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Lanthanide/Proline Cotransport across Rabbit Renal Brush Borders

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Summary. It has been suggested previously that La^{3+} can replace Na^+ on various cotransport systems in renal brush border membranes. In the present study, we used rabbit renal brush border membrane vesicles to examine the specificity and kinetics of Ln^{3+}/proline cotransport. Experiments were carried out under zero-*trans*, voltage clamped conditions using a rapid-mix/filtration technique. Initial experiments confirmed that La^{3+} produced the classical overshoot phenomenon. The initial rates of proline uptake relative to Na^+ were Eu^{3+}, Tb^{3+}, Nd^{3+}, Pr^{3+}, Ho^{3+} (3.3) > Na^+ (1.0) > La^{3+} (0.86) > choline$^+$ (0.1). At a saturating salt concentration, uptake saturated with increasing proline concentration: the K_t and J_{max} were 0.05 mM and 17 pmol mg^{-1} sec^{-1} in Na^+; and 0.28 mM and 73 pmol mg^{-1} sec^{-1} in Tb^{3+}. The higher J_{max} in Tb^{3+} indicates that the Tb^{3+}-proline loaded carrier is more effective than the Na^+-proline loaded carrier in overcoming some rate-limiting barriers in the transport process. Na^+ activated proline uptake with a Hill coefficient of 1.6 and a $K_{0.5}$ of 21 mM, while Tb^{3+} activated with a Hill coefficient of 0.88 and a $K_{0.5}$ of 28 mM. The Hill coefficient for Na^+ suggests two binding sites, whereas the Hill coefficient for Tb^{3+} may indicate negative cooperativity between the trivalent ligands at the binding sites. We conclude that lanthanides are able to substitute for Na^+ on the brush border proline carrier and that the lanthanides may serve as useful probes for the ligand binding sites.

Key Words brush border membranes · lanthanides · cotransporters · sodium · amino acids

Introduction

A striking feature of Na^+ cotransport systems is their specificity for sodium. No other monovalent or divalent cations are able to substitute for Na^+ to any significant degree in a wide variety of tissues (*see* Schultz & Curran, 1970). However, a recent electrophysiological study of cotransporters in renal brush border membrane vesicles provided some evidence that La^{3+} was able to substitute for Na^+ (Schell & Wright, 1985). To examine this possibility further, we have measured the ability of the lanthanides (Ln^{3+}) to stimulate the uptake of labeled substrates. We have confirmed that Ln^{3+} support cotransport of L-proline and probably also D-glucose and succinate. In the case of L-proline transport across renal brush border membranes, we have measured the selectivity of six lanthanides, and compared and contrasted the kinetics of Tb^{3+}/proline cotransport with Na^+/proline cotransport. A preliminary account of some of our findings has already been presented (Birnir, Hirayama & Wright, 1987). These results provide unique information about cation interactions with the proline cotransporter.

Materials and Methods

Renal cortical brush border membrane vesicles were prepared from rabbits by a Ca^{2+} precipitation method (Wright et al., 1980). The membranes were washed once in the appropriate preincubation buffer (detailed in figure legends) containing 25 μg/ml of valinomycin, pelleted, resuspended in a small volume of the same buffer and stored in liquid nitrogen until use. The brush border marker enzyme alkaline phosphatase was enriched at least 10-fold with respect to the initial homogenate. Transport measurements were done under zero-*trans*, voltage clamped conditions using a rapid mix and rapid filtration technique (Wright et al., 1983). A 40–90 μl aliquot of transport buffer, containing radiolabeled substrate and appropriate concentrations of unlabeled substrate and salts (detailed in figure legends), was rapidly mixed with a 10-μl aliquot of the brush-border suspension. The transport reaction was stopped with 1 ml of an ice-cold isosmotic KCl quench solution containing 10 mM labeled substrate and 10 mM Mes/Tris at pH 6.0. This pH was selected to facilitate filtering of the quenched solution. The quenched solution was filtered using prewetted 0.45 μm cellulose ester filters (type GN-6, Gelman) and quickly rinsed with an additional 4 ml of the cold quench solution. The filters were dissolved in scintillation cocktail (Liquiscint, National Diagnostics) and counted. Values for nonspecific retention of radioactivity by the filters and vesicles were obtained from zero time uptakes and were subtracted from total filter radioactivity. Timing for the short intervals was done with an electronic metronome. All experiments were performed at room temperature (20–23°C).

The preincubation buffer and the Na^+-containing uptake medium were at pH 7.5 as detected by a pH meter, whereas the La^{3+} medium was pH 7.0, and the Tb^{3+} medium was pH 6.5 as determined by pH indicator paper. The effect of pH (5.5–7.5) on

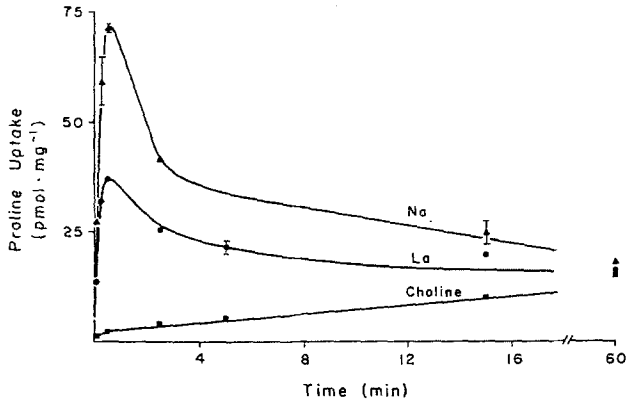

Fig. 1. La^{3+} time course. Time course of L-proline uptake into brush-border membrane vesicles. Vesicles were suspended in 490 mM mannitol, 10 mM HEPES/Tris, pH 7.5, 50 mM KCl, and 25 μg/ml valinomycin. The uptake medium consisted of 40 μM labeled L-proline, 10 mM HEPES/Tris, 50 mM KCl, 25 μg/ml valinomycin, and 100 mM NaCL, $LaCl_3$, or choline chloride. Isosmolarity was maintained with mannitol. Data are given as mean ± SE (n = 3 or 4). SE bars are shown when the SE is larger than the size of the symbol

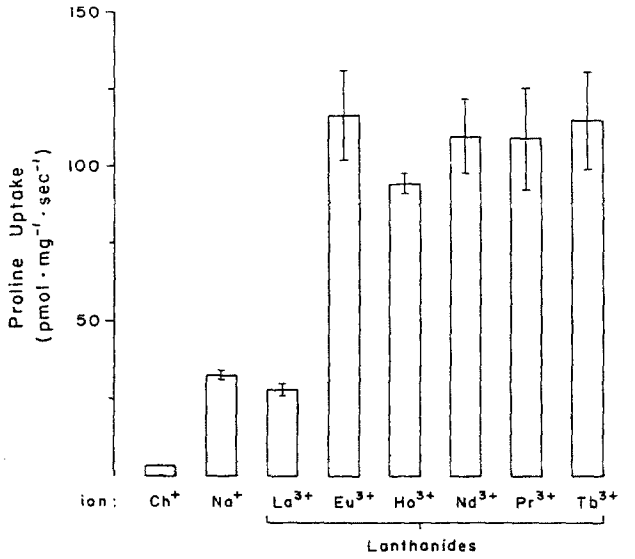

Fig. 2. Lanthanides selectivity. Initial rates of L-proline uptakes. Vesicles were suspended in 490 mM mannitol, 10 mM HEPES/Tris, pH 7.5, 50 mM KCl, and 25 μg/ml valinomycin. The uptake medium consisted of 0.85 mM labeled L-proline, 10 mM HEPES/Tris, 50 mM KCl, 25 μg/ml valinomycin, and 100 mM NaCl, $LaCl_3$, $EuCl_3$, $HoCl_3$, $NdCl_3$, $PrCl_3$, $TbCl_3$, or choline chloride. Isosmolarity was maintained with mannitol, and the proline uptakes were measured using 5-sec time points. Data are given as mean ± SE (n = 3 or 4)

transport was tested both under pH gradient conditions and pH nongradient conditions. In neither case did pH in this range affect Na^+-dependent proline transport (*see also* Hammerman & Sacktor, 1977).

Data were analyzed by computer as described previously (Wright et al., 1983).

D-(6-^{3}H(N)) glucose, > 25 Ci/mmol; L-(U-^{14}C) lactate, 150 mCi/mmol; L-(2, 3, 4, 5-^{3}H) proline, > 60 Ci/mmol; and (2, 3-^{14}C) succinate, 42 mCi/mmol were purchased from Amersham, ICN, or New England Nuclear. The lanthanides were from Aldrich Chemical Company. All chemicals were of the highest grade commercially available.

Results

Time Course of Proline Uptake

The time courses for ion-dependent proline uptake into rabbit renal cortical brush border vesicles are shown in Fig. 1. In the presence of an inwardly directed Na^+ or La^{3+} concentration gradient, there was a rapid accumulation of proline within the vesicles which peaked at around 30 sec and then declined towards a common equilibrium value. The absolute magnitude of the peak is greater in the presence of Na^+. In the absence of Na^+ or La^{3+}, the initial rates of uptake were reduced, and there was no overshoot. The magnitude and time course of the uptakes in Na^+ and choline are comparable to those reported previously (Hammerman & Sacktor, 1977; Mircheff et al., 1982). We then examined whether

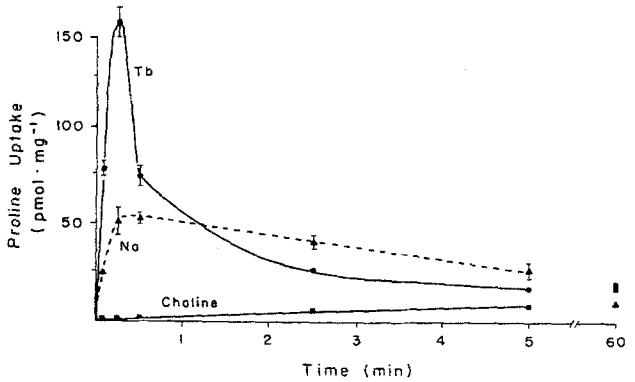

Fig. 3. Tb^{3+} time course. Time course of L-proline uptake into brush-border membrane vesicles. Vesicles were suspended in 590 mM mannitol, 10 mM HEPES/Tris, pH 7.5, 50 mM KCl, and 25 μg/ml valinomycin. The uptake medium consisted of 50 μM labeled L-proline, 10 mM HEPES/Tris, 50 mM KCl, 25 μg/ml valinomycin, and 140 mM NaCl, $TbCl_3$, or choline chloride. Isosmolarity was maintained with mannitol. Data are given as mean ± SE (n = 3 or 4). SE bars are shown except where SE was less than the size of the symbol

any of the other lanthanides could substitute for Na^+. The lanthanides tested were: europium (Eu^{3+}), holmium (Ho^{3+}), neodymium (Nd^{3+}), praseodymium (Pr^{3+}), and terbium (Tb^{3+}). Initial rates of proline uptake for the five different lanthanides (Fig. 2) are three to four times higher than in the

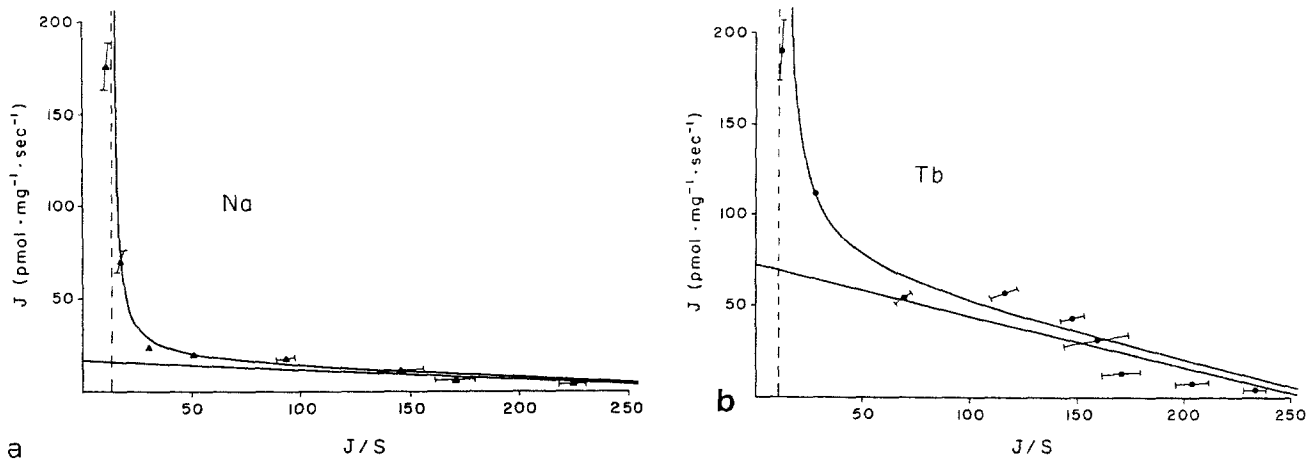

Fig. 4. Proline uptake kinetics. Kinetics of total L-proline uptake. Conditions were identical to those described for Fig. 3, except the labeled L-proline concentration varied from 20 μM to 16 mM. The curved line represents the best fit of the data by nonlinear regression analysis to an equation with one saturable and one diffusional component. The slanted line depicts the saturable component of the uptake, whereas the vertical line depicts the diffusive process. The same vesicle preparation was used in the Na^+ (*a*) and the Tb^{3+} (*b*) experiment. Uptakes were measured using 5-sec time points. Data are given as mean ± SE (n = 3 or 4). SE bars are shown except where SE was less than the size of the symbol. The correlation coefficient of the fit is 0.988 for Na^+ and 0.986 for Tb^{3+}

presence of Na^+ or La^{3+}. Figure 3 shows a time course in the presence of an inwardly directed Tb^{3+} concentration gradient where the overshoot characteristics of ion-coupled transport into an intravesicular space is observed. The Tb^{3+}-supported proline transport differs from the uptake in Na^+ in the greater absolute magnitude of its peak and its earlier time to peak (15 *vs.* 30 sec).

Proline Uptake Kinetics

The kinetics of L-proline transport in the presence of either *cis* NaCl or *cis* $TbCl_3$ were measured using 5-sec time points (uptake of 40 μM L-proline was linear from 0–6 sec), under zero-*trans*, voltage clamped conditions. Figure 4 shows the initial proline uptake rates as Woolf-Augustinsson-Hofstee plots. The curves depict the best fit of the data to an equation with one saturable and one diffusive component,

$$J^t = J^i + J^P$$

$$J^t = (J_{max}[Pro])/(K_t + [Pro]) + P[Pro]$$

where J^t = total proline uptake, J^i = proline uptake due to a saturable transport system, J^P = proline uptake due to diffusion, J_{max} = maximum velocity of transport for the saturable transport system, K_t = half saturation concentration of proline uptake, P = permeability coefficient, and [Pro] = proline concentration. The vertical line depicts the diffusive process with a permeability coefficient P equal to the X intercept. If the diffusive component of the uptake is subtracted from the total uptake, transport conforming to Michaelis-Menten-type kinetics is observed and is linear with the Y intercept equal to J_{max} and a slope equal to $-K_t$. The kinetic parameters are given in Table 1. In the presence of the Tb^{3+} gradient, J_{max} was four times higher than in the presence of the Na^+ gradient, but the apparent affinity for proline was about six times lower.

Table 1.

Ion	Proline kinetics		
	J_{max} (pmol mg^{-1} sec^{-1})	K_t (mM)	P (μl mg^{-1} sec^{-1})
Na^+	17 ± 3	0.05 ± 0.03	13 ± 1
Tb^{3+}	73 ± 10	0.28 ± 0.07	10 ± 2

The kinetic constants were calculated by nonlinear regression analysis of the total L-proline uptake, depicted in Fig. 4*a* and *b*, when the data were fitted to an equation with one saturable and one diffusional component. J_{max} is the maximum velocity of transport for the saturable transport system. K_t is the half saturation concentration of proline uptake, and P is the permeability coefficient. The correlation coefficient of the fit is 0.988 and 0.986 for Na^+ and Tb^{3+}, respectively.

Ion-Activated Uptakes

Figure 5 shows Na^+ and Tb^{3+} activation curves. L-proline uptakes were measured using 5-sec time points and zero-*trans*, voltage clamped conditions.

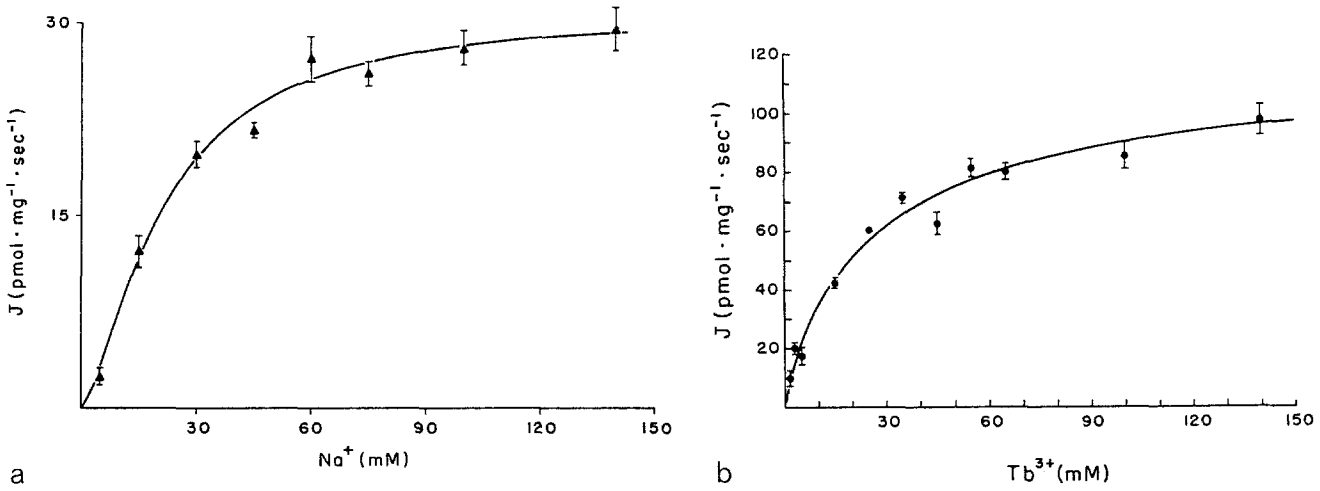

Fig. 5. Ion activated L-proline uptakes. Conditions were identical for those described for Fig. 3. except the labeled L-proline concentration was 0.85 mM and the Na^+ or Tb^{3+} concentration varied from 0–140 mM. The ionic strength was maintained with choline chloride. The activation curve was calculated by fitting the data by nonlinear regression analysis to the Hill equation. Uptakes were measured using 5-sec time points. Data are given as mean ± SE (n = 4). SE bars are shown except where SE was less than the symbol. The correlation coefficient of the fit is 0.995 for Na^+ and 0.994 for Tb^{3+}

Table 2.

Ion	Ion kinetics		
	J'_{max} (pmol mg^{-1} sec^{-1})	$K_{0.5}$ (mM)	n
Na^+	31 ± 2	21 ± 2	1.6 ± 0.1
Tb^{3+}	120 ± 12	28 ± 8	0.88 ± 0.04

The apparent maximum velocity of proline uptake, J'_{max}, and the ion half-saturation constant, $K_{0.5}$, were calculated by nonlinear regression analysis of the proline uptake, depicted in Fig. 5*a* and *b*, when the data were fitted to the Hill equation. The correlation coefficient of the fit is 0.995 and 0.994 for Na^+ and Tb^{3+}, respectively. The Hill constant, n, was obtained from the slope of a line calculated by linear regression analysis of a Hill plot of the data. The correlation coefficient of the fit was 0.952 and 0.938 for Na^+ and Tb^{3+}, respectively.

The uptake rates are plotted as a function of the ion concentration and the data fitted by nonlinear regression analysis to the Hill equation:

$$J = (J'_{max}[\text{ion}]^n)/(K^n_{0.5} + [\text{ion}]^n)$$

where, J = total proline uptake, J'_{max} = maximum velocity of proline uptake at a fixed proline concentration, $K_{0.5}$ = ion concentration giving 0.5 J'_{max}, n = Hill coefficient, and [ion] = ion concentration. The Na^+ activation curve is sigmoidal, whereas the Tb^{3+} activation curve appears hyperbolic. The kinetic constants determined from the experiments in Fig. 5 are given in Table 2. There is no significant difference in the affinity of the carrier for the two ions, $K_{0.5}$ being 21 ± 2 for Na^+ and 28 ± 8 for Tb^{3+}, but the Hill coefficients are different. The Hill coefficient for Na^+ (1.6) indicates a minimum of two Na^+ binding sites, whereas the Hill coefficient for Tb^{3+} (0.88) may suggest negative cooperativity between the trivalent cations at the ligand binding sites.

Ion-Dependent Transport of Substrates

Previous experiments using voltage-sensitive dyes had indicated that La^{3+} was able to support uptake of glucose, lactate, proline, and succinate in renal cortical brush borders (Schell et al., 1985). Table 3 shows initial rates of ion-dependent uptake of these substrates using radioactive tracers. Tb^{3+} was able to support transport of glucose and succinate in addition to proline. However, the Tb^{3+}-dependent glucose and succinate transport are only 4 and 8%, respectively, of the Na^+-dependent uptake as compared to 127% for the Tb^{3+}-supported proline transport (Table 3). Tb^{3+} did not support lactate uptake.

Effects of Voltage

Figure 6 shows the voltage dependence of the Tb^{3+}-supported and Na^+-supported proline uptake at 30 sec with a proline concentration close to its K_t value. When the membrane potential was changed from 0 to −59 mV (intravesicular space with respect to the outside solution), there was an increase in uptake in both cases and an indication of a greater effect in Tb^{3+} than in Na^+. The initial rates of uptake at saturating proline concentrations, as well as

Table 3.

Substrate	Ion-dependent transport of substrates		
	Uptake (pmol mg^{-1} sec^{-1})		
	Na^+	Tb^{3+}	Choline
Glucose	14.5 ± 0.4 (n = 4)	0.61 ± 0.13 (n = 4)	0.11 ± 0.01 (n =4)
Lactate	37.0 ± 0.6 (n = 4)	0.18 ± 0.04 (n = 4)	0.56 ± 0.03 (n = 4)
Proline	6.0 ± 0.3 (n = 4)	7.5 ± 0.9 (n = 4)	0.36 ± 0.10 (n = 3)
Succinate	22.6 ± 1.7 (n = 4)	1.9 ± 0.2 (n = 5)	0.003 ± 0.03 (n = 4)

Ion-dependent transport of substrates. Conditions were identical to those described for Fig. 3, except the substrate varied. It was either glucose, lactate, proline, or succinate. Data are given as mean ± SE.

at concentrations well below the K_ts, showed the same trend, but the difference in uptake between 0 and −59 mV was smaller. These results, together with the observations that L-proline in the presence of Na^+ or La^{3+} depolarizes the brush-border membrane (Schell, Stevens & Wright, 1983; Schell & Wright, 1985), indicate electrogenic $Na^+(Ln^{3+})$/proline cotransport.

Discussion

In this study, we have shown that lanthanides are able to substitute for Na^+ in driving cotransport of amino acids, sugars, and carboxylic acids across renal brush border membranes. In the case of L-proline, which is handled mainly by an IMINO carrier (*see* Hammerman & Sacktor, 1977; Mircheff et al., 1982; Stevens & Wright, 1985), we provide five criteria to support this conclusion: (i) La^{3+} and Tb^{3+} gradients produce the diagnostic overshoot phenomenon only seen previously with Na^+ gradients (Figs. 1 and 3); (ii) uptake at a fixed cation concentration is saturable with respect to the L-proline concentration (Fig. 4*a* and *b*); (iii) uptake at a fixed proline concentration is a saturable function of the Tb^{3+} and Na^+ concentration (Fig. 5*a* and *b*); (iv) proline uptake in both Tb^{3+} and Na^+ solutions is sensitive to the membrane potential (Fig. 6); and (v) proline depolarizes the membrane potential in Na^+ and La^{3+} solutions (Schell & Wright, 1985).

The existence of secondary active transport is most commonly demonstrated in vesicles by showing that an ion gradient alone can drive concentrative uptake of substrate. Here we have shown that Na^+, La^{3+}, and Tb^{3+} gradients, but not a choline gradient, drive the concentrative uptake of proline into renal vesicles. As the Na^+ and Ln^{3+} gradients dissipate, the driving force dissipates, and the proline in the vesicle falls back towards the equilibrium value obtained in choline. It is the physical coupling of the Na^+ or Ln^{3+} flux to the proline flux that is the primary cause of the overshoot (Hill & Eisenberg, 1981). Hence, we conclude that there is electrogenic Ln^{3+}/proline transport across rabbit renal brush border membranes. Preliminary experiments also suggest that Ln^{3+} can substitute for Na^+ on the brush-border glucose and proline cotransporters in both rabbit and human intestine (B.R. Stevens, *personal communication*).

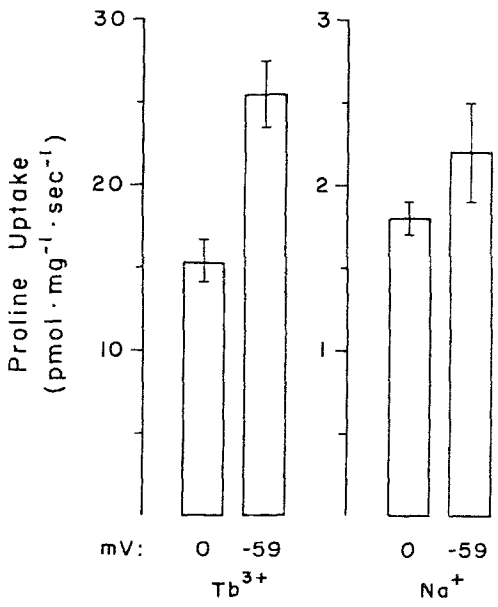

Fig. 6. Voltage dependency of L-proline uptake. Conditions were similar to those described for Fig. 3. The labeled L-proline concentration was 4/5 of its K_t value: 0.04 mM in the presence of Na^+ and 0.22 mM in the presence of Tb^{3+}. The membrane potential was clamped at either 0 or −59 mV, vesicle interior with respect to the uptake medium, using K and valinomycin (25 μg/ml). In both experiments the intravesicular KCl concentration was 50 mM, but the uptake medium concentration was either 50 mM (0 mV) or 5 mM (−59 mV). Uptakes were measured at 30 sec, and data are given as mean ± SE (n = 3 or 4)

Detailed comparison of the kinetics of Tb^{3+}/proline and Na^+/proline transport provides some unique information about the IMINO carrier. First,

our observation that the maximal velocity of L-proline uptake at an infinite activator concentration (140 mM, Fig. 5) is fourfold greater in Tb^{3+} than in Na^+ indicates that the translocation of the Tb^{3+} loaded carrier across the membrane is faster than the Na^+ loaded form. This suggests that the rate-limiting barrier is lower for translocation of the Tb^{3+}/proline/carrier complex. Second, the higher K_t for proline transport in Tb^{3+} (0.28 *vs.* 0.05 mM, Table 1) further suggests that the Tb^{3+} loaded carrier is not in the optimal conformation for proline binding. Finally, the Hill analysis of the cation activation of proline uptake (Fig. 5 and Table 2) yields clues about the ligand binding sites. In Na^+, the Hill coefficient indicates that there are at least two Na^+ binding sites on the renal IMINO carrier as there are on the intestinal IMINO carrier (Stevens & Wright, 1987). On the other hand, in Tb^{3+}, the Hill coefficient is significantly less than 1. If there is a single class of proline transport proteins with two ligand binding sites, a Hill coefficient of less than 1 may be due to: (i) a difference in the intrinsic affinity of the two binding sites, and/or (ii) Tb^{3+} binding to one site decreasing the affinity of the vacant site. Whether one or two Tb^{3+} ions are transported across the membrane with proline is unclear at this time, and further elucidation of this point awaits more direct measurement of Tb^{3+}/proline coupling.

While there is agreement between our uptakes into vesicles and previous voltage-sensitive dye experiments (Schell & Wright, 1985), there is one major exception. Lactate produced a small depolarization of the membrane potential in La^{3+}, but did not exhibit Tb^{3+} cotransport. We have no ready explanation for this discrepancy.

A unique property of Na^+-cotransporters is the specificity of the ligand binding sites for Na^+. In both cation-substitution and cation-competition experiments, there has been little compelling evidence that cations other than Na^+ bind to these transporters. Apart from the lanthanides, there is one report suggesting that H^+ gradients can drive glucose transport across intestinal brush borders in the absence of Na^+ (Hoshi et al., 1986). Inward H^+ gradients stimulated stereospecific glucose uptake, but there was no concentrative uptake (overshoot). In addition, D-glucose depolarized the membrane potential in Na^+-free solutions with an inward H^+ gradient, and this was blocked by the specific inhibitor of Na^+/glucose-cotransport phlorizin. The apparent affinity for D-glucose was almost two orders of magnitude greater in Na^+ than in H^+, whereas in renal membranes, the affinity for L-proline in Na^+ was only about sixfold higher than in Tb^{3+}. These results suggest that H^+ can replace Na^+ on one cotransporter, but much less efficiently than Ln^{3+}.

The lanthanides are quite well-known substitutes for Ca^{2+} in many biological systems, but there are at least two systems, transferrin and conalbumin, where Ln^{3+} substitutes for a monovalent metal cation (Martin & Richardson, 1979). One reason for the similarity may be the close approximation of the ionic radii of the ions. La^{3+} is slightly larger than Na^+, but as the atomic number of the lanthanide series increases at a constant coordination number the radius decreases, and for each lanthanide the effective ionic radius increases with coordination number. In minerals size appears more important than charge in the substitution of divalent cations by trivalent cations. Thus, at the sodium sites on the cotransporters, a lanthanide may be accommodated by a change in size due to a change in the coordination number, which in turn may be produced by a rearrangement of functional groups at the active site and/or a change in hydration at the site. These alterations at the active site must be such that the conformational changes that underlie the increase in the affinity of the cotransporter for the organic substrate are still allowed (Wright & Peerce, 1985; Peerce & Wright, 1987).

An important implication of our observation that lanthanides can mimic Na^+ on these transporters is that the spectral and magnetic properties of these cations may be used to probe the structure of the protein (Horrocks, 1982). For example, Tb^{3+} luminescence by indirect excitation from nearby (within 10 Å) aromatic side-chain chromophores may be used as a sensitive environmental probe of Na^+ binding sites.

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