimajiri & Inouye, 1977; Iwatsuki & Petersen, 1980*a*,*b*; Bergman & Bergman, 1981, 1985; Frömter, 1982; Gunter-Smith, Grasset & Schultz, 1982; Jung, Schwarz & Passow, 1984; Singh, 1984).

In the following we describe the application of the technique of tight-seal whole-cell recording (Hamill, Marty, Neher, Sakmann & Sigworth, 1981; Marty & Neher, 1983; Iwatsuki & Petersen, 1985) to the study of sodium-coupled alanine transport in pancreatic acinar cells. In whole-cell recording experiments the cell is attached to the tip of a glass micropipette with a high seal resistance (>10 GΩ) between glass and cell membrane, whereas the series resistance between cell interior and preamplifier is only about 20 MΩ. A major advantage of the whole-cell recording technique is the free diffusional exchange between the solution inside the pipette and the cell interior. By internal perfusion of the pipette (Soejima & Noma, 1984) the intracellular concentrations of Na^+ and amino acid can be easily controlled. Pancreatic acinar cells which have a diameter of 15-30 μm are particularly suitable for studying Na^+-coupled amino acid transport since they have a high rate of protein synthesis and accumulate amino acids with high efficiency (Wheeler, Lukins & György, 1949; Hokin, 1951; Bégin & Scholefield, 1964, 1965*a*,*b*; Cheneval & Johnstone, 1974, 1976; Petersen, 1976; Tyrakowski, Milutinović & Schulz, 1978; Schulz & Ullrich, 1979; Iwamoto & Williams, 1980; Iwatsuki & Petersen, 1980*a*,*b*; Laugier & Petersen, 1981; Mann & Peran, 1986)

Depending on the direction of the driving force,

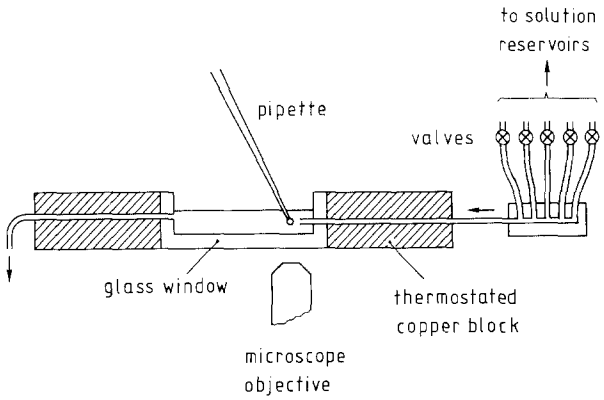

Fig. 1. Experimental set-up for whole-cell recordings. The Teflon tube connecting the solution reservoirs to the measuring chamber was enclosed in the thermostated copper block on a length of about 20 cm in order to ensure temperature equilibration. Solutions of different composition were separated by a small air bubble which was injected into the manifold

both inward- and outward-directed alanine-dependent currents have been recorded. In this way, by measuring the current as a function of the intra- and extracellular concentrations of Na^+ and alanine, kinetic models of the cotransport systems can be tested. Furthermore, since the electrical conductance of the cell membrane strongly increases upon addition of alanine, information on the current-voltage characteristic of the cotransporter can be obtained from current measurements in the presence and in the absence of the amino acid.

Materials and Methods

Materials

Reagents were obtained from the following sources: Collagenase (high purity), tris(hydroxymethyl)aminomethan (Tris), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and ethylene bis(oxyethyleneitrilo)tetraacetic acid (EGTA) from Sigma; 2-(methylamino)-isobutyric acid (MeAIB) from Alrich; N-methylalanine from Serva (Heidelberg); L- and D-alanine and (2,2,1)heptane-2-carboxylic acid (BCH) from Sigma; Eagle basal medium from Serva. Electrolytes and buffers were analytical grade.

Cell Preparation

Single cells and small cell clusters were prepared by enzymatic dissociation of mouse pancreatic tissue (Amsterdam & Jamieson, 1972; Schulz, Heil, Kribben, Sachs & Haase, 1980; Schultz et al., 1980; Iwatsuki & Petersen, 1985). A small piece of pancreas from a white laboratory mouse was injected with a solution containing 140 mM NaCl, 5 mM KCl, 10 mM HEPES (pH 7.3) and 100 U/ml high-purity collagenase (Sigma) and incubated with 1 ml of the same solution at 37°C for 30 min. During the incubation period mild shearing forces were applied by repeated gentle pipetting. The resulting suspension of rounded-up cells and small cell clusters was diluted fivefold with collagenase-free buffer medium and the cellular material was allowed to settle. The supernatant was removed and replaced with buffer medium. The cell suspension could be stored in Eagle basal medium at 3°C for 24 hr without significant change in the electrical properties of the cells.

A number of control experiments were carried out in order to test the possibility that the collagenase treatment impairs the cotransport system under study. The magnitude of alanine-driven currents remained virtually unchanged when the duration of the collagenase incubation was increased from 30 to 90 min.

Whole-Cell Recordings

The suspension of single cells and small cell clusters was transferred to a perspex chamber in a thermostated copper block which was mounted on the stage of an inverted microscope (Zeiss Invertoscope D). The chamber was made as a 15-mm long slit with a width of 2 mm in a 2-mm thick perspex plate, and bottom of the chamber was a glass slide sealed to the perspex plate. The chamber could be perfused by different electrolyte solutions from a number of reservoirs (Fig. 1). By placing the cell at the tip of the micropipette close to the inlet of the solution, the solution composition in the vicinity of the cell could be changed within less than 1 sec. The Teflon tube connecting the reservoirs with the chamber was enclosed in the thermostated copper block on a length of about 20 cm. The temperature in the vicinity of the cell could be kept constant within ±0.2°C while solution was flowing through the chamber. The temperature was measured with an electronic miniature thermometer with a tip size of 1 × 2 mm.

Pipettes were pulled from glass capillaries (Micro-Hematocrits 101-PS, Birkerød, Denmark) using a vertical microelectrode puller (David Kopf, Tujunga, CA, Mod. 700C) as described by Hamill et al., 1981. Pipettes had internal tip diameters of 1-2 μm; when filled with 150 mM NaCl solution they exhibited resistances of 4 to 8 MΩ. The pipette was connected to the amplifier head stage of the L/M-EPC-5 patch-clamp system (List-Electronic, Darmstadt, F.R.G.). The head stage was mounted on a Narishige (Tokyo, Japan) hydraulic micromanipulator. The patch-clamp system allowed either to clamp the current to a predetermined value and measure voltage or to clamp the voltage and monitor current. The current signals were recorded on tape (Ampex Corp., Redwood City, Cal,; Mod. PR2200). Silver-silver chloride electrodes were used inside the pipette and in the bath. The lower part of the pipette was filled with a chloride-free sulfate solution (*see below*) and the upper part (in which the electrode is located) with a solution containing 150 mM chloride. This resulted in a nearly time-independent diffusion potential which did not affect the measurements. Volume changes by perfusion of the pipette were small so that the silver-chloride electrode remained always in contact with the chloride solution. The external silver-silver chloride electrode was connected to the bath medium by a salt bridge.

The performance of the amplifying system was checked with a test circuit consisting of a resistance (10 MΩ) in series with a parallel combination of a resistance (10 MΩ-10 GΩ) and a capacitance (5-300 pF). From the current signals resulting from

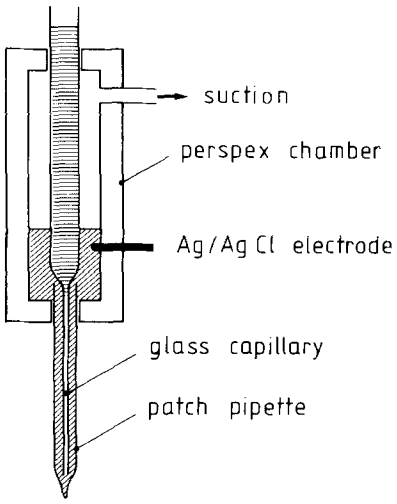

Fig. 2. Internal perfusion of the patch pipette (not drawn to scale). The glass capillary had a length of about 5 cm and an internal diameter of 20-40 μm. The distance between the tip of the capillary and the tip of the patch pipette was 100-200 μm

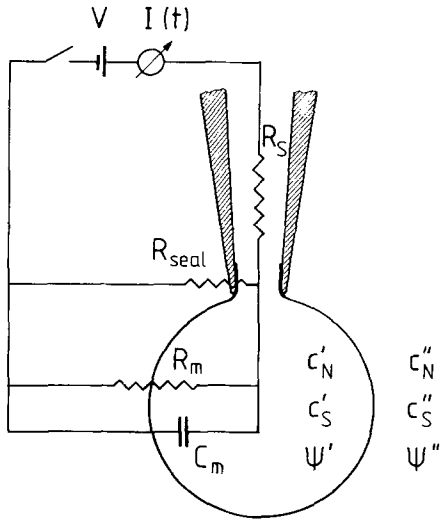

Fig. 3. Circuit parameters of a whole-cell recording experiment. R_S: series resistance of the pipette; R_{seal}: pipette-membrane seal resistance; R_m and C_m: resistance and capacitance of the cell membrane. c'_N, c''_N, c'_S, c''_S are the intra- and extracellular concentrations of sodium (N) and amino acid (S), and ψ is the electrical potential

the application of rectangular voltage-pulses to the test circuit, the rise time of the amplifier was determined to be about 20 μsec.

Before contacting the cell membrane with the pipette tip, a small positive pressure difference (~10 mbar) was applied between pipette and bath in order to maintain a flow of solution out of the pipette. The electrical resistance between pipette and bath was monitored by applying voltage pulses of ~2 mV. A gigaohm seal between cell membrane and pipette was formed by slight suction, and thereafter a whole-cell configuration was established by further brief suctions and rupturing the membrane patch under the pipette (Hamill et al., 1981). In the whole-cell configuration seals were usually mechanically stable enough so that the cell (or the cell cluster) could be detached from the bottom of the chamber by slowly lifting the pipette. Under the usual experimental conditions seals maintained their high electrical resistance for times up to 2 hr. Voltages as high as ±200 mV could be applied for brief periods (~100 msec); however, voltage pulses above 150 mV sometimes resulted in a transient decrease of the seal resistance.

The pipette solution always contained 1 mM EGTA and the external medium 2 mM $CaSO_4$. In order to minimize leakage conductance of the cell membrane, SO_4^{2-} was used as anion instead of Cl^- in most experiments. Since the solubility product of $CaSO_4$ is $\approx 6 \times 10^{-5}$ M^2, the concentration of free Ca^{2+} was about 0.6 mM at the highest sulfate concentration (100 mM). If not otherwise indicated, the experiments were carried out at 37°C and at a pH value of 7.25-7.30 which was stabilized by addition of 10 mM $Tris_2SO_4$.

Internal Perfusion of the Pipette

In order to control the composition of the intracellular solution, the pipette was internally perfused using a slightly modified version of the method of Soejima and Noma (1984). A glass capillary of an internal diameter of 30-50 μm was introduced coaxially into the pipette, with the tip of the capillary located 100-200 μm away from the tip of the pipette (Fig. 2). A flow of solution through the capillary at a rate of 0.4-3 nl/sec was maintained by a hydrostatic pressure difference of ~10 mbar. The pipette-cell exchange time, as estimated from the time course of the zero-current membrane potential in the presence of an alanine-concentration difference between pipette and external medium, was found to be 3-5 min for single cells. The perfusion medium could be exchanged within 1-2 min during the whole-cell recording experiment. This was done by inserting a fine plastic capillary with the tip located above the constriction of the inner glass tube.

Results

Electrical Cell Parameters

After formation of a seal between cell membrane and micropipette, the electrical resistance between the interior of the pipette and the bath medium was typically of the order of 10-100 GΩ; this value represents a lower limit for the pipette-membrane seal resistance (R_{seal}, Fig. 3). After a whole-cell configuration has been established by breaking the membrane patch under the pipette, the current I was clamped to zero. The membrane potential V under the condition $I = 0$ and in the absence of extracellular K^+ was initially about −40 mV (cell interior negative). This value agrees with the resting potential of acinar cells in intact mouse pancreatic tissue recorded with conventional microelectrodes (Iwatsuki & Petersen, 1980*a*,*b*). Under normal experimental conditions, when the perfusion medium in

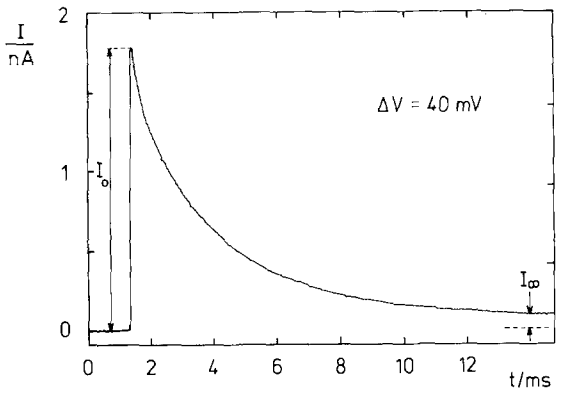

Fig. 4. Current transient $I(t)$ after a stepwise voltage change V, recorded from a cluster of seven cells. Pipette solution: 100 mM Na_2SO_4, 10 mM $Tris_2SO_4$, 1 mM EGTA. External solution: 100 mM Na_2SO_4, 10 mM $Tris_2SO_4$, 2 mM $CaSO_4$; pH = 7.3, T = 37°C. Using Eqs. (1)-(3), the following circuit parameters are obtained from $I(t)$: R_S = 25 MΩ; R_m = 520 MΩ; C_m = 160 pF

the pipette and the bath medium had the same composition, V decreased to zero in the course of 3-15 min (depending on the size of the cell cluster). This decline of membrane potential is likely to result from the equilibration of the cell interior with the pipette solutions. (Occasionally, the equilibration time was much longer, presumably as a result of partial clogging of the pipette tip. In this case the experiment was continued with another cell or cell cluster.) The value of $V \approx -40$ mV remained stable for >30 min when the solution inside the pipette contained 140 mM K^+.

A stepwise change of voltage, ΔV, in the whole-cell configuration resulted in a current signal as shown in Fig. 4. From the time constant τ of the current decline and from the initial (I_0) and final (I_∞) current amplitude the circuit parameters R_s, R_m and C_m of Fig. 3 were determined according to

$$I_0 = \Delta V/R_s \tag{1}$$

$$I_\infty = \frac{\Delta V}{R_s + R_m} \approx \frac{\Delta V}{R_m} \tag{2}$$

$$\tau = \frac{R_s C_m}{1 + R_s/R_m} \approx R_s C_m. \tag{3}$$

These relations hold under the condition $R_s \ll R_m \ll R_{seal}$. As seen from Table 1, the series resistance R_s was in the range of 10-30 MΩ, about 2.5 times as large as the pipette resistance R_{pip} before touching the cell. The effect of R_s on the observed transmembrane currents was always negligible. The specific membrane resistance $R_M = R_m A$ was calculated from R_m and C_m assuming a specific membrane capacitance of $C_m/A = 1\ \mu F/cm^2$ (A is the area of the cell membrane). The values of R_M as given in Table 1 ($R_M \approx 50$ kΩ cm²) have been measured in the nominal absence of potassium and at low calcium concentration inside the pipette with SO_4^{2-} as anion; under physiological conditions R_M is of the order of 10 kΩ cm² (McCandless, Nishiyama, Petersen & Philpott, 1981).

Table 1. Electrical parameters of whole-cell recordings from single pancreatic acinar cells[a]

Pipette resistance R_{pip}	4-8 MΩ[b]
Seal resistance R_{seal}	10-100 GΩ[c]
Series resistance R_s	10-30 MΩ
Membrane capacitance C_m	6-30 pF
Membrane resistance R_m	2-8 GΩ
Specific membrane resistance R_M	50 kΩ cm²[d]
Cell diameter	15-30 μm

[a] Pipette solution: 100 mM Na_2SO_4; 1.5 mM EGTA; 10 mM Tris sulfate; pH 7.25 Bath solution: 75 mM Na_2SO_4; 2 mM $CaSO_4$; 50 mM Tris sulfate; pH 7.25. T = 37°C.
[b] Pipette resistance before touching the cell.
[c] Lower limit of pipette-membrane seal resistance before rupturing the membrane patch under the pipette.
[d] Calculated from R_m and C_m assuming a specific membrane capacitance of 1 μF/cm².

In many cases small clusters consisting of 2 to 5 cells were used instead of single cells for measuring alanine-driven currents. By electrophysiological studies it is well established that cells in mouse pancreatic acini are electrically coupled (Iwatsuki & Petersen, 1978*a*,*b*). After enzymatic dissociation of pig or mouse pancreatic acini, cells within a cluster remain coupled (Maruyama, Petersen, Flanagan & Pearson, 1983; I. Findlay & O.H. Petersen, *unpublished observations*). Direct evidence that the electrically coupled state persists under the conditions of our whole-cell recording experiments comes from the observation that the membrane capacitance C_m increases with the number of cells in the cluster (*compare* Fig. 7). This finding would be difficult to explain if the cell under the pipette were not electrically coupled to its neighbors.

Electrical coupling of cells within a cluster could be abolished in a controlled and reversible fashion by treatment with diphenylmethanol. In a typical experiment in which a 10-cell cluster was used, the time constant $\tau \approx R_s C_m$ of the current transient after a voltage jump (Fig. 4) was found to decrease about 10-fold within 30 sec when 10 mM diphenylmethanol was added to the medium, corresponding to an approximately 10-fold decrease of membrane capacitance C_m. After superfusion of the cell cluster with a diphenylmethanol-free medium, the relaxation time returned to very nearly the origi-

nal value within 1 min. Similar uncoupling effects have been observed previously with a number of alkanols (Johnston, Simon & Ramon, 1980; Bernardini, Peracchia & Peracchia, 1984). In experiments with cell clusters spontaneous uncoupling was occasionally observed after long times (about 30-60 min after the start of whole-cell recording). For the evaluation of kinetic data only experiments with time-invariant relaxation time τ were used.

When a voltage V is applied between pipette and bath and a current I flows through the pipette and the cell membrane, a voltage drop of magnitude IR_s occurs across the series resistance R_s. The transmembrane voltage V_m across the cell membrane is then given by

$$V_m = V - IR_s. \tag{4}$$

The total current I may be represented as the sum of the current I_a carried by the sodium-alanine transporter plus the current $I_m = V_m/R_m$ through other conductive pathways in the cell membrane (R_m is the membrane resistance in the absence of amino acid). Combining the relation $I = I_a + V_m/R_m$ with Eq. (4) one obtains:

$$I_a = (I - I^*)(1 + R_s/R_m) \tag{5}$$

where $I^* = V/(R_s + R_m)$ is the current in the absence of amino acid. Implicit in Eq. (5) is the assumption that the amino acid has no other electrical effects apart from activating the cotransport system. Eqs. (4) and (5) will be used later in order to evaluate I_a and V_m from the experimental quantities I and V. The correction term R_s/R_m is usually rather small; for instance, for a cluster of four cells where R_m is about 500 MΩ, R_s/R_m is of the order of 0.02-0.06.

When alanine-driven currents are recorded from a cluster of cells, imperfect voltage clamping within the cluster is a potential source of error. Specific resistances of cell-to-cell junctions (referred to total contact area between cells) have been found to be in the range of 1-10 Ω cm^2 (Peracchia, 1985). Taking the contact area of cells of radius r to be of the order of r^2, the coupling resistance R_c will be about 1-10 MΩ for $r = 10$ μm. Since R_c is considerably lower than the membrane resistance of a single cell ($R_m \simeq$ 2-8 GΩ), spatial variations of voltage within the cell cluster are likely to be small. More direct information on the degree of electrical coupling may be obtained from the shape of the current transient after a voltage-jump (Fig. 4). From simple circuit analysis it may be predicted that the current transient $I(t)$ is a single exponential function in the case of ideal coupling, whereas $I(t)$ contains two or more exponential terms when the cells in the cluster are not perfectly coupled (Maruyama & Petersen, 1983). From the analysis of relaxation curves such as shown in Fig. 4 it is found that the decline of current after the voltage jump is always described by a single exponential function within experimental error limits.

Alanine-Driven Currents

Inward and Outward Currents Recorded from a Single Cell Cluster

In the experimental represented in Fig. 5 identical solutions containing 200 mM Na^+ were present in the pipette and in the bath at the beginning. After the zero-current membrane potential had dropped to ≈0 mV (about 5 min after the establishment of the whole-cell configuration), the medium flowing around the 2-cell aggregate was changed to a solution containing 5 mM L-alanine. This resulted in a transient inward current with an initial value of 28 pA. After the current started to decline, the pipette was perfused with a medium containing 5 mM L-alanine. Within about 6 min the current decreased to less then 3pA. When the extracellular medium was then changed back to zero alanine concentration, a transient outward current with a peak value of 26 pA was observed which again declined to zero.

The experiment represented in Fig. 5 shows that the transport system can carry both inward and outward currents. The initial inward current is driven by an inwardly-directed concentration gradient of alanine. Increase of intracellular alanine concentration (which results from transmembrane transport as well as from equilibration with the pipette solution) leads to a decline of inward current. When the concentration gradient of alanine is reversed, an outward current is observed.

In the experiment of Fig. 5 the pipette was perfused in order to speed up the equilibration of intracellular and extracellular alanine concentrations. In experiments without perfusion of the pipette, in which equilibration of alanine concentration occurred exclusively by transmembrane transport, the current signal was similar to that shown in Fig. 5, but the decay time was prolonged. The rate of decline of inward and outward currents varied considerably from experiment to experiment. This was observed in experiments with and without perfusion of the pipette. These variations are likely to result from differences in the surface-to-volume ratio of the cell or cell cluster and from variations in the diffusional resistance of the pipette tip. Since the

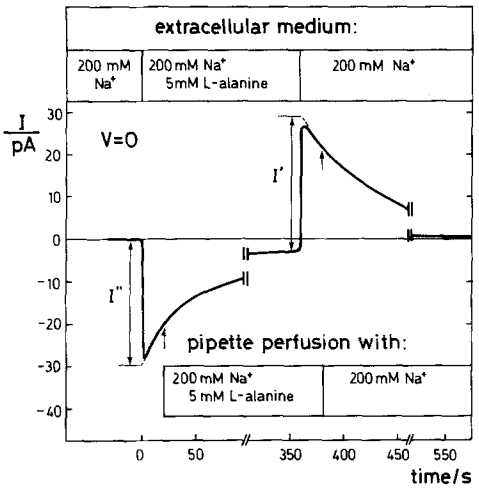

Fig. 5. Alanine-driven inward and outward currents recorded from an aggregate of two cells under short-circuit conditions. At time $t \leq 0$ both the pipette solution and the external medium were alanine-free (pipette solution: 100 mM Na_2SO_4; 1 mM EGTA; 10 mM $Tris_2SO_4$; pH 7.3. External solution: 100 mM Na_2SO_4; 2 mM $CaSO_4$; 10 mM $Tris_2SO_4$; pH 7.3). At time $t = 0$ the medium superfusing the cell cluster was changed to a solution containing, in addition, 5 mM L-alanine; at $t \leq 20$ sec after the inward current had passed through the peak value, the pipette was perfused with a solution containing 5 mM L-alanine and 100 mM Na_2SO_4 in order to shorten the equilibration time (the start of the perfusion is marked by an arrow). At $t = 360$ sec the superfusing medium was changed back to an alanine-free solution; after the outward current had reached the peak, the pipette was perfused with a solution containing 100 mM Na_2SO_4 and 0 mM L-alanine. The membrane capacitance was 50 pF, corresponding to a membrane area of 5×10^{-5} cm^2. $T = 37$°C. I' and I'' are the (extrapolated) peak outward and inward currents. The current trace was redrawn from the original record

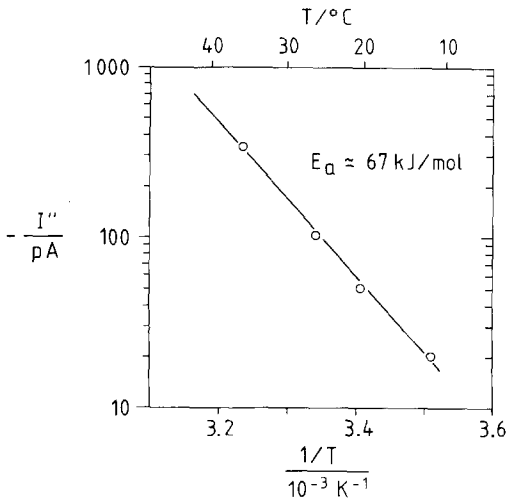

Fig. 6. Peak inward current I'' (*compare* Fig. 5) recorded from a cluster of about 10 cells after addition of 2 mM L-alanine to the external medium, as a function of reciprocal temperature. Pipette solution: 145 mM NaCl; 1 mM EGTA; 10 mM HEPES, pH 7.3. External solution: 145 mM NaCl; 2 mM $CaCl_2$; 10 mM HEPES; pH 7.3

kinetic analysis described below is based on the initial currents (I' and I'' in Fig. 5), variations in the time behavior of the current do not affect the results.

A number of control experiments was carried out in which sodium was replaced by $Tris^+$ or by K^+. In this case L-alanine had virtually no effect. Furthermore, with D-alanine instead of L-alanine the current signal was at least five times smaller.

Passive, Na^+-independent transport of alanine may occur by diffusion through the lipid bilayer or by permeation via the "L-system" which is known to occur as an electroneutral amino-acid transport system in animal cells (Christensen, 1979, 1984; Mircheff, van Os & Wright, 1980; Kilberg, 1982). In order to test the latter possibility, experiments were carried out (without perfusion of the pipette) in the presence and in the absence of 2-aminobicyclo (2,2,1)heptane-2-carboxylic acid (BCH), a competitive inhibitor of amino acid transport through the L-system (Christensen, 1984). With 10 mM BCH in the external medium, the peak amplitude of alanine-induced inward current (Fig. 5) was virtually unchanged, but the time constant of the current decline was significantly increased. This finding indicates that the L-system is present in the membrane in addition to the Na^+, alanine transporter under study.

The alanine-induced inward current I'' strongly increases with temperature T. From the Arrhenius plot (Fig. 6) an activation energy of $E_a \simeq 67$ kJ/mol ($\simeq$16 kcal/mol) was determined using the relation $d\ln I''/d(1/T) = -E_a/R$ (R is the gas constant). A similar value ($E_a \simeq 63$ kJ/mol) has been reported by LeCam and Freychet (1977) for the transport of neutral amino-acids through the "A-system" of hepatocytes, which is comparable to the transport system in pancreatic acinar cells (*see below*).

Proportionality between Current and Membrane Area

Figure 7 summarizes experiments in which alanine-driven inward currents were recorded from clusters of 2 to 15 cells under otherwise identical conditions (200 mM Na^+ on both sides, 8 mM L-alanine in the extracellular medium). Prior to each current recording, the membrane capacitance C_m of the cell cluster was measured. As shown by Fig. 7, the current amplitude I'' increases more or less in proportion to C_m, with maximum deviations from linearity of about 20%. Since C_m may be assumed to be proportional to the area of membrane surface which is in

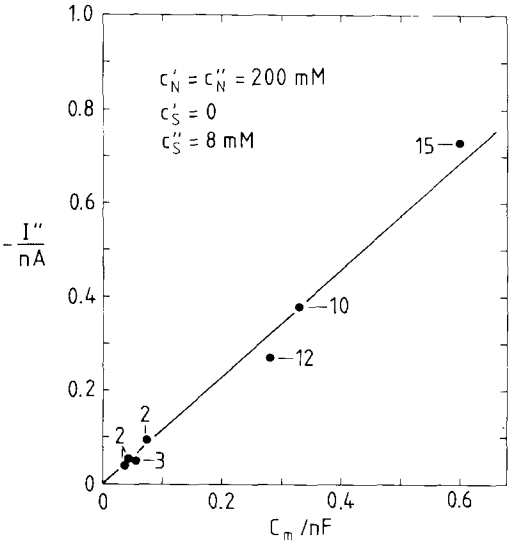

Fig. 7. Correlation between membrane capacitance C_m and alanine-driven inward current I'', recorded from small cell clusters. The number of cells in the cluster is indicated for each experiment. The cells were obtained from different pancreata. Pipette solution: 100 mM Na_2SO_4; 1 mM EGTA; 10 mM $Tris_2SO_4$; pH 7.3. External solution: 100 mM Na_2SO_4; 2 mM $CaSO_4$; 10 mM $Tris_2SO_4$; 0 or 8 mM L-alanine; pH 7.3; $T = 37$°C. I'' is the initial current (*compare* Fig. 5) recorded immediately after exchanging the external medium for a solution containing 8 mM L-alanine. With a specific membrane capacitance of 1 μF/cm^2, the slope of the straight line corresponds to a current density of 11.4 mA/m^2

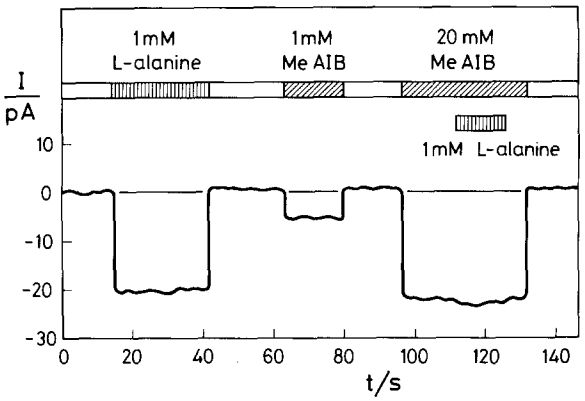

Fig. 8. Short-circuit current induced by L-alanine and 2-(methylamino)-isobutyric acid (MeAIB). The pipette was perfused with a solution containing: 100 mM Na_2SO_4; 10 mM $Tris_2SO_4$; 1 mM EGTA; pH 7.3. External solution: 100 mM Na_2SO_4; 10 mM $Tris_2SO_4$; 2 mM $CaCl_2$; pH 7.3, plus various additions of amino acids, as indicated. 3-cell cluster ($C_m \simeq 50$ pF), $T = 37$°C

contact with the external solution, the (approximately) linear relationship between C_m and I'' indicates that the surface density of transporter molecules does not vary appreciably from cell to cell. With a specific membrane capacitance of $\simeq 1$ μF/cm^2, the straight line in Fig. 7 corresponds to a current density of $\simeq 12$ mA/m^2. The (approximate) proportionality between alanine-driven currents and membrane capacitance may be used to correlate current measurements carried out with different cells from the same preparation.

A similar correlation with capacitance C_m is found for the membrane conductance $1/R_m$, corresponding (in 100 mM Na_2SO_4) to a specific membrane resistance $R_M \simeq 50$-100 kΩ cm^2.

Comparison with Amino-Acid Transport Systems Described from Other Cell Types

Two different Na^+-coupled transport systems for alanine and related neutral amino-acids have been described from animal cells (Kilberg, 1982; Christensen, 1984). The "A-system" is strongly pH-dependent and accepts N-methylated amino acids such as 2-(methlyamino)-isobutyric acid (MeAIB), whereas the "ASC-system" is only weakly pH-dependent around pH 7 and is insensitive to MeAIB. Furthermore, amino-acid transport via the ASC-system has been reported to be voltage independent (Valdeolmillos, Garcia-Sancho & Herreros, 1986).

As shown by Fig. 8, 1 mM MeAIB added to the external medium induces an inward current which is about a fourth of the current observed with 1 mM L-alanine. The half-saturation concentrations of MeAIB and L-alanine are almost the same (about 1.7 with 145 mM Na^+ on both sides), but MeAIB has a 4-5 times lower I''_{max} (Jauch, Maruyama, Petersen, Kolb & Läuger, 1986). In the presence of 20 mM MeAIB, addition of 1 mM L-alanine does not further increase the current, which suggests that MeAIB competes with alanine for the same transport site. Furthermore, N-methylalanine, which is accepted by the A-system but not by the ASC-system, is found to induce a current similar to that evoked by alanine.

The dependence of alanine-induced inward current on external pH is represented in Fig. 9. The current amplitude exhibits a strong decrease below pH 7, which is taken to be a characteristic property of the A-system (Kilberg, 1982). Near pH 7.0 the current reaches a maximum and declines toward more alkaline pH values.

Thus, the experiments with N-methylated amino acids, as well as the observed pH and voltage dependence of alanine-driven current indicate that the transport system under study is similar to (or identical with) the A system which has been described in other animal cells.

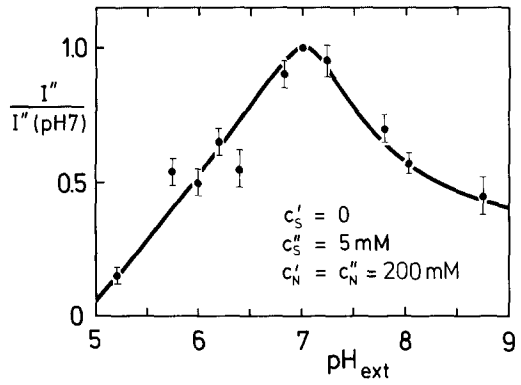

Fig. 9. Inward current I'' after addition of 5 mM L-alanine to the external medium, as a function of external pH. Pipette solution: 100 mM Na_2SO_4; 10 mM $Tris_2SO_4$; 1 mM EGTA; pH 7.3; external solution: 100 mM Na_2SO_4; 10 mM $Tris_2SO_4$; 2 mM $CaSO_4$; 0 or 5 mM L-alanine. The external pH was adjusted with H_2SO_4 or NaOH. The experiments have been carried out with six different cell clusters at 37°C. For each cell cluster the current at pH = 7.0 was used as a reference value. As a test for the reversibility of the pH effects, a current measurement at pH 7 was carried out after each series of recordings. The bars indicate the standard deviations of the current amplitudes. The curve has been drawn to guide the eye

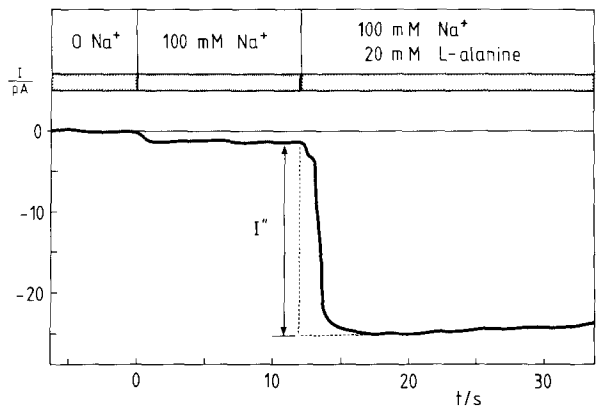

Fig. 10. Inward current under short-circuit conditions, driven by gradients of Na^+ and L-alanine. 2-cell cluster, T = 37°C. The pipette was perfused with a solution containing 1 mM EGTA and 100 mM $Tris_2SO_4$. The external solution contained 2 mM $CaSO_4$ and either 100 mM $Tris_2SO_4$ or (50 mM Na_2SO_4 + 50 mM $Tris_2SO_4$) and 0 or 20 mM L-alanine (as indicated). The pH was 7.25 on both sides. The current trace was redrawn from the original record

Inward Current as a Function of Extracellular Concentrations of Sodium and Alanine

In order to test predictions from theoretical models and to evaluate kinetic parameters of the transport system, it is advantageous to carry out experiments under "zero-*trans*" conditions, i.e., with finite concentrations of substrate and driving ion on one side and vanishing concentrations on the other. A measurement of alanine-dependent inward current at vanishing intracellular concentrations of Na^+ and amino acid is described in Fig. 10. Sodium-free and alanine-free solutions of $Tris_2SO_4$ were present in the pipette and in the bath at the beginning. When the external solution was replaced by a solution containing 100 mM Na^+ (but no alanine), a small inward current was recorded. This alanine-independent current is likely to result from passage of Na^+ through unspecific leakage pathways and/or from uncoupled Na^+ flow through the Na^+, alanine cotransporter. A much larger current was observed after exchanging the external medium for a solution containing 100 mM Na^+ and 20 mM L-alanine (Fig. 10). With external alanine alone (in the absence of sodium) no inward current could be detected. Accordingly, it is assumed in the following that the difference of inward currents in the presence and in the absence of L-alanine represents the current associated with Na^+-coupled amino-acid transport.

In the double-reciprocal plot of Fig. 11 the peak inward current I'' (Fig. 10) is plotted as a function of extracellular L-alanine concentration c''_S for different values of extracellular sodium concentration c''_N. The intracellular concentrations of sodium and L-alanine were maintained at zero. $1/I''$ is found to vary linearly with $1/c''_S$; this means that the current can be represented by a Michaelis-Menten equation:

$$I'' = I''_{\infty,S} \frac{c''_S}{c''_S + L''_S} \tag{6}$$

$I''_{\infty,S}$ is the current for $c''_S \to \infty$ and L''_S is the half-saturation concentration of alanine at the external site. The experimentally observed dependence of I'' on c''_S is consistent with the assumption that a single amino-acid molecule is transported per turnover. As seen from Fig. 11, L''_S depends on sodium concentration c''_N, decreasing from $\simeq$ 18 mM at c''_N = 5 mM to $\simeq$ 2.9 mM at c''_N = 150 mM. The value of 2.9 mM at 150 mM Na^+ may be compared with the half-saturation concentration ($L''_S \simeq$ 1.6 mM) determined previously from microelectrode experiments with intact cells in 126 mM Na^+ (Iwatsuki & Petersen, 1980*b*). The difference between the two L''_S values may result from the different intracellular concentrations of Na^+ and L-alanine.

In Fig. 12 the inward current I'' is plotted as a function of external sodium concentration c''_N at constant external alanine concentration. The shape of the function $I'' = I''(c''_N)$ contains information on

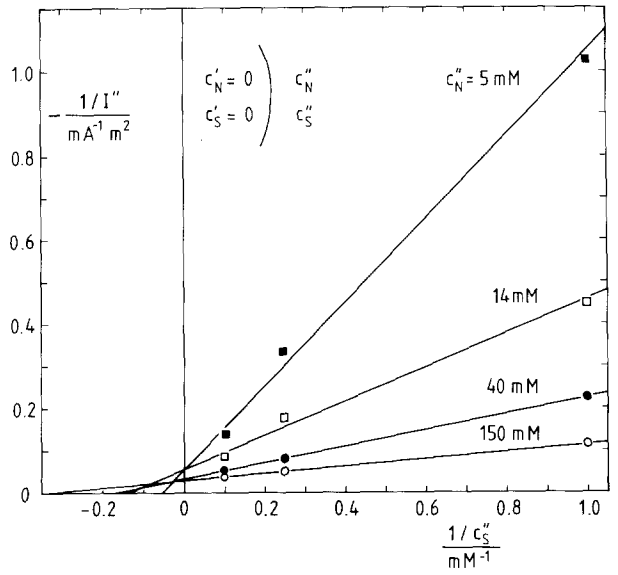

Fig. 11. Double reciprocal plot of peak inward current I'' (Fig. 10) as a function of external L-alanine concentration c''_S for different values of the external sodium concentration c''_N. I'' is referred to unit area of the cell membrane. The intracellular concentrations of sodium and L-alanine were maintained at zero. I'' is the difference of the inward current (measured under short-circuit conditions) with and without L-alanine. Experimental results obtained from different cell clusters were normalized by comparison of I'' values recorded in a standard experiment (c''_N = 150 mM, c''_S = 4 mM). Each data point represents an average from several measurements. The pipette was perfused with a solution containing 1 mM EGTA and 100 mM $Tris_2SO_4$, pH 7.3; external solution: 2 mM $CaCl_2$, 100 mM (Na_2SO_4 + $Tris_2SO_4$), pH 7.3, plus various concentrations of L-alanine; T = 37°C. The straight lines were obtained by linear regression with I'' as weighting factor. The abscissa intercepts correspond to the following values of the half-saturation concentration L''_S [Eq. (6)]: 18.4 mM (c''_N = 5 mM); 6.8 mM (c''_N = 14 mM); 5.8 mM (c''_N = 40 mM); 2.9 mM (c''_N = 150 mM)

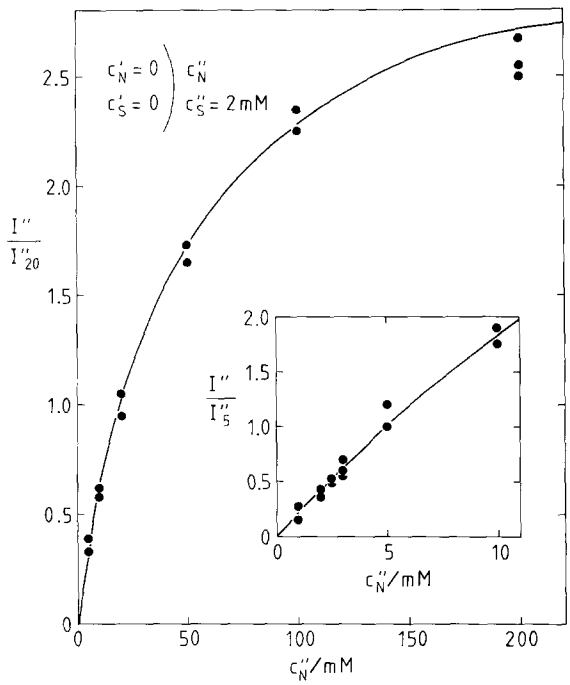

Fig. 12. Peak inward current I'' under short-circuit conditions as a function of external sodium concentration c''_N. I'' is the difference of the inward current with and without L-alanine (Fig. 10). The pipette was perfused with a solution containing 1 mM EGTA and 100 mM $Tris_2SO_4$, pH 7.25; external medium: 100 mM (Na_2SO_4 + $Tris_2SO_4$); 2 mM L-alanine; 2 mM $CaSO_4$, pH 7.25. The temperature was 37°C. I''_5 and I''_{20} are the values of I'' at c''_N = 5 mM and c''_N = 20 mM, respectively, which were used for normalization of current amplitudes recorded from different cell clusters. The curve has been calculated according to Eq. (7) with L''_N = 47 mM. Current amplitudes at low c''_N are given in the inset

the Na^+/alanine stoichiometry. If the stoichiometry is 1 : 1, the current should obey the equation

$$I'' = I''_{\infty,N} \frac{c''_N}{c''_N + L''_N} \tag{7}$$

where $I''_{\infty,N}$ and L''_N are independent of c''_N. On the other hand, any deviation from a linear relationship between I'' and c''_N at low c''_N would indicate that the stoichiometry is different from 1 : 1. A comparison of the theoretical curve in Fig. 12 with the experimental points show that Eq. (7) fits the results within close limits. In particular, no deviation from $I'' \propto c''_N$ can be seen at low c''_N (inset in Fig. 12). While this finding is consistent with a 1 : 1 ratio of sodium binding sites to alanine binding sites, it does not exclude other stoichiometries, since a transport system with n : 1 stoichiometry ($n > 1$) may approximately obey Eq. (7) in the experimental range of c''_N, if the binding sites for Na^+ have strongly different affinities. A more stringent method for distinguishing between different stoichiometries consists in measuring the reversal potential V_r of transmembrane current under the condition $c'_S \neq c''_S$ $c''_N = c''_N$. As will be shown below, the observed dependence of V_r *on* c'_S/c''_S is strongly in favor of a stoichiometric ratio of 1 : 1.

The data presented in Fig. 12 may be compared with the results of a recent study of the Na^+-coupled alanine transport system in *Xenopus* oocytes (Jung et al., 1984). These authors observed a distinctly sigmoid dependence of inward current on sodium concentration and interpreted this finding as evidence for the participation of at least two Na^+ ions in the translocation of a single alanine molecule.

The sodium-concentration dependence of inward current I'' measured at a given c''_S is summa-

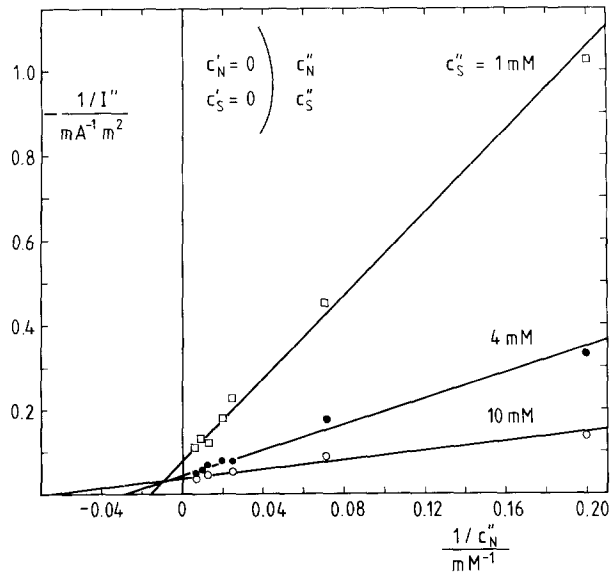

Fig. 13. Double reciprocal plot of peak inward current I'' (Fig. 10) at zero voltage as a function of external sodium concentration c''_N for different values of the external L-alanine concentration c''_S. I'' is referred to unit area of the cell membrane. The experimental conditions were the same as described in the legend of Fig. 11. The straight lines were obtained by linear regression with I'' as weighting factor. The abscissa intercepts correspond to the following values of the half-saturation concentration L''_N [Eq. (7)]: 63 mM (c''_S = 1 mM); 36 mM (c''_S = 4 mM); 14 mM (c''_S = 10 mM)

rized in the double reciprocal plot of Fig. 13. At all values of c''_S the current can be represented by a Michaelis-Menten relation (Eq. 7). The half-saturation concentration of sodium, L''_N, is found to decrease from 63 mM at c''_S = 1 to 14 mM at c''_S = 10 mM.

The functions $L''_S(c''_N)$, $L''_N(c''_S)$, $I''_{\infty,S}(c''_N)$ and $I''_{\infty,N}(c''_S)$ which can be evaluated from Figs. 11 and 13 contain information on the kinetic parameters of the cotransport system. An analysis of the experimental data in terms of microscopic transport models is carried out in part II of the paper (Jauch & Läuger, 1986).

Outward Currents

A series of experiments has been carried out in which both inward and outward currents have been measured under "zero-*trans*" conditions on the same cell (or cell cluster). In Table 2 the ratio I'/I'' of outward to inward currents is given for equal but opposite concentration gradients; the outward current I' has been measured under the condition $c'_N = c_N$, $c'_S = c_S$, $c''_N = c''_S = 0$ and the inward current I'' under the condition $c'_N = c'_S = 0$, $c''_N = c_N$, $c''_S = c_S$. It is seen from Table 2 that in the whole experimental range of c_N and c_S, I' and I'' are nearly equal, the

Table 2. Ratio I'/I'' of outward to inward currents measured under "zero *trans*" conditions with equal but opposite concentration gradients[a]

c_N/mM	24	24	24	80	80	200	200
c_S/mM	2	4	8	2	4	2	4
I'/I''	1.3	1.2	1.5	0.79	0.75	0.83	0.90

[a] The outward current I' has been measured under the condition $c'_N = c_N$, $c'_S = c_S$, $c''_N = c''_S = 0$ and the inward current I'' under the condition $c'_N = c'_S = 0$, $c''_N = c_N$, $c''_S = c_S$. The intra- and extracellular media contained 100 mM ($Tris_2SO_4$ + Na_2SO_4), pH 7.3. In experiments with 100 mM Na_2SO_4 on one side, 10 mM $Tris_2SO_4$ were present on the same side. The pipette solution contained in addition 1 mM EGTA, and the external medium 2 mM $CaSO_4$. I' and I'' have been corrected for the currents in the presence of a Na^+-gradient alone (*compare* Fig. 10). For each single value of I'/I'' given in the Table, I' and I'' have been measured on the same cell cluster (1 to 3 cells). T = 37°C. Further experiments at c_N = 20 and 100 mM with c_S = 2 and 8 mM gave similar results (I'/I'' between 0.75 and 1.4).

maximum deviation of I'/I'' from unity being 50%. No systematic variation of I'/I'' with c_N and c_S can be detected within the limits of experimental error. This result indicates that the Na^+, alanine cotransporter is functionally nearly symmetric.

Degree of Coupling

From the experiment represented in Fig. 10, the ratio $\rho''_{I,\mathrm{exp}}$ of alanine-dependent to alanine-independent inward current can be determined:

$$\rho''_{I,\mathrm{exp}} \equiv \frac{I''(c''_S > 0)}{I''(c''_S = 0)}. \tag{8}$$

As will be discussed in more detail in part II of the paper, $\rho''_{I,\mathrm{exp}}$ provides an estimate for the degree of coupling of the cotransport system with respect to Na^+. Incomplete coupling is associated with an intrinsic leakage permeability of the cotransporter for sodium. In the experiment represented in Fig. 10, $\rho''_{I,\mathrm{exp}}$ was about 12*. $\rho''_{I,\mathrm{exp}}$ was found to depend on

* The experiment of Fig. 10 has been carried out under the condition of constant ionic strength, keeping the total concentration of Na_2SO_4 plus $Tris_2SO_4$ the same on both sides. This means that an inward-directed Na^+ gradient is associated with an outward-directed $Tris^+$ gradient, which in turn could give rise to a current component from a net outward flow of $Tris^+$. In order to test for this possibility, an experiment was carried out with identical concentrations (100 mM) of $Tris_2SO_4$ on both sides and, in addition, 10 mM Na_2SO_4 on the external side. The current was found to be virtually the same as in the corresponding experiment performed at constant ionic strength. This means that current components from $Tris^+$ permeation are negligible.

c''_N and c''_S; at small c''_N and saturating c''_S the observed values of $\rho''_{I,\text{exp}}$ ranged between 10 and 30. The variation of $\rho''_{I,\text{exp}}$ from experiment to experiment mainly resulted from variations in the magnitude of uncoupled current, $I''(c''_S = 0)$, whereas the relative change of alanine-dependent current was small. For this reason the largest value of $\rho''_{I,\text{exp}}$ ($\simeq$30) was taken as a lower limit for the ratio ρ''_I of coupled to uncoupled sodium flow through the cotransporter. The estimated value $\rho''_I > 30$ indicates that the cotransport system is tightly coupled with respect to sodium.

Reversal Potential

An alternative method for obtaining information on the stoichiometry consists in measuring the reversal potential, i.e., the voltage at which the gradient-driven, cotransporter-mediated current I vanishes. If the system is tightly coupled and if ν sodium ions and σ neutral substrate molecules S are transported per turnover, the reversal potential is given by the thermodynamic relation:

$$V_r \equiv (\psi' - \psi'')_{I=0} = \frac{RT}{\nu F}\left(\sigma \ln \frac{c''_S}{c'_S} + \nu \ln \frac{c''_N}{c'_N}\right). \qquad (9)$$

ψ' and ψ'' are the electrical potentials on the cytoplasmic and extracellular side, respectively; R is the gas constant, T the absolute temperature and F the Faraday constant. Implicit in the derivation of Eq. (9) is the assumption that the amino acid is transported as an (electrically neutral) zwitterion. Since the membrane has a finite leakage resistance R_l, the reversal potential V_r is, in general, smaller than the actually observed potential V_0 which is measured under the condition of vanishing total transmembrane current I_t:

$$V_0 \equiv (\psi' - \psi'')_{I_t=0} \simeq \frac{V_r}{1 + R_i/R_l}. \qquad (10)$$

Equation (10) is based on the assumption that the cotransport system may be represented, to a first approximation, by a combination of an electromotive force V_r in series with an internal resistance R_i (*see* inset of Fig. 14). The leakage resistance R_l includes contributions from the finite membrane-pipette seal resistance. The ratio R_i/R_l may be estimated by measuring the membrane conductance in the presence ($c_S > 0$) and in the absence ($c_S = 0$) of amino acid:

$$\frac{I_t(c_S > 0)}{I_t(c_S = 0)} \simeq 1 + \frac{R_l}{R_i}. \qquad (11)$$

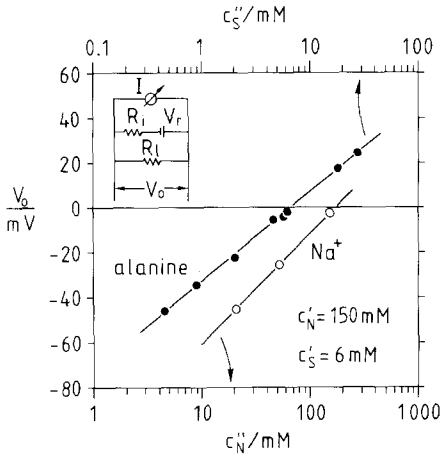

Fig. 14. Zero-current membrane potential V_0. The curve labeled "alanine" was measured with symmetrical sodium concentrations ($c'_N = c''_N = 150$ mM) and variable external alanine concentration c''_S; the curve labeled "Na^+" was measured with symmetrical alanine concentrations ($c'_S = c''_S = 6$ mM) and variable external Na^+ concentration c''_N. The pipette was perfused with a solution containing 75 mM Na_2SO_4, 6 mM L-alanine, 1 mM EGTA and 25 mM $Tris_2SO_4$, pH 7.25; external solution: 100 mM (Na_2SO_4 + $Tris_2SO_4$), 2 mM $CaSO_4$, various concentrations of alanine, pH 7.25. At the beginning of the experiment, the sodium and alanine concentrations were the same on both sides ($c'_N = c''_N = 150$ mM $c'_S = c''_S = 6$ mM); after establishing the whole-cell configuration, the current was clamped to zero and the decline of the membrane potential V to zero was followed. The external superfusion medium was then exchanged for a solution of different sodium or alanine concentration. A quasistationary voltage (V_0) was reached within $\simeq$5 sec. Thereafter the external medium was again exchanged for the reference solution (containing 6 mM alanine and 150 mM Na^+) in order to check for $V = 0$. Then the external medium was changed to a new composition and so forth. The test with the reference solution was repeated after each measurement. All data points have been recorded from the same 2-cell cluster at 37°C. The membrane (plus seal) resistance in the presence of alanine ($c'_S = c''_S = 6$ mM, $c'_N = c''_N = 150$ mM) was 0.7 GΩ; after equilibration with an alanine-free solution ($c'_S = c''_S = 0$) the resistance increased to 2.5 GΩ. After correction for the finite leakage resistance R_l according to Eq. (10), the slope of the alanine curve is 56.9 mV per decade and the slope of the Na^+ curve 68.0 mV per decade. *Inset*: equivalent circuit of the membrane; V_r and R_i are the reversal potential and the internal resistance of the cotransport system, R_l is the leakage resistance of the membrane (including the pipette-membrane seal resistance)

Since both R_l and R_i are, in general, voltage dependent, Eqs. (10) and (11) are only approximately valid.

The zero-current potential V_0 was measured under the condition $c'_N = c''_N = 150$ mM, $c'_S = 6$ mM and variable external alanine concentration c''_S. A semilogarithmic plot of V_0 *vs.* c''_S yielded a straight line with a slope of $\simeq$41 mV per decade (Fig. 14). In a second series of measurements performed with the same 2-cell cluster, the zero-current potential was determined for symmetrical alanine concentrations and variable external sodium concentration c''_N ($c'_S =$

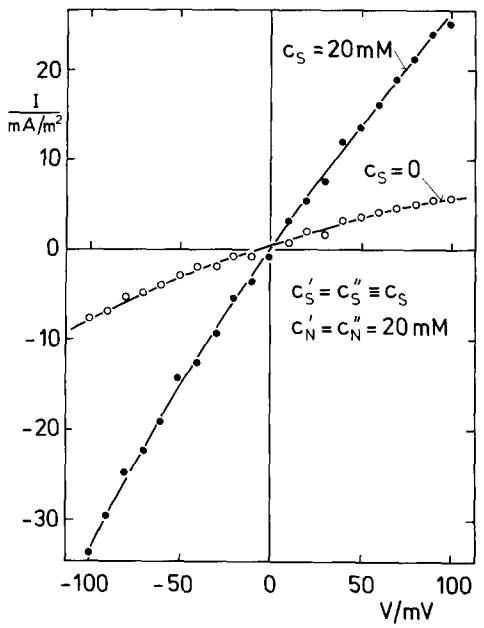

Fig. 15. Current as a function of transmembrane voltage V in the absence and in the presence of 20 mM L-alanine. Both curves have been obtained from the same cell cluster (containing three cells) at 37°C. The pipette was perfused with a solution containing 10 mM Na_2SO_4; 1 mM EGTA and 10 mM $Tris_2SO_4$; pH 7.25; external medium: 10 mM Na_2SO_4; 2 mM $CaSO_4$; 10 mM $Tris_2SO_4$; pH 7.25. The current was measured by applying short voltage pulses (pulse length 60 msec). The first series of current measurements was performed with alanine-free solutions in the pipette and in the bath. Thereafter the cell cluster was equilibrated for 10 min with a solution containing 20 mM L-alanine. When at the end of the experiment the cell cluster was again equilibrated with an alanine-free solution, virtually the same current amplitudes were observed as in the first series of measurements. After completing a sequence of voltage pulses of increasing amplitudes, the sequence was repeated in order to check for reversibility. The current amplitudes in the first and second pulse sequence were virtually indistinguishable

c''_S = 6 mM, c'_N = 150 mM). In this case the slope was ≈49 mV per decade (Fig. 14). With 6 mM L-alanine and 150 mM Na^+ on both sides, the current, at a voltage of 30 mV, was 43 pA; after equilibration of the cell cluster with an alanine-free solution the current (at the same voltage) was 12 pA. This yields, according to Eq. (11), $R_l/R_i \simeq 2.6$. Using Eq. (10), the reversal potential V_r may be obtained by multiplying V_0 with the correction factor $1 + R_i/R_l \simeq 1.4$. The slopes of V_r then become:

$$h_S \equiv \frac{\partial V_r}{\partial(\log c''_S)} \simeq 56.9 \text{ mV}$$

$$h_N \equiv \frac{\partial V_r}{\partial(\log c''_N)} \simeq 68.0 \text{ mV}.$$

Very similar values of h_S and h_N were obtained in additional experiments with two other cell clusters. The finding that h_S is close to $(RT/F)\ln 10 \approx 61.5$ mV (at 37°C) indicates that σ/ν in Eq. (9) is unity. This conclusion, of course, depends on the validity of Eq. (9), which is derived under the assumption that the fluxes of Na^+ and substrate are tightly coupled. However, as shown in part II of the paper (Jauch & Läuger, 1986), incomplete coupling always tends to *diminish* the absolute magnitude of V_r. The experimental results are thus incompatible with a sodium-alanine stoichiometry of 2:1. Excluding the less likely possibility $\sigma, \nu > 1$, we assume in the following that the Na^+/alanine stoichiometry is 1:1.

The determination of h_N has been carried out mainly as a consistency check for the measurement of the reversal potential. The fact that the corrected experimental value of h_N (68 mV) is larger than the theoretical value $(RT/F)\ln 10 \simeq 61.5$ mV is not unexpected since part of the "leakage" conductance results from sodium permeation, leading to an overestimation of the correction factor.

It is pertinent to discuss the results of this section also from a more general point of view. The finding that extracellular addition of L-alanine results in an inward current in intact cells under physiological conditions (Iwatsuki & Petersen, 1980*b*) could be explained, in principle, by the assumption that the amino acid opens a sodium-specific channel, leading to a passive influx of Na^+. This possibility can be excluded by the observation of a nonvanishing reversal potential when solutions of unequal alanine concentration but equal sodium concentration are present on both sides of the membrane (Fig. 14).

Current-Voltage Behavior

The current-voltage characteristic of an ion-driven cotransport system contains information on the nature of the elementary charge-translocations associated with substrate transport (Läuger & Jauch, 1986). The current I associated with Na-coupled alanine transport may be approximately determined from the difference $I(c_S > 0) - I(c_S = 0)$ of transmembrane current in the presence and in the absence of L-alanine. This procedure is based on the assumption that alanine has no effect on other conductive pathways in the membrane. Furthermore, part of the "leakage" current $I(c_S = 0)$ may actually result from uncoupled Na^+ transport mediated by the cotransporter; this current component is automatically eliminated by taking the difference $I(c_S > 0) - I(c_s = 0)$.

In Fig. 15 current-voltage curves are shown for identical Na^+ concentrations on both sides ($c'_N = c''_N$

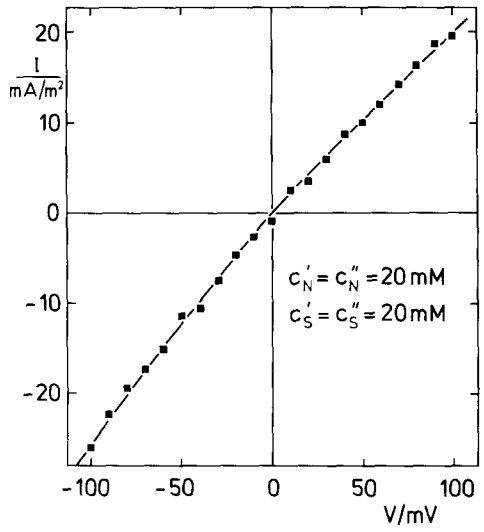

Fig. 16. Difference $I \equiv I(c_S > 0) - I(C_S = 0)$ of the current with and without L-alanine, from Fig. 15. $I(V)$ is thought to represent an approximation to the current-voltage characteristic of the cotransport system

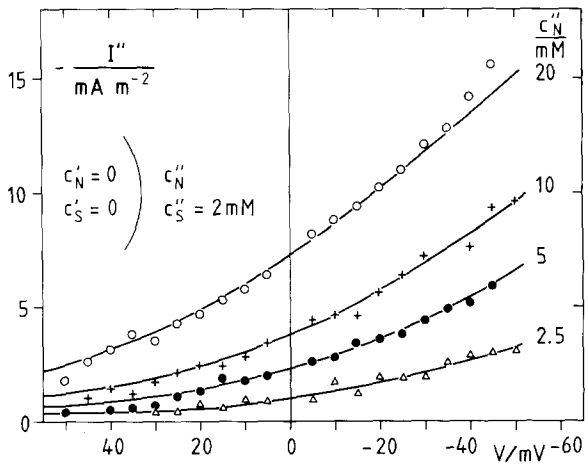

Fig. 17. Current-voltage characteristic measured with sodium- and alanine-free intracellular solutions, as a function of extracellular sodium concentration c''_N. The current was measured by applying voltage pulses of 60 msec duration. $I'' \equiv I''(c''_S > 0) - I''c''_S = 0)$ is the difference of the currents in the presence and in the absence of extracellular L-alanine (*compare* Fig. 10). $I''(c''_S = 0)$ was about 5% of $I''(c''_S > 0)$ at $c''_N = 20$ mM and zero voltage. All data have been obtained from the same cell cluster (five cells) at 37°C. The pipette was perfused with a solution containing 1 mM EGTA and 100 mM $Tris_2SO_4$, pH 7.25; external solution: 2 mM $CaSO_4$, 100 mM ($Tris_2SO_4$ + Na_2SO_4), pH 7.25, 2 mM L-alanine. The curves have been drawn according to Eq. (7) with L''_N = (50 mM) · exp(0.65 FV/RT) and $I''_{\infty,N} = -22.2$ mA/m²

= 20 mM) in the presence ($c'_S = c''_S = 20$ mM) and in the absence of L-alanine. Both curves have been obtained from the same cell cluster. Upon addition of alanine the conductance is found to increase by a factor of about 3.7 (at 50 mV). The difference $I \equiv I(c_S > 0) - I(c_s = 0)$ of the current with and without L-alanine is plotted in Fig. 16; $I(V)$ is thought to represent an approximation to the current-voltage characteristic of the cotransporter. As seen from Fig. 16, the current-voltage curve is almost linear between −100 and +100 mV, with a slight tendency for superlinear behavior at negative membrane potentials. Apart from this small nonlinearity, the $I(V)$ characteristic is nearly symmetrical with respect to $V = 0$. This finding is consistent with the results of inward- and outward-current measurements presented above, indicating again that the cotransporter is functionally nearly symmetrical.

For the quantitative analysis of $I(V)$-data it is advantageous to study the current-voltage characteristic under conditions where substrate and driving ion are present only on one side of the membrane. Current-voltage curves measured with sodium- and alanine-free intracellular solutions ($c'_N = c'_S = 0$) are represented in Fig. 17 for $c''_S = 2$ mM and different extracellular sodium concentrations c''_N. As expected for $c'_N = c'_S = 0$, only inward currents ($I'' < 0$) are observed; I'' approaches zero for increasingly positive membrane potentials $V \equiv \psi' - \psi''$.

The data of Fig. 17 are replotted in Fig. 18 in the form of a Lineweaver-Burk diagram in which $-1/I''$ is represented as a function of $1/c''_N$ for four different voltages V. An important result which is apparent from Fig. 18 is the increase of half-saturation concentration L''_N [Eq. (7)] with increasing (inside positive) voltage. This finding is consistent with the assumption that the Na^+-binding site is located inside the membrane dielectric so that a Na^+ ion approaching the site from the extracellular medium has to cross part of the transmembrane electric field. L''_N can be approximately represented by $L''_N = \tilde{L}''_N \exp(0.65 \cdot FV/RT)$, where $\tilde{L}''_N = 50$ mM is the half-saturation concentration at zero voltage. A more detailed discussion of the voltage dependence of L''_N will be given in part II of the paper.

In the plot of $(1/I'')$ versus $(1/c''_N)$ the intercept of the straight line with the $(1/I'')$-axis yields $1/I''_{\infty,N}$ [Eq. (7)]. It is seen from Fig. 18 that, within the experimental error limits, the maximum current $I''_{\infty,N}$ is virtually the same for all four voltages (−40, −20, 0 and 20 mV).

Discussion

Most investigations of sodium-coupled cotransport systems have been carried out so far by measuring isotope fluxes in membrane vesicles or intact cells

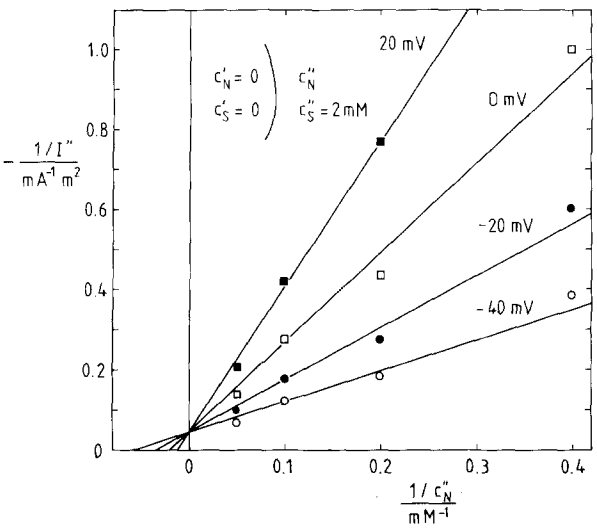

Fig. 18. Lineweaver-Burk representation of the data of Fig. 17. The reciprocal current is plotted as a function of reciprocal extracellular Na^+ concentration for four different transmembrane voltages V. The straight lines have been drawn according to Eqs. (7) with $I''_{\infty,N} = -22.2$ mA/m² and the following values of the half-saturation concentration L''_N: $L''_N = 83$ mM (20 mV); $L''_N = \bar{L}''_N = 50$ mM (0 mV); $L''_N = 30$ mM (−20 mV); $L''_N = 18$ mM (−40 mV). The voltage dependence of L''_N can be approximately described by $L''_N = \bar{L}''_N \exp(0.65\, FV/RT)$

(Kessler & Semenza, 1983; Turner, 1983; Kaunitz & Wright, 1984; Koepsell et al., 1984; Restrepo & Kimmich, 1985). In this study we have used the whole-cell recording technique for measuring electric currents associated with sodium-driven alanine transport. Pancreatic acinar cells that exhibit high rates of amino-acid transport are particularly suitable for this purpose; maximum alanine-dependent Na^+ currents are of the order of 100 pA per cell. The method of tight-seal whole-cell recordings with internal perfusion of the pipette allows us to control the composition of the intracellular medium and to measure transport rates as a function of intra- and extracellular concentrations of Na^+ and amino acid. Moreover, the transmembrane voltage may be varied over a wide range, a condition that cannot easily be realized in experiments with cells or membrane vesicles.

In order to extract mechanistic information from the experiments described above, certain conditions must be met. Current components from other transport systems such as ion pumps or passive ionic channels must be small; furthermore, it must be known whether the observed currents result from a single amino-acid transport system. In whole-cell recording experiments with internal perfusion of the pipette, intracellular low-molecular-weight solutes are almost completely replaced by solutes from the pipette medium. Since it is known that metabolites up to a molecular mass of 500-1000 g/mol can easily permeate across cell-to-cell junctions (Loewenstein, 1981; Finbow & Pitts, 1981), solute exchange with the pipette may be assumed to be fast in the experiments with small cell clusters. In most experiments, the only intracellular low-molecular solutes (apart from Na^+ and alanine) were $Tris^+$, SO_4^{2-} and EGTA. Under this condition current contributions from ion pumps such as the Na,K-ATPase are negligible. The remaining currents are likely to be alanine-independent leakage currents.

From other studies it is known that many cell preparations contain more than one sodium-coupled transport system for neutral amino-acids (Christensen, 1979, 1984; Mircheff et al., 1980; Kilberg, 1982). Evidence that a single transport system is responsible for the observed alanine-dependent currents comes from the finding (Fig. 11) that the sodium- and alanine-concentration dependence of the current exhibits simple Michaelis-Menten behavior. (If two Na^+, alanine transporters are present in the membrane, they are likely to differ in their values of $I_{\infty,S}$ and L_S so that the current will no longer be given by Eq. (6).) Two principal Na^+-coupled transport systems for alanine and similar amino acids have been characterized from a variety of cells, usually referred to as the "A-system" and the "ASC-system." As discussed above, both the pH-dependence of current, as well as the fact that 2-(methylamino)isobutyric acid (MeAIB) competes with L-alanine argue against the participation of the "ASC-system" but are compatible with the assumption that the transport system under study is similar to (or identical with) the "A-system," which is widespread in animal cells.

The stoichiometric ratio ν/σ is defined as the ratio of the number of sodium binding sites (ν) and amino-acid binding sites (σ) participating in the transport reaction. ν/σ has to be distinguished from the ratio Φ_N/Φ_S of the fluxes of sodium and amino acid; while ν/σ is a fixed number, the flux ratio, in general, depends on the concentrations of N and S. Information on the stoichiometric ratio has been obtained from two kinds of experiment, from the dependence of inward current I'' on the extracellular concentrations of sodium (c''_N) and amino acid (c''_S) and from the reversal potential V_r as a function of alanine concentration ratio c''_S/c'_S. The observed slope of V_r versus ln (c''_S/c'_S) indicates that the stoichiometric ratio ν/σ is unity. While from V_r alone the possibility $\nu = \sigma > 1$ cannot be excluded, the most likely assumption is that the transporter has a stoichiometry of 1 : 1.

Information on the stoichiometry of sodium-

alanine cotransport in other cell types is scanty so far (Johnstone, 1979). Evidence for a 1 : 1 stoichiometric ratio in the case of rat hepatocytes has been presented by Kristensen and Folke (1983, 1986). On the other hand, in experiments with *Xenopus* oocytes, Jung et al. (1984) observed sigmoid dependence of alanine-induced inward current on external sodium concentration, which indicates a Na^+/alanine stoichiometric ratio larger than 1 : 1.

The concentration dependence of current has been measured with finite concentrations of sodium and alanine on one side and vanishing concentrations on the other. Such "zero-*trans*" experiments are particularly suitable for comparison with predictions from transport models and for the evaluation of kinetic parameters. The inward current as a function of extracellular concentrations of Na^+ and alanine was found to exhibit a simple Michaelis-Menten behavior. According to Figs. 11 and 13, the half-saturation concentration L''_X of substrate X ($X = N, S$) as well as the maximum current $I'_{\infty,X}$ for $c''_X \to \infty$ depend on the concentration of the other substrate Y. The mechanistic implications of these findings will be discussed in part II of the paper (Jauch & Läuger, 1986).

Information on the sidedness of the cotransporter has been obtained by measuring inward and outward currents under "zero-*trans*" conditions for equal but opposite concentration gradients. The ratio I'/I'' of outward to inward currents was found to be close to unity in the whole experimental range of sodium and alanine concentrations. Moreover, the current-voltage characteristic of the cotransporter for symmetric concentrations of Na^+ and substrate was found to be nearly symmetric (Fig. 16). These results are consistent with (but do not prove) the assumption that the cotransporter is nearly symmetric with respect to its left-sided and right-sided kinetic parameters; see part II of the paper for further discussion of this point.

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Lapointe, Hudson and Schultz (1986) have recently used the reversal-potential method for determining the stoichiometric ratio of Na, sugar cotransport in *Necturus* small intestine.