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Sodium-Alanine Cotransport in Oocytes of *Xenopus laevis*: Correlation of Alanine and Sodium Fluxes with Potential and Current Changes

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Summary. The sodium-dependent L-alanine transport across the plasma membrane of oocytes of *Xenopus laevis* was studied by means of [^{14}C]-L-alanine, $^{22}Na^+$ and electrophysiological measurements. At fixed sodium concentrations, the dependence of alanine transport on alanine concentration follows Michaelis-Menten kinetics; at fixed alanine concentrations, the transport varies with sodium concentration with a Hill coefficient of 2. In the presence of sodium the uptake of alanine is accompanied by a depolarization of the membrane. Under voltage-clamp conditions this depolarization can be compensated by an inward-directed current. Assuming that this current is carried by sodium we arrive at a 2:1 stoichiometry for the sodium-alanine cotransport. The assumption was confirmed by direct measurements of both sodium and alanine fluxes at saturating concentrations of the two substrates, which also yielded a stoichiometry close to 2:1. The sodium-L-alanine cotransport is neither inhibited by furosemide (0.5 mmol/liter) nor by N-methyl amino isobutyric acid (5 mmol/liter). A 20-fold excess of D-alanine over L-alanine caused about 60% inhibition.

Key Words *Xenopus* · oocyte · cotransport · alanine flux · sodium flux · sodium current

Introduction

Since the first discovery of cotransport of sodium and amino acids in tumor ascites cells (Oxender & Christensen, 1963; Christensen, Liang & Archer, 1967), the nearly universal occurrence of cotransport systems has been demonstrated and many papers have appeared that deal with the analysis of their kinetics (for review, *see* Guidotti, Borghetti & Gazzola, 1978). One of the central questions concerns the stoichiometrical ratio between sodium and solute transport. In a number of instances, notably in experiments with membrane vesicles (Kinne, Barac & Murer, 1980) and red cells (Vidaver, 1964*a, b*), it was possible to measure directly the influx of sodium and solute. The results suggest stoichiometrical ratios of 1:1 between sodium and sugars or certain amino acids, and of 2:1 between sodium and certain other amino acids or inorganic phosphate. In experiments with intact cells this technique frequently encounters difficulties and the information available only allows one to make suggestions based on indirect reasoning and circumstantial evidence (e.g. Samarzija & Frömter, 1982).

In the present paper an attempt is made to determine the stoichiometrical ratio for the cotransport of alanine and sodium in the plasma membrane of the oocytes of *Xenopus laevis*. A saturable alanine transport system (Bravo, Salazar & Allende, 1976) has previously been demonstrated to exist in these cells. We have shown that this system requires sodium, similar to leucine transport (Bellé, Marot & Ozon, 1976), and have examined the sodium dependence of the influx of alanine by means of the radioactively labeled amino acid. These measurements were compared with voltage-clamp determinations of the membrane current that is known to be associated with the cotransport (Bergman & Bergman, 1981; Petersen & Singh, 1981; Frömter, 1982). This permits a direct estimate of the stoichiometry of the number of charges that are moved together with the amino acid.

Materials and Methods

Ovaries of *Xenopus laevis* were removed from the females under anaesthesia with MS 222 (Sandoz) and the oocytes were defolliculated by enzymatic treatment with collagenase (0.5 units/ml Barth's medium, 8 to 16 hr, 18 °C) and subsequent washings in Ca^{++}-free Barth's medium.

To measure alanine uptake, prophase-arrested, full grown oocytes were incubated at 21 °C in Barth's solution containing [^{14}C]-L-alanine. After suitable lengths of time, 5 oocytes were removed from the medium and carefully washed in Barth's solution. Each of the 5 oocytes was dissolved separately in sodium dodecyl sulfonate solution and the radioactivity was determined by liquid scintillation counting. The rates of uptake were derived from the slopes of the straight line through the data points obtained at 4 different sampling times within one hour of incubation. $^{22}Na^+$ uptake was measured essentially similar except that 60 oocytes were counted in pairs of two per vial.

Barth's solution had the following compositon (in mmol/liter): 88 NaCl, 1.0 KCl, 2.4 $NaHCO_3$, 0.82 $MgSO_4$, 0.33 $Ca(NO_3)_2$, 0.41 $CaCl_2$, 5.0 Hepes, pH 7.6. Barth's solutions with variable sodium concentrations were obtained by isosmotic substitution of NaCl by choline chloride and of $NaHCO_3$ by Li_2CO_3. The voltage-clamp experiments were performed by the conventional 2-microelectrode technique. Micropipettes contained 1 mol/liter KCl and had electrical resistances of about 10^7 Ω. The membrane resting potentials given as inside minus outside potentials, were -67.8 ± 6.5 mV ($n = 28$). During the time course of the measurements, the oocytes were kept in a chamber which was continuously perfused with unmodified or modified Barth's solution. The temperature was always 20 °C.

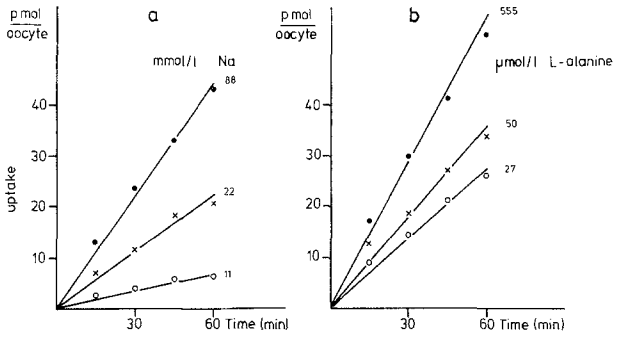

Fig. 1. Time course of uptake of [^{14}C]-alanine by full-grown oocytes. Each data point represents the mean of uptake into 4 to 5 separately counted oocytes. *a*) Alanine uptake at constant concentration of 558 μmol/liter alanine at three different Na^+ concentrations (11, 22 and 88 mmol/liter). *b*) Alanine uptake at a constant concentration of 88 mmol/liter Na^+ at three different alanine concentrations (27, 50, 555 μmol/liter)

Results

The uptake of alanine by the prophase-arrested, full grown oocytes follows a linear time course for at least the first 60 min after exposing the cells to the alanine containing Barth's solution (Fig. 1 *a*, *b*). The Figure indicates that the data point at the first sampling time is in the majority of the experiments situated above the straight line that passes through the origin. This suggests that the inward transport of the amino acid may be preceded by some rapid binding to the outer cell surface. We have calculated the uptake rates from straight lines that were forced through zero as well as from the best straight lines that can be plotted through the data points without this constraint. We found that the results of the evaluation of the kinetics of alanine transport were similar regardless of how the uptake rates were obtained. In all subsequent Figures we show data that are based on the assumption that binding prior to transport can be neglected and that the curves start at the origin.

Figure 2 *a* shows that with increasing alanine concentration the rate of uptake increases. The relationship between concentration and rate of uptake is a function of the sodium concentration in the medium. In the absence of sodium, the relationship is linear up to the highest alanine concentration tested. The addition of sodium enhances the rate of uptake. After subtraction of the sodium-insensitive uptake, the curves show simple saturation kinetics. Both V_{max} and K_m vary with the sodium concentration: V_{max} increases with increasing sodium while K_m decreases (Fig. 2 *b*).

In a second set of experiments, we studied the influence of the variations of the sodium concentration on alanine uptake in a more systematic manner. We chose an alanine concentration at which the transport system is saturated when the sodium concentration produces maximal effect. Figure 3 shows an S-shaped relationship between alanine uptake and sodium concentration. An Eadie-Hofstee plot shows deviations from linearity when we plot on the abscissa $v/[Na]$. However, when we plot $v/[Na]^2$ we obtain straight-line relationships indicating that the uptake takes place with a Hill coefficient of 2. When a lower than maximally saturating alanine concentration is chosen, one again obtains an S-shaped curve, which again yields a straight-line relationship in an Eadie-Hofstee plot where $v/[Na]^2$ is plotted on the abscissa. Again, the Hill coefficient is 2, but the maximal rate of transport is reduced.

In order to explore whether the sodium dependence of alanine transport represents an activation process without or with cotransport, we studied the effects of alanine and sodium on membrane potential and membrane current. Figure 4 shows that the addition of alanine to oocytes that are suspended in sodium-containing Barth's solution leads to a depolarization of the membrane. The extent of this depolarization varies with the alanine concentration in the medium. The potential drop reflects a current that can be measured under voltage-clamp conditions (Fig. 5). The potential change can only be seen when both alanine and sodium are present (Fig. 6). This suggests that we are dealing with a cotransport of the two substances, uncharged alanine and, presumably, sodium.

In a set of experiments with the oocytes of 3 different animals, an attempt was made to determine the stoichiometrical relationship between sodium and alanine transport. For this purpose, V_{max} was measured by means of [^{14}C]-alanine and the sodium-dependent current was determined at constant membrane potential. The Table shows that after conversion of the current into fluxes, the ratio between the movements of sodium and alanine are close to 2:1. This applies not only to the oocytes

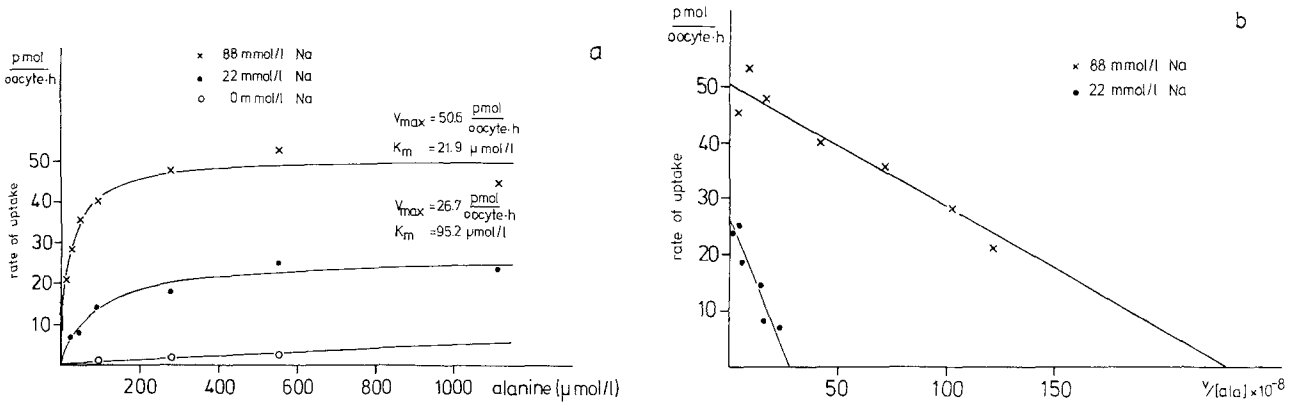

Fig. 2. *a*) Rate of alanine uptake as a function of alanine concentration in the medium. 0, 22 and 88 mmol/liter refer to the Na^+ concentration in the medium at which the rates of uptake were measured. The data at 22 and 88 mmol/liter Na^+ are corrected for the Na^+-independent alanine uptake. The solid lines with the parameters V_{max} and K_m are least-squares fits of Michaelis-Menten kinetics to the data. *b*) Eadie-Hofstee plot of the data in Fig. 2*a*

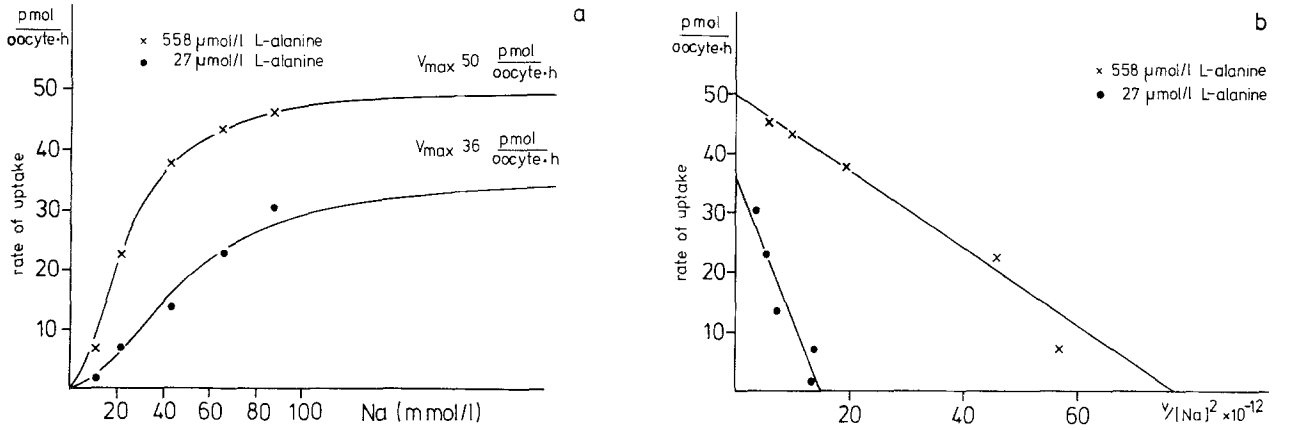

Fig. 3. *a*) Rate of alanine uptake as a function of Na^+ concentration in the medium. 27 and 558 µmol/liter refer to the alanine concentration in the medium. The data are corrected for the Na^+-independent alanine uptake. The solid lines represent least-squares fits of $V_{max} \cdot [Na^+]^2/(K_m + [Na^+]^2)$, with fixed V_{max} obtained from Fig. 3*b*. *b*) Modified Eadie-Hofstee plot of the data in Fig. 3*a*; on the abscissa is plotted $v/[Na^+]^2$ instead of $v/[Na^+]$ as in the original version of the plot

from the two animals with nearly equal V_{max} values, but also to the oocytes of the third animal with a V_{max} that is only 60% of the value found in the oocytes of the other two animals. It appears, therefore, that the cotransport of alanine and sodium in the oocytes takes place at a sodium/alanine ratio of 2:1.

The stoichiometrical ratio of 2 sodium ions/1 alanine inferred from the electrochemical measurements was confirmed by direct determinations of sodium and alanine influx, using $^{22}Na^+$ and [^{14}C]-alanine for double labeling. The fluxes were measured in 30 pairs of oocytes of the same female in Barth's solution containing 558 µmol/liter alanine and 44 mmol/liter Na^+. Under these conditions the cotransport system is nearly saturated with both substrates. Sodium influx, as measured

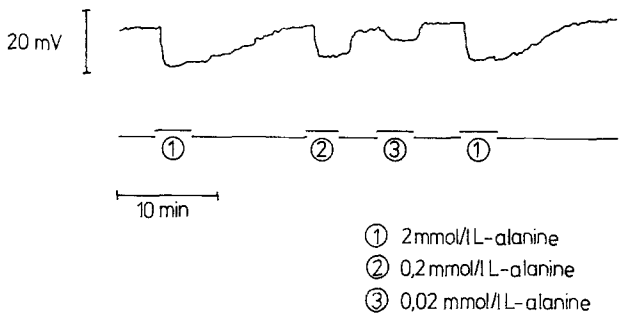

Fig. 4. Changes of membrane potential induced by changes of the alanine concentration. Upper trace: membrane potential; downward deflections indicate depolarizations of the resting potential ($E_r = -75$ mV). The chamber containing the oocyte is perfused with alanine-free Barth's solution. Upward deflections of the lower trace indicate a replacement of the Barth's solution by an otherwise identical medium containing alanine of one of the concentrations indicated in the Figure

during 60 min of incubation in the presence of the radioactively labeled substrates, was 91.0±10 pmol/oocyte/hr; the alanine influx was 48.7±5.0 pmol/oocyte/hr corresponding to a stoichiometrical ratio of 2:1. Rather similar results were obtained when the fluxes were measured in the presence of furosemide (0.5 mmol/liter): The sodium influx was 79.7±10.5 pmol/oocyte/hr, the alanine influx was 48.7±12.5 pmol/oocyte/hr, giving again a stoichiometrical ratio of 2:1. The latter set of data does not only confirm the former but also shows that furosemide neither affects the sodium-alanine cotransport nor the alanine-independent sodium transport. Measurements of the alanine-induced current were performed parallel to the measurements of the cotransport by means of radioisotopes. When the membrane potential was clamped at the resting potential seen before initiation of cotransport, the alanine-induced current was 3.0 nA, which corresponds to a flux of 114 pmol/oocyte/hr. This is close to the isotopically measured sodium flux of 91.0 pmol/oocyte/hr that accompanies the alanine movements.

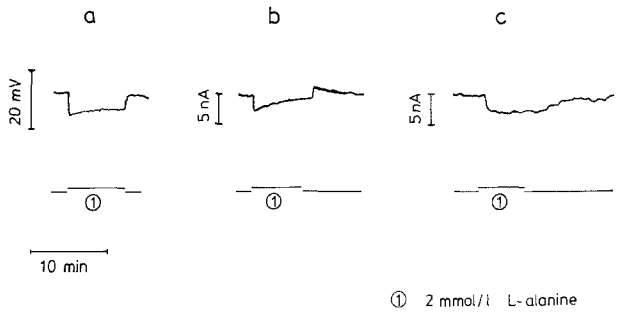

Fig. 5. Changes in membrane potential and current in response to perfusion with 2 mmol/liter L-alanine. The periods of perfusion with alanine-containing media are indicated by the lower traces. *a*) Membrane potential; a downward deflection indicates a depolarization of the resting potential ($E_r = -62$ mV). *b*) and *c*) Membrane currents during constant voltage clamp to the resting potential E_r; downward deflections indicate inward currents of positive charges. *a* and *b* are obtained from the same oocyte. The gradual current decline seen in part *b* was observed in many experiments and reflects an additional transmembrane charge movement that is not attributed to Na^+ movements. In some experiments this decline of inward current was not observed (part *c*). Comparing steady-state currents as in part *c* or peak currents as in part *b* during alanine application with measurements of the alanine flux give the same stoichiometrical ratio of 2:1 for the sodium-alanine cotransport

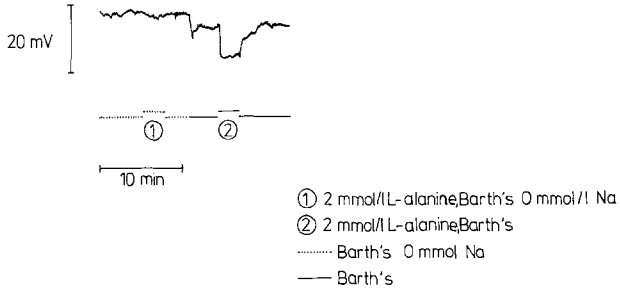

Fig. 6. Addition of alanine in the absence of Na^+ (Na^+ substituted by tetramethylammonium (TMA)) produces no potential drop (1); when added in the presence of Na^+, the potential drop is induced (2). Note: the change from TMA-substituted barth's medium to Na^+-containing Barth's medium is accompanied by a potential drop of no more than 5 mV; resting potential in Barth's solution: −50 mV

Table. Comparison of isotopically measured maximal velocity (V_{max}) of alanine uptake with current induced by exposure of the oocyte to a saturating alanine concentration (I_m)

Animal	V_{max}	I_m	Ratio I_m/V_{max} after conversion of I_m in pmol/oocyte/hr[a]		
	(pmol/oocyte/hr)	(nA)	1:1	2:1	3:1
1	58.9±3.2	3.1±0.6 ($n=4$)	28.4± 5.1	56.8±10.3	85.2±15.4
2	32.4±4.6	2.3±0.4 ($n=5$)	20.8± 3.7	41.6± 7.3	62.4±11.0
3	54.7±4.3	3.2±0.5 ($n=8$)	29.8± 4.4	59.5± 8.7	89.3±13.0
	% of measured V_{max}				
1	100± 5.4		48.2± 8.7	96.4±17.5	144.7±26.0
2	100±14.2		64.2±11.4	128.4±22.5	192.6±34.0
3	100± 7.8		54.5± 8.0	108.8±15.9	163.3±23.8
Mean	100± 7.6		55.6± 7.3	111.2±14.5	166.9±21.7

[a] These 3 columns give values of V_{max} to be expected from I_m for three different stoichiometrical ratios of I_m/V_{max}. It is evident that the ratio 2:1 gives the best agreement with the determinations of V_{max}

Differences of V_{max} values do not only represent biological variations between the oocytes from different animals. When the oocytes in the same animal are removed from their follicles and incubated *in vitro,* an increase with time of V_{max} at nearly constant K_m is observed (Fig. 7). The difference between the oocytes from different animals reflects, perhaps, differences of the developmental stages of the oocytes.

To classify the transport system of the oocyte of *Xenopus,* with respect to the other hitherto known amino acid transport systems, we studied the sensitivity of alanine transport to D-alanine and N-methyl amino isobutyric acid. The latter produced no inhibition, even when added at a large excess over the alanine. D-alanine produced about 60% inhibition at a 20-fold excess (Fig. 8). In the presence of sodium, D-alanine was also found to induce a sodium-dependent voltage drop, although the effects were only about one-half as large as with L-alanine.

Discussion

The dependence of alanine flux on sodium concentration is sigmoidal and yields a straight-line relationship when the flux is plotted against $v/[Na]^2$. This strongly suggests that the binding of two sodium ions is required for the transport of one amino acid molecule. The measurement of the membrane current shows that at complete saturation of the transport system with both sodium and the amino acid, 2 positive charges are moved inward together with 1 amino acid molecule. On the basis of the electrical measurements alone the possibility cannot be ruled out that this cotransport process may be associated with the movements of other anions or cations that electrically balance each other out and thus do not contribute to the current. The stoichiometrical ratio 2:1 represents, therefore, a minimum estimate. The simultaneous measurements of sodium and alanine uptake into single oocytes by double labeling with $^{22}Na^+$ and [^{14}C]-alanine confirms, however, that the ratio refers indeed to the coupled transport of 2 sodium ions and 1 alanine molecule.

The sigmoidal dependence of the alanine influx on sodium seems to represent a cooperative effect of sodium ions that cannot be related to membrane depolarization and the concomitant reduction of the driving force for sodium. There are two reasons that support this view: (i) large changes of the sodium concentration exert only minor effects on membrane potential (*see* Fig. 6 and Kusano, Miledi & Stinnakre, 1982); (ii) experiments with metaphase-arrested, shed oocytes (Jung & Richter, 1983) with resting potentials of -15 to -18 mV gave the same dependence on sodium concentration of the alanine transport as the experiments with the full-grown oocytes used in this work with resting potentials of about -68 mV.

Among the various transport systems for neutral amino acids that have been described so far there are 3 that are dependent on sodium. Two of the systems are designated by the letters ASC and A. Both are widely distributed in different types of cells. The third system prefers glycine as a substrate, and it has been found in red blood cells. The ASC system can be distinguished from

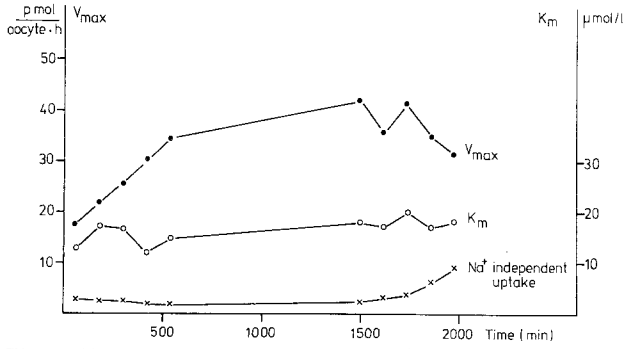

Fig. 7. Change of V_{max}, of the rate of Na^+-independent alanine uptake (left ordinate), and of K_m (right ordinate) with time after defolliculation. The incubation medium is standard Barth's solution. V_{max} and K_m are corrected for the Na^+-independent uptake

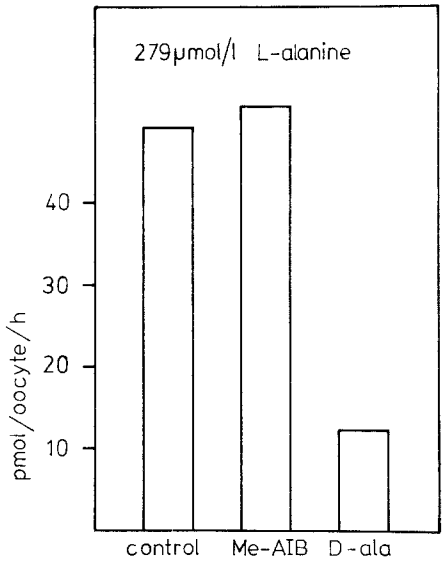

Fig. 8. Na^+-dependent rate of uptake of 279 µmol/liter L-alanine in control medium containing 88 mmol/liter Na^+, and in the presence of either 5 mmol/liter N-methyl amino isobutyric acid or 5 mmol/liter D-alanine added to the control medium

the others by its stereospecificity, its insensitivity to inhibition by N-methyl amino butyric acid or other methylated amino acids, and pH changes. The rate of amino acid transport is hyperbolically related to the sodium concentration in the medium. The ASC system has been shown to transport alanine in a number of tissues. Deviations from hyperbolic relationship between alanine flux and sodium concentration, toleration of an N-methyl group as a substituent, and its sensitivity to pH changes are important properties of the A system. It has been suggested that this system and the glycine transport system may just be variants of each other (Guidotti et al., 1978). The rate of glycine transport is linearly related to the reciprocal of the square of the sodium concentration in the medium (Vidaver, 1964*a*; Al-Saleh & Wheeler, 1982).

As shown in Fig. 3, the dependence of alanine transport in *Xenopus* oocytes on the square of the sodium concentration is similar to that of the glycine transport in red blood cells (Vidaver, 1964*a*). In unpublished experiments a strong pH dependence in the range of pH 4.4 to 8.1 was observed, similar to the pH dependence for the A system in Ehrlich ascites tumor cells described by Christensen et al. (1967). This suggests that our results with *Xenopus* oocytes refer to the A system. The lack of inhibition by N-methyl amino isobutyric acid and the slight-inhibition by D-alanine (Fig. 8) is similar to that observed in the ASC-system (Guidotti et al., 1978). We believe, therefore, that the stereospecifity and the specifity with respect to the methyl group in the A system of *Xenopus* oocytes is higher than in other types of cells. The existence of the A system has been demonstrated previously in *Xenopus* embryos developed to stages 23 to 25 (Mlot, Prahlad & Hampel, 1978).

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