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SODIUM GRADIENT DEPENDENCE OF PROLINE AND GLYCINE UPTAKE IN RAT RENAL BRUSH-BORDER MEMBRANE VESICLES

PAMELA D. McNAMARA, LOUISE M. PEPE and STANTON SEGAL

Division of Biochemical Development and Molecular Diseases, Children's Hospital of Philadelphia, and Departments of Pediatrics and Medecine, University of Pennsylvania School of Medicine, Philadelphia, PA (U.S.A.)

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Summary

The sodium-dependent entry of proline and glycine into rat renal brush-border membrane vesicles was examined. The high K_m system for proline shows no sodium dependence. The low K_m system for glycine entry is strictly dependent on a Na^+ gradient but shows no evidence of the carrier system having any affinity for Na^+. The low K_m system for proline and high K_m system for glycine transport appear to be shared. Both systems are stimulated by a Na^+ gradient and appear to have an affinity for the Na^+. The effect of decreasing the Na^+ concentration in the ionic gradient is to alter the K_m for amino acid entry and, at low Na^+ concentrations, to inhibit the V for glycine entry.

Introduction

Recently, a number of reports utilizing isolated renal brush-border membrane vesicles have been published which demonstrate Na^+ gradient dependence for uptake of amino acids [1—5]. We have previously reported the Na^+-dependent entry of 0.06 mM L-proline and 0.06 mM glycine in rat renal brush-border membrane vesicles. Both amino acids were seen to exhibit dual systems for entry. At 0.06 mM concentrations, uptake of L-proline occurred primarily via the low K_m system for entry while about 25% of 0.06 mM glycine uptake occurred via its low K_m system. We have further examined the Na^+ dependence of these two amino acids at concentrations where uptake is mediated by both the dual systems for entry in each amino acid. The results presented in this

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

report indicate that the effect of Na^+ is different on each of the dual systems for entry of proline as it is on each of the dual systems for glycine entry in brush-border membrane vesicles.

Materials and Methods

Rat renal brush-border membrane vesicles were prepared from adult male Sprague-Dawley rats (Charles River) using a Booth and Kenny [6] method modified as previously described [5]. The final membrane pellet was suspended in Tris/Hepes/mannitol buffer, pH 7.4 (2 mM Tris/Hepes + 100 mM mannitol) to a protein concentration of 0.3—0.4 mg/ml as determined by the method of Lowry et al. [7].

The measurement of amino acid uptake using Millipore filtration on HAWP filters (0.45 μm) was performed using the techniques described by McNamara et al. [2]. Trapped and diffused space was measured using L-[^{3}H]glucose or 3-*O*-[^{3}H]methyl-D-glucose as previously described [2,5] and results are expressed as uptake in excess of diffusion unless otherwise stated. The brush-border vesicle preparation used is osmotically active, and no breakdown of labeled substrates was seen to occur upon incubation with the membrane preparation for 60 min.

All materials were of the highest quality available. Unlabeled amino acids were obtained from Mann Research Laboratories. Radiolabeled compounds purchased from New England Nuclear Corporation were [U-^{14}C]glycine (102 Ci/M), L-[U-^{14}C]proline (248 Ci/M), 3-*O*-[^{3}H]methyl-D-glucose (3.6 Ci/M), and L-[U-^{3}H]glucose (17.5 Ci/mM). Hepes buffer was purchased from Calbiochem.

Results

Fig. 1 shows the time-dependent uptake of 0.02 mM and 1.85 mM L-proline and glycine under conditions of a sodium gradient (100 mM NaCl). The typical Na^+ gradient-dependent 'overshoot' is seen for the lower concentration of proline (Fig. 1A), where 91% of the uptake observed is mediated by the low K_m system, but not for the higher proline concentration (Fig. 1B). Initial uptake of 1.85 mM proline is more rapid under conditions of a Na^+ gradient then when Na^+ is equilibrated across the membrane; however, at 1.85 mM proline and a 100 mM NaCl gradient, only 54% of the uptake in excess of diffusion is mediated by the high K_m system. Thus, it is probable that any Na^+ gradient stimulation observed could be due to the 46% contribution from the low K_m system which appears to be greatly stimulated by a 100 mM NaCl gradient (Fig. 1A).

For glycine uptake, under conditions of a 100 mM NaCl gradient, a small overshoot in the time course of uptake was observed for the lower substrate concentration (Fig. 1A), while a more obvious overshoot was seen at the higher substrate concentration (Fig. 1B).

To determine the effect of Na^+ concentration on the uptake of proline and glycine, two types of studies were performed: (1) the effect of different Na^+ concentration gradients (where choline replaced Na^+ in the total ionic

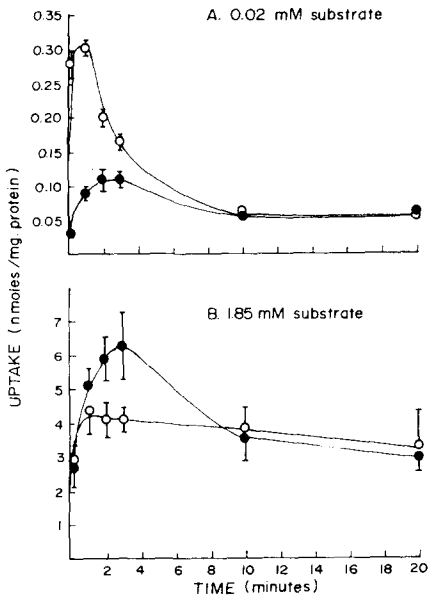

Fig. 1. Uptake of 0.02 mM (A) and 1.85 mM (B) proline (○) and glycine (●) by isolated rat renal brush-border vesicles. The time course of uptake by membranes in Tris/Hepes/mannitol buffer incubated with proline (○) or glycine (●) was measured under conditions of a 100 mM NaCl gradient as previously described [2]. Values shown are the means of 8—16 determinations. Standard errors are indicated by brackets. Where no bracket appears, the standard error lies within the size of the symbol used to designate the mean.

component) on the substrate concentration dependence of amino acid uptake, and (2) the effect of stepwise replacement of Na^+ with choline$^+$ on the 'initial' (15 s) uptake of a single amino acid concentration. The results of the first type of studies which show the effect of different NaCl concentrations (where choline chloride was used to replace NaCl) on the concentration-dependent uptake of proline (0.018—3.914 mM) and glycine (0.019—7.629 mM) can be seen in Fig. 2. For proline (Fig. 2A), no statistical difference between the uptake values at 100 mM and 50 mM NaCl was observed. Under 30 mM and 10 mM NaCl gradients, the values for 15-s uptake studies at lower proline concentrations differed significantly from those at 50 and 100 mM NaCl. The data in Fig. 2A have been used to draw the Lineweaver-Burk plots in Fig. 3A. The double-reciprocal plots of the data indicate that the NaCl concentration, under gradient conditions, affects the K_m but not the V of proline entry in the low proline concentration range (0.0184—0.208 mM, low K_m system). The values for the observed kinetic parameters are presented in Table I. The observed effect of Na^+ on the low K_m for proline is substantiated by calculations of the K_m and V values for a two component system performed using non-linear regression analysis of the curves from Fig. 2A obtained under different Na^+ concentration gradients. A Digital PDP-12 computer was used to determine the calculated parameters which would best-fit the observed data using the equation below for a two component system:

$$V_{total} = \frac{V_1[S]}{[S] + K_{m(1)}} + \frac{V_2[S]}{[S] + K_{m(2)}} \quad (1)$$

The kinetic parameters thus determined are presented in Table II. By sub-

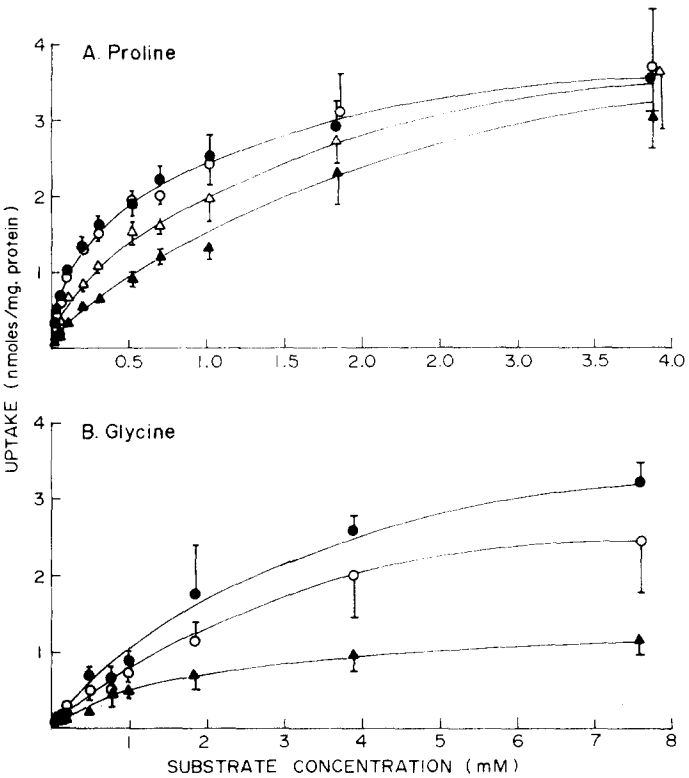

Fig. 2. Influence of $[Na^+]$ on the concentration dependence of L-proline (A) and glycine (B) uptake by brush-border vesicles. Freshly prepared rat renal brush-border membrane vesicles were incubated in 0.5 ml Tris/Hepes/mannitol buffer with 0.1 μCi ^{14}C-labeled amino acid and 0.1 μCi 3-*O*-[^{3}H]methyl-D-glucose for 15 s under gradient conditions. The ionic gradients consisted of 100 mM NaCl (●), 50 mM NaCl + 50 mM choline chloride (○), 30 mM NaCl + 70 mM choline chloride (○), and 10 mM NaCl + 90 mM choline chloride (▲). The values given are means of 4—12 determinations/point. Standard errors are indicated by brackets. Where no bracket appears, the standard error lies within the symbol used.

stituting the values in Table II into Eqn. 1, the uptake theoretically mediated by each of the two transport components,

$$\frac{V_1[S]}{[S] + K_{m(1)}} \quad \text{or} \quad \frac{V_2[S]}{[S] + K_{m(2)}},$$

can be determined for any substrate concentration desired. These contributions may then be expressed as a percent of total uptake. Such relative contributions for each system to the total uptake observed at two selected substrate concentrations (0.02 mM and 1.85 mM for both proline and glycine) under differing conditions are presented in Table III. The parameters for best-fit of all data demonstrate that varying the NaCl concentration between 10 and 100 mM by replacement with choline chloride has no effect on the high K_m system for proline entry, while the primary effect of decreasing the Na^+ concentration in the ionic gradient (by choline replacement) on the low K_m system is to raise the K_m for proline entry. At 10 mM NaCl, there is also a slight decrease in the V over the low proline concentration range. Thus, the high K_m system for proline is independent of Na^+ while the low K_m system shows a rigid dependence on the Na^+ gradient.

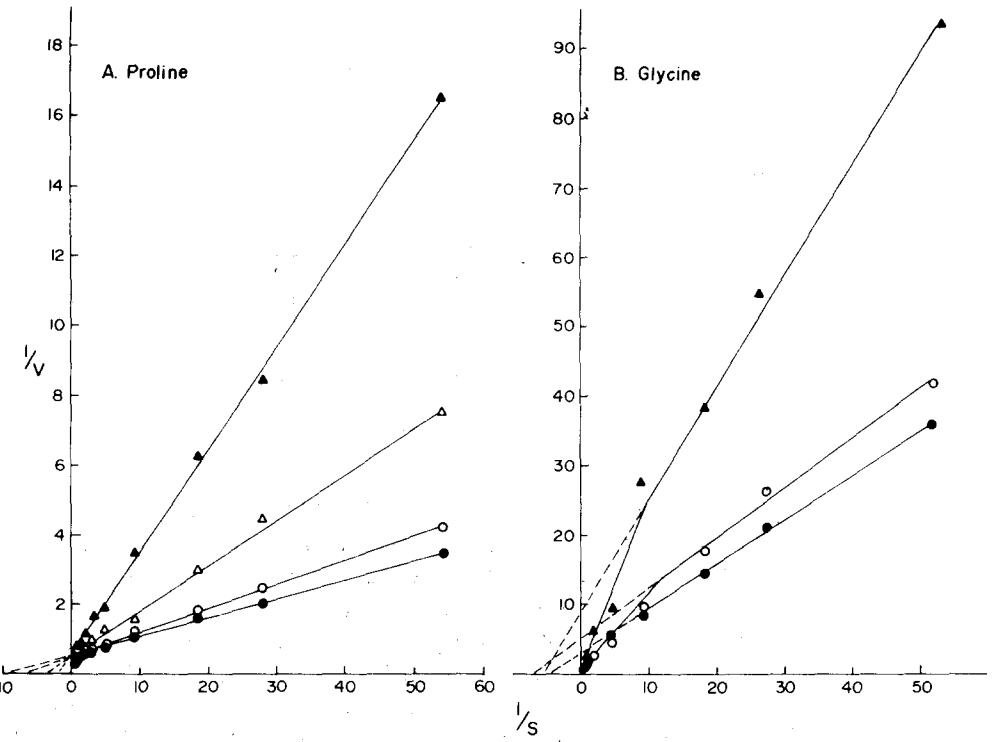

Fig. 3. The double-reciprocal plot of the influence of [Na^+] on the concentration-dependent uptake of proline (A) and glycine (B). Reciprocals of the data in Fig. 2 are plotted for gradients of 100 mM NaCl (●), 50 mM NaCl (○), 30 mM NaCl (△), and 10 mM NaCl (▲).

With glycine, the concentration dependence of 'initial' (15 s) uptake may be seen in Fig. 2B. At low glycine concentrations, no statistical difference between uptake under a 100, 50, or 10 mM NaCl gradient (with choline chloride replacing NaCl in the latter two) occurred. At higher glycine concentrations, the uptake under conditions of a 10 mM NaCl gradient differed from that under 50 or 100 mM NaCl gradients. The double-reciprocal plot of the data from Fig. 2B is presented in Fig. 3B. The kinetic parameters determined from

TABLE I

OBSERVED KINETIC PARAMETERS FOR PROLINE AND GLYCINE UPTAKE

Values for K_m are in mM and values for V are in nmol/mg protein per 15 s. The values given represent the mean ± S.E. of 4—8 determinations from experiments performed as described in Fig. 2.

Amino acid	[NaCl] (mM)	K_m (1)	V_1	K_m (2)	V_2
Proline	100	0.11 ± 0.008	2.11 ± 0.061	0.78 ± 0.080	4.00 ± 0.352
	50	0.16 ± 0.005	2.39 ± 0.086	1.26 ± 0.161	5.91 ± 0.592
	30	0.29 ± 0.017	2.25 ± 0.122	1.46 ± 0.088	4.63 ± 0.251
	10	0.45 ± 0.032	1.47 ± 0.133	2.02 ± 0.261	4.29 ± 0.492
Glycine	100	0.26 ± 0.013	0.39 ± 0.018	5.47 ± 0.622	5.92 ± 0.596
	50	0.14 ± 0.025	0.19 ± 0.028	4.76 ± 0.390	3.92 ± 0.196
	10	0.18 ± 0.023	0.11 ± 0.012	1.82 ± 0.154	1.36 ± 0.057

TABLE II

CALCULATED PARAMETERS FOR PROLINE AND GLYCINE UPTAKE

K_m values are given as mM and V as nmol/mg protein per 15 s. The parameters were calculated as described in the text from the data in Fig. 2.

Amino acid	[NaCl] (mM)	K_m (1)	V_1	K_m (2)	V_2
Proline	100	0.075	1.40	2.2	3.50
	50	0.100	1.40	2.3	3.80
	30	0.190	1.10	2.3	3.80
	10	0.60	0.90	2.4	3.65
Glycine	100	0.25	0.10	5.5	5.50
	50	0.25	0.08	4.6	4.00
	10	0.25	0.01	2.6	1.60

this graphical presentation are presented in Table I and indicate that the primary effect of reducing the low Na^+ concentration of the ionic gradient (by choline replacement) on the K_m system for glycine is to lower the V for glycine entry. Reducing the Na^+ concentration in the ionic gradient (by choline replacement) appears to affect both the K_m and V for glycine entry in the high glycine concentration range (0.528—7.629 mM). By using Eqn. 1 to obtain calculated parameters for the observed data on glycine uptake, the observed effect of the Na^+ concentration is substantiated (Table II). Thus, the low K_m system as well as the high K_m system for glycine uptake in rat renal brush-border membrane vesicles are Na^+ gradient dependent. For the low K_m glycine system, the effect of varying the Na^+ concentration in the ionic gradient by

TABLE III

RELATIVE CONTRIBUTION OF EACH TRANSPORT SYSTEM TO TOTAL UPTAKE UNDER VARYING NaCl CONCENTRATION

Relative contribution was calculated according to Eqn. 1 in the text using the kinetic parameters in Table II.

Amino acid concentration (mM)	[NaCl] (mM)	Calculated contribution of each component (%)	
		Low K_m system	High K_m system
Proline (0.02)	100	91	9
	50	88	12
	30	76	24
	10	47	53
Proline (1.85)	100	46	54
	50	44	56
	30	37	63
	10	30	70
Glycine (0.02)	100	27	73
	50	25	75
	10	6	94
Glycine (1.85)	100	6	94
	50	6	94
	10	2	98

choline replacement is on the V for glycine uptake, while the effect on the high K_m system was determined to be on both the K_m and V for glycine uptake.

The second type of study examining the effect of stepwise replacement of NaCl by choline chloride on uptake of 0.02 mM and 1.85 mM proline and glycine can be seen in Fig. 4. The double-reciprocal plots of the data obtained under NaCl and substrate gradients indicate an apparent affinity for Na^+ in the 0.02 mM proline system of 107 mM and an apparent Na^+ affinity in the 1.85 mM proline system of 18 mM. For the 0.02 mM glycine system, the apparent sodium K_m equals 61 mM, while for the 1.85 mM glycine system the apparent sodium K_m equals 13 mM. In addition to these studies where NaCl and the amino acids both formed gradients, experiments were performed where tracer amino acid uptake was measured under conditions of NaCl equilibration and an unlabeled amino acid extravesicular to intravesicular gradient, under conditions of unlabeled amino acid equilibration and an extravesicular to intra-

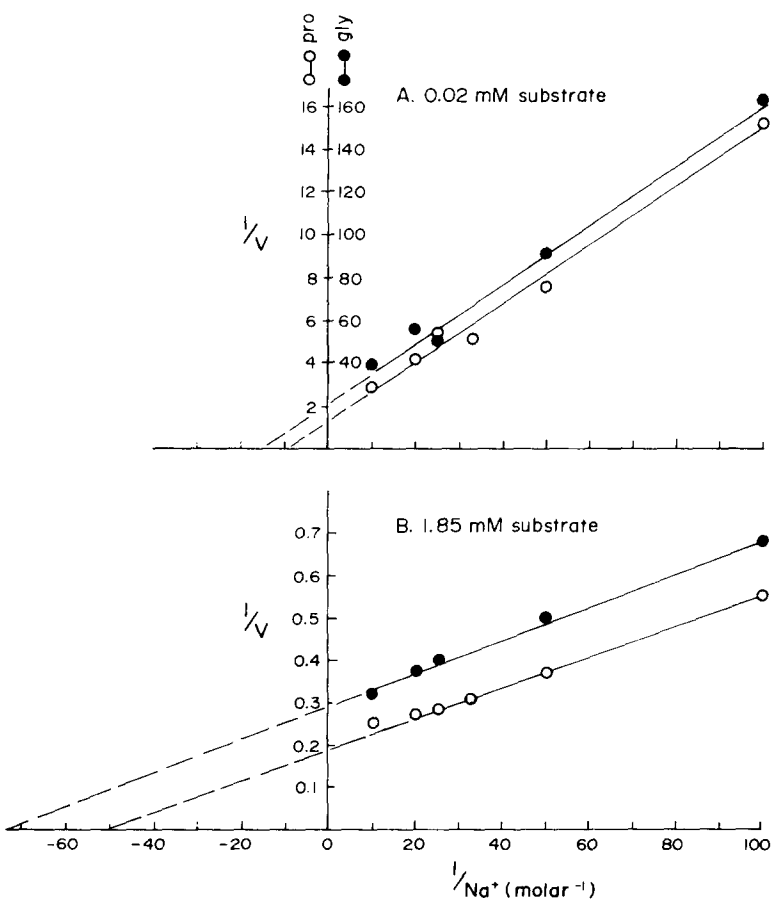

Fig. 4. Effect of [Na^+] on initial rate of 0.02 mM (A) and 1.85 mM (B) L-proline (○) and glycine (●) uptake by brush-border vesicles. Uptake was measured after 15 s as described in Fig. 2 under gradient conditions where 100 mM NaCl was replaced stepwise by choline chloride in the standard incubation medium. Values shown are the reciprocal of means of 8—16 determinations.

vesicular NaCl gradient, as well as when both NaCl and unlabeled amino acid were equilibrated across the membrane. In the absence of a NaCl gradient, no Na^+ dependence was observed for the 1.85 mM proline uptake system whether or not proline was equilibrated across the membrane, while an apparent K_m for Na^+ of 4.5 mM was observed for the 0.02 mM proline system. For glycine, no Na^+ dependence was observed in the absence of a Na^+ gradient for the 0.02 mM glycine system; however, an apparent K_m for Na^+ of 25 mM was observed for the 1.85 mM glycine system in the absence of an electrochemical gradient. Chemical gradients of glycine and proline had no effect on uptake.

Discussion

We have presented evidence to support the conclusion that the dual systems for proline and for glycine uptake have different sodium requirements. For proline, uptake by brush-border membrane vesicles in the concentration range of the high affinity, low K_m system is characterized by the transient accumulation of the substrate against a concentration gradient under conditions of a sodium gradient, an observation consistent with the Na^+ gradient hypothesis for electrogenic transport originally proposed by Crane [8]. Transport mediated by the low affinity, high K_m system for proline does not appear to be electrogenic in nature. Three basic pieces of evidence support these claims. (1) At low proline concentrations, stepwise replacement of NaCl by choline chloride results in the determination of a high apparent K_m for Na^+ while, at high proline concentrations, the requirement for Na^+ is low and can be shown to be due to the contribution of the low K_m system to total uptake. (2) The appearance of an 'overshoot' in the time curve for proline uptake occurs only at low proline concentrations. (3) The kinetic parameters determined by non-linear regression analysis of the curves for concentration dependence of initial uptake of proline under ionic gradients of decreasing Na^+ concentration are changed only for the high affinity, low K_m system and not for the low affinity, high K_m system.

For glycine, the data indicate that both the high and low affinity systems for uptake have Na^+ gradient requirements. Time curves for uptake of both low and high concentrations of glycine show evidence of the 'overshoot' phenomenon. However, the effect of the stepwise replacement of Na^+ by choline$^+$ indicates that the high affinity, low K_m system for glycine uptake has a higher affinity for Na^+ under gradient conditions than the low affinity, high K_m system. Regression analysis of the concentration dependence curves for gradient-dependent glycine uptake under differing Na^+ concentrations indicate that the requirement for Na^+ by the high affinity system is not a true binding requirement since the effect of Na^+ is on the V for glycine entry. Under conditions where Na^+ is equilibrated across the membrane, no apparent K_m for Na^+ is observed at low glycine concentrations. The effect of Na^+ on the low affinity, high K_m system for glycine uptake is more complex and seems to involve both a change in binding affinity for glycine and in the V of uptake for glycine.

We have previously demonstrated that the high K_m system for glycine and low K_m system for proline are shared. The fact that the substrate binding affinity for both these systems is affected by Na^+ provides further support for

this theory. In addition, the differing Na^+ requirement for the dual transport systems for each amino acid lend credence to the evidence previously presented [2] that they are, indeed, two different systems for uptake and may serve differing functions in situ. Evidence for the existence of multiple transport systems for individual amino acids has been presented previously using renal cortical slices and isolated tubules [9—11]. Finding two systems in isolated brush-border membrane vesicles suggests that the transport observed in cortical slices in vitro does not represent uptake via the basolateral membrane but through the luminal membrane of the tubule cell. The brush-border vesicles may, however, be derived from different lengths along the proximal tubules and the two systems observed thus may represent two populations of vesicles. Lingard et al. [12], using microperfusion, have demonstrated a lower K_m system for histidine in the luminal membranes of proximal tubule cells adjacent to the glomerulus while luminal membranes of cells further down the proximal tubule have a higher K_m for amino acid entry.

The data presented here and in a previous report [2] correlate well with the observations of Hillman et al. [9], Mohyuddin and Scriver [10] and Holtzapple et al. [13] that glycine and proline share a transport system (the high K_m for glycine and low K_m for proline). Intravenous proline infusion augments glycine excretion, also supporting the shared system theory. The occurrence of inherited iminoglycinuria (combined glycine-prolinuria) in man may represent a derangement in this shared transport system and is usually a benign disorder clinically. Hyperglycinuria without prolinuria has been reported [14] indicating that two distinct transport mechanisms for glycine do indeed exist in man.

The examination of the Na^+ effect on amino acid uptake by brush-border vesicles is complicated by the fact that uptake is measured under experimentally imposed gradient conditions for both NaCl and the non-electrolytic substrate. We have examined the effect of Na^+ concentration on tracer uptake by the 0.02 mM and 1.85 mM proline and glycine systems when no ionic gradient existed across the membrane, when no non-electrolytic substrate gradient existed across the membrane, and when both or neither gradients occurred. From these studies, it became evident that, on the low K_m system for proline, there is a definite Na^+ requirement for proline binding in the absence of an ionic gradient, and that the chemical gradient imposed by proline itself had no effect on uptake. At 1.85 mM proline, no requirement for Na^+ could be detected. Similar studies with glycine showed that there was no Na^+ requirement for glycine affinity on the low K_m system in the absence of an ionic gradient but that a definite Na^+ requirement for glycine affinity existed on the high K_m glycine system in the absence of the ionic gradient. Again, the chemical gradient created by glycine itself had no effect on uptake. Thus, for the shared system for proline and glycine transport, Na^+ plays a dual role. First, it has a direct effect on the affinity of the system for the two non-electrolyte substrates, and second, the inward electrochemical gradient for Na^+ provides the energy for amino acid uptake against a concentration gradient. The latter role appears to apply also to the low K_m unshared system for glycine.

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