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TRANSPORT OF β-ALANINE IN RENAL BRUSH BORDER MEMBRANE VESICLES

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Summary

The findings that the equilibrium uptake of β-alanine decreased with increasing medium osmolarity and preincubation with β-alanine increased uptake of the amino acid indicate that the uptake of β-alanine by rabbit renal brush border membranes represents transport into membrane vesicles. A Na^+ electrochemical gradient (extravesicular > intravesicular) stimulated the initial rate of β-alanine uptake about three times and effected a transient accumulation of the amino acid twice the equilibrium value. Stimulation of the uptake was specific for Na^+. Gramicidin abolished the overshoot, presumably by dissipating the gradient by accelerating the electrogenic entrance of Na^+ into the vesicle via a pathway not coupled to uptake of β-alanine. In K^+-loaded vesicles, valinomycin enhanced the Na^+ gradient-dependent uptake of β-alanine. These findings indicate that the Na^+ gradient-dependent transport of β-alanine is an electrogenic process and suggest that the membrane potential is a determinant of β-analine transport. Uptake of β-aniline, at a given concentration, reflected the sum of contributions from Na^+ gradient-dependent and -independent transport systems. The dependent system saturated at 100 μM. The independent system did not saturate. At physiological concentrations the rate of the Na^+ gradient-dependent uptake was four times that in the absence of the gradient. The Na^+ gradient-dependent rate of β-alanine uptake was strongly inhibited by taurine, suggesting that β-amino acids have a common transport system. α-Amino acids, i.e. L-alanine, L-arginine, L-glutamate, L-proline, and glycine, representing previously reported specific α-amino acid transport systems in the brush border membrane, did not inhibit the uptake of β-alanine. These findings indicate that the brush border membrane has a distinct transport system for β-amino acids.

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

Introduction

The transport of neutral amino acids, i.e. L-α-alanine [1] and L-α-phenylalanine [2], L-α-proline [3—5], and L-α-cystine [6] in renal proximal tubule brush border membrane vesicles were recently reported. These studies demonstrated a Na^+ electrochemical gradient-dependent uptake for α-amino acids analogous to that described for D-glucose [7,8]. It was also found that the membrane preparations distinguished between neutral, basic, and acidic L-α-amino acids as well as L-α-imino acids and glycine [9], suggesting that the ability of the proximal tubule to transport amino acids selectively may be ascribed to an intrinsic property of its luminal membrane. This is particularly relevant to the understanding of metabolic disorders characterized by specific hyperaminoaciduria [10]. The uptake of β-amino acids by membrane vesicles has heretofore not been reported. Information on this transport system takes on added significance because of hyper-β-alaninemia and hyper-β-alaninuria found in man [11], in patients undergoing transplant rejection crises following renal allotransplantation [12], and in hypertaurinuric mutant strains of mice [13—15]. In the present study we describe a Na^+ electrochemical gradient-dependent β-alanine transport system in renal brush border membrane vesicles and show its specificity for β-amino acids.

Materials and Methods

Rabbit renal brush border membranes were isolated as described previously [16,8]. Following the last centrifugation the membranes were suspended in a medium containing 300 mM mannitol, buffered with 1 mM Tris/HEPES (1 mM HEPES adjusted with Tris hydroxide) to pH 7.5 and the protein concentration was adjusted approximately to 10 mg per ml. The quality of the preparations was randomly evaluated by specific enzyme markers [16] and occasionally by electron microscopy. Trehalase and γ-glutamyltranspeptidase activities averaged 1.2 and 5.9 μmol/mg of protein per min, respectively, approx. 12—18-fold those in the cortical homogenate [9].

Uptake of β-alanine was measured by the Millipore filtration technique detailed previously [17,8]. In the present study 10 μl of membrane suspension were preincubated for 1 min at 20°C and incubation at 20°C was initiated by the addition of 40 μl of buffered mannitol medium containing β-[^{3}H]alanine (approx. 0.4 μCi) and other constituents as indicated. Unless otherwise specified, the additions replaced mannitol isotonically. Incubations were terminated and the filters washed with 154 mM NaCl containing 1 mM Tris/HEPES buffer, as previously described [8]. Values for the non-specific retention of radioactivity on the filters (0.03—0.04% of the total radioactivity in the incubation mixture) were subtracted from the values of the incubated samples. All incubations were carried out in triplicate with freshly prepared brush border membrane vesicles. Each experiment was repeated at least three times with different membrane preparations.

Protein concentrations were determined by a standard procedure [18], using bovine serum albumin as the standard.

β-[^{3}H]Alanine (36 Ci/mmol) was obtained from New England Nuclear. The

radioactive amino acid was tested for purity by thin-layer silica gel chromatography with a solvent system comprised of isopropanol/formic acid/water (40 : 2 : 10, v/v) and essentially all radioactivity appeared in a single spot having the same R_F as authentic β-alanine. Other chemicals were of the highest purity available from commerical sources. All water used for preparing media and reagents was triple distilled and deionized, and all solutions were filtered through 0.45 μm Millipore filters prior to use [19].

Results

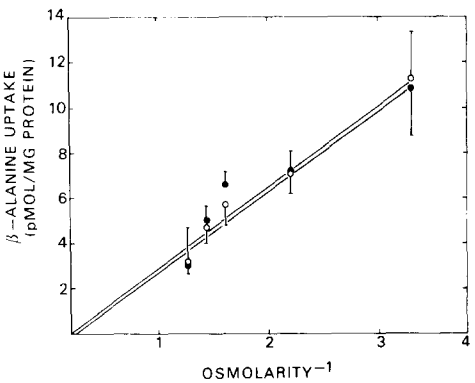
Transport of β-alanine into renal brush border membrane vesicles

The initial rate of 25 μM β-alanine uptake by renal brush border membranes, in the presence or absence of a Na^+ gradient, was proportional to the concentration of membrane protein, at least within the range of from 40 to 150 μg per 50 μl of incubation reaction mixture. Moreover, the membrane protein content per incubation was maintained relatively constant from day to day and always well within this range.

The β-alanine taken up by the brush border membrane vesicles was recovered and identified. Membranes were incubated with 25 μM β-[^{3}H]alanine and the brush borders retained on Millipore filters by the standard procedure. The membranes on 10 filters were extracted with 10 ml of water in a boiling water bath for 30 min. The extract was centrifuged, lyophilized, and chromatographed on thin-layer silica gel plates. Approx. 98 and 94% of the radioactivity taken up at 30 s and 60 min, respectively, had a R_F identical with that of authentic β-alanine. No other discrete radioactive spot was detected. These findings indicate that the β-alanine taken up by the membranes was accumulated and not metabolized.

The question as to whether the uptake of β-alanine by brush border membranes represents transport into membrane vesicles or simply binding to the membrane was examined by determining the effect of the osmolarity of the medium on uptake. Since, as will be shown below, the uptake of β-alanine reaches equilibrium after about 60 min of incubation, the amount of amino acid taken up at this time should, if it were transported, be dependent on the intravesicular volume. Intravesicular space was decreased by increasing the medium osmolarity with sucrose, a relatively impermeable solute which is not hydrolyzed in the kidney [20]. As shown in Fig. 1, uptake of 25 μM β-alanine was inversely proportional to medium osmolarity from 300 to 860 mM, thus, directly related to intravesicular space. Moreover, the relationship between β-alanine uptake at equilibrium and medium osmolarity was independent of the presence initially of a Na^+ gradient. Little, if any, uptake was estimated by extrapolation to infinite medium osmolarity. Therefore, β-alanine uptake into the renal brush border membranes can largely be accounted for by transport across the membrane into an intravesicular space, in agreement with the uptakes of D-glucose [21], L-α-alanine [1], and L-proline [3] by these membrane vesicles.

Additional evidence that the uptake of β-alanine by renal brush border membranes represents transport of the amino acid into membrane vesicles rather than binding to the membrane was obtained from experiments



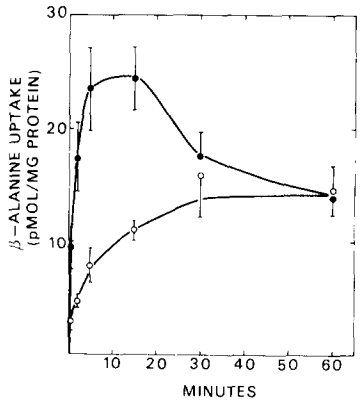


Fig. 1. Effect of the osmolarity of the medium on the uptake of β-alanine by brush border membrane vesicles. Uptake of 25 μM β-alanine was measured after 60 min of incubation in the absence (○) or presence (●) initially of a 100 mM Na^+ gradient. The medium osmolarity was adjusted with sucrose. Each point represents the mean ± S.E. of three experiments each carried out in triplicate.

Fig. 2. The time course of uptake of β-alanine by brush border membrane vesicles in 300 mM buffered mannitol (○) or in a medium in which mannitol was replaced isosmotically by 100 mM NaCl (●) at the initiation of incubation. The concentration of β-alanine was 25 μM. Each point represents the mean ± S.E. of three experiments each carried out in triplicate.

measuring exchange diffusion. Membranes were preloaded with 1 mM β-alanine in 300 mM buffered mannitol or with the mannitol medium in the absence of amino acid (control). At zero time, traces of β-[^{3}H]alanine were added and the initial (30 s) rate of β-alanine uptake measured. Relative to the uptake in the control, 38.8 ± 2.5 pmol/mg of protein per min, there was a marked stimulation, 160%, in the initial rate of β-alanine uptake when the brush borders were preloaded with β-alanine. This demonstration of accelerated exchange diffusion provides strong support for the view that the uptake of β-alanine by the membranes represents transport into membrane vesicles because preincubation with unlabeled β-alanine would occupy binding sites and, if anything, would have inhibited the uptake of β-[^{3}H]alanine. In contradistinction, models to explain accelerated exchange diffusion infer facilitated transport across a membrane [22,23].

Na^+ gradient-dependent uptake of β-alanine

The uptake of 25 μM β-alanine by renal brush border membrane vesicles as a function of time of incubation, either in a 300 mM buffered mannitol medium or in a medium in which mannitol was replaced isosmotically by 100 mM NaCl at the initiation of incubation, is illustrated in Fig. 2. In the absence of the Na^+ gradient, steady-state levels were reached in about 60 min. The presence of the salt gradient between the external medium and the intravesicular medium stimulated uptake of the amino acid. The initial (30 s) rate was greater than three times the initial rate in the absence of the gradient, 9.4 compared to 2.9 pmol/mg of membrane protein. Accumulation of β-alanine in the membrane vesicles was maximal at about 5 min. Afterwards the amount of amino acid in the vesicles decreased, indicating efflux. The final level of uptake of β-alanine in the presence and absence of the Na^+ gradient was the

same, 14 pmol/mg of protein, suggesting that equilibrium was established. At equilibrium the intravesicular space for β-alanine uptake in the brush border membrane vesicles approximated those previously reported for D-glucose [8], Na^+ [3], L-α-alanine [1], and L-proline [3].

At the peak of the "overshoot" the accumulation of β-alanine was approximately twice the final equilibrium value. These results suggest that the imposition of a large extravesicular to intravesicular electrochemical gradient of Na^+ provided the driving force to effect the transient movement of β-alanine into renal brush border membrane vesicles against its concentration gradient (uphill or active transport). When gramicidin was added to an incubation containing a Na^+ gradient (extravesicular > intravesicular) the "overshoot" of β-alanine was abolished (Fig. 3). The ionophore, which enhances membrane conductance for Na^+ [24,25], presumably dissipated the gradient by accelerating the electrogenic entrance of Na^+ into the membrane vesicle via a pathway not coupled to the uptake of β-alanine. Gramicidin had no effect on the equilibrium value; the uptakes of β-alanine at 60 min were identical in the presence and absence of the ionophore, suggesting that the action of gramicidin in abolishing the "overshoot" was not due to a non-specific effect on the brush border membrane. These findings indicate that it is not the concentration of Na^+ per se but the Na^+ electrochemical gradient that is crucial in driving the uphill transport of β-alanine into the brush border membrane vesicle. To be noted, however, was the substantial stimulation in the rate of β-alanine uptake due to the presence of Na^+, even in the absence of the Na^+ electrochemical gradient, i.e. compare the uptake in the presence of Na^+ and gramicidin (Fig. 3) with the uptake in buffered mannitol (Fig. 2).

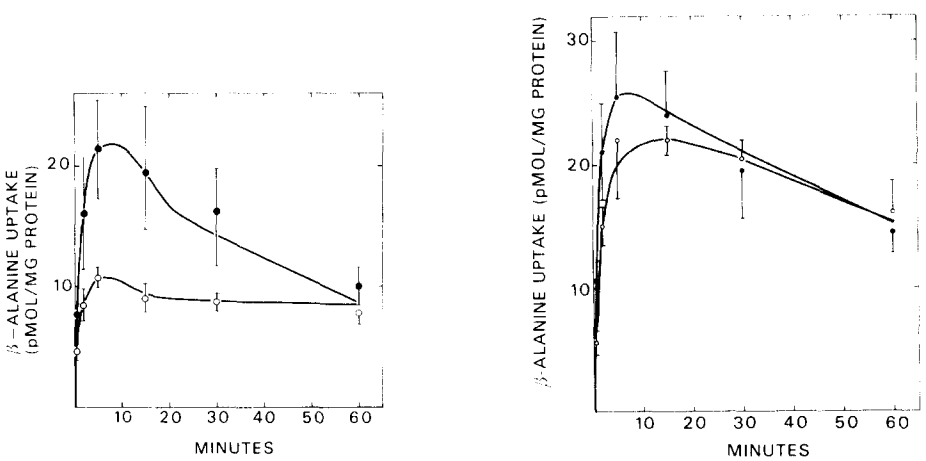


Fig. 3. Effect of gramicidin on the Na^+ gradient-dependent uptake of β-alanine in brush border membrane vesicles. The incubation medium contained 25 μM β-alanine, 100 mM NaCl, 100 mM buffered mannitol, without (●) or with (○) gramicidin, 8 μg per mg of membrane protein. Each point represent the mean ± S.E. of three experiments each carried out in triplicate.

Fig. 4. Effect of valinomycin on the Na^+ gradient-dependent uptake of β-alanine in brush border membrane vesicles. Membrane vesicles were preloaded with 100 mM K^+ as described in the text and incubated in 25 μM β-alanine, 100 mM Na^+ in buffered mannitol, with and without ionophore (8 μg/mg protein). Control (○) and valinomycin (●). Each point represents the mean ± S.E. of three experiments each carried out in triplicate.

In contrast to the action of gramicidin, the ionophore, valinomycin, which mediates the electrogenic movement of K^+ down its concentration gradient [25—27], enhanced the "overshoot" of β-alanine when the brush border membrane vesicles were preloaded with K^+ and then assayed for Na^+-dependent β-alanine uptake (Fig. 4). In the presence of valinomycin, the initial rate of transport was doubled and the peak of the "overshoot" was higher. Valinomycin added to K^+-loaded membrane vesicles presumably induced the efflux of K^+ down its concentration gradient with concomitant generation of an electrochemical potential, interior negative. The development of this membrane potential would accelerate the influx of Na^+ and β-alanine into the vesicle resulting in the larger transient accumulation of the amino acid. These findings suggest that the coupled translocation of β-alanine and Na^+ is electrogenic, i.e. the co-transport of the amino acid and Na^+ across the brush border membrane is associated with a net transfer of a positive charge, as was shown previously for D-glucose, L-proline and neutral L-α-amino acids [1—3,21,28].

The specificity of Na^+ in stimulating the rate of β-alanine uptake by brush border membrane vesicles was tested. The membranes were incubated with 25 μM β-alanine and 300 mM buffered mannitol (control) or mannitol replaced isosmotically with 100 mM Na^+, K^+, Li^+ or choline, as chloride salts. The uptake in the mannitol control was 4.77 pmol/mg of protein per min. Neither K^+ nor choline influenced the uptake. Li^+ did effect a relatively slight stimulation, 128 ± 15% that of the control. However, Na^+ increased the rate about three times. These results demonstrate the specificity of the β-alanine transport system for Na^+, in contrast to other monovalent cations. They also eliminate the addition of Cl^- and alterations in ionic strength as factors that may have accounted for the observed stimulation with NaCl. In other experiments (data not shown) it was found that a concentration of 10 mM NaCl, added to the extravesicular medium stimulated β-alanine uptake about 40% and that increasing the concentration further enhanced the rate of uptake. However, even at the highest concentration of salt tested, 100 mM, only partial saturability with respect to Na^+ was found.

Kinetics of the Na^+ gradient-dependent uptake of β-alanine

The effect of different concentrations of β-alanine on the rate of amino acid uptake, in the absence and presence of a 100 mM Na^+ gradient, is illustrated in Fig. 5. In the absence of Na^+, the rate of β-alanine uptake increased linearly with increasing β-alanine concentration throughout a range of from 1 to 200 μM. Thus, the Na^+-independent transport system for β-alanine appeared not to saturate. The simplest interpretation of this finding might suggest that the Na^+-independent system represents passive diffusion. However, the possibility that this system is comprised of a passive diffusion component and one or more carrier-mediated systems, saturable at very high concentrations of β-alanine was not ruled out. In the presence of a Na^+ gradient, the relationship between β-alanine concentration and uptake was non-linear, providing evidence for saturability. At higher β-alanine concentrations, however, uptake was more linearly related to amino acid concentration, proceeding with a pattern similar to that found in the absence of Na^+. If at each β-alanine concentration the Na^+-free uptake was subtracted from the uptake measured in the presence of Na^+,

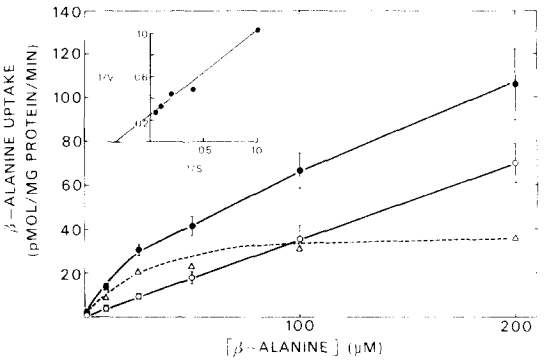


Fig. 5. The relationship between β-alanine concentration and the Na^+ gradient-dependent and -independent initial rates of uptake of the amino acid. Incubations were for 30 s in the absence (○) and presence (●) of 100 mM NaCl replacing the buffered mannitol isosmotically at the initiation of incubation. Each point represents the mean ± S.E. of three experiments each carried out in triplicate. The dashed line represents the Na^+ — Na^+-free values obtained by subtracting the Na^+-free uptake from the uptake in the presence of 100 mM NaCl, at each concentration of β-alanine. The inset shows a double reciprocal plot of the Na^+ gradient-dependent transport system.

a curve (Fig. 5, dashed line) was obtained which describes a completely saturable Na^+-dependent β-alanine transport process. This system was saturated at about 100 μM β-alanine. A double reciprocal plot of the rate of the Na^+-dependent uptake as a function of β-alanine concentration (Fig. 5, inset) describes a straight line relationship with a calculated V of 40 pmol/mg of protein per min and an apparent K_m of 30 μM.

To determine the effect of Na^+ on the kinetics of β-alanine transport, the initial rates of uptake of β-alanine were measured using amino acid concentrations of 1—50 μM in the absence or presence of 25, 50, or 100 mM NaCl, added at the start of the incubation. Double reciprocal plots of the rate of the net Na^+ gradient-dependent uptake (uptake in the absence subtracted from that in the presence of Na^+) as a function of β-alanine concentration for the different salt concentrations are presented in Fig. 6. Increasing Na^+ in the extravesicular medium increased the affinity for the β-amino acid. In these experiments the calculated apparent K_m values were 57, 15, and 8 μM β-alanine at 25, 50, and 100 mM NaCl, respectively; the V remained relatively constant.

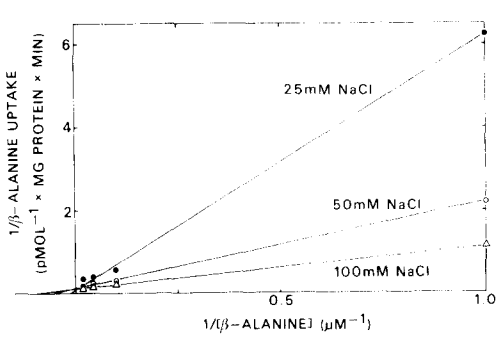


Fig. 6. Effect of Na^+ on the kinetics of β-alanine uptake. The incubation time was 30 s. NaCl replaced mannitol isosmotically.

TABLE I

EFFECT OF DIFFERENT AMINO ACIDS ON THE NET Na^+ GRADIENT-DEPENDENT RATE OF β-ALANINE UPTAKE

The concentration of β-[^{3}H]alanine was 25 μM and the test compound was 1 mM. The incubation period was 30 s. Net Na^+ gradient-dependent uptakes were obtained by subtracting the Na^+-free uptake from the uptake measured in the presence of a 100 mM Na^+ gradient. The net Na^+ gradient-dependent uptake of β-alanine in the absence of other test inhibitors (control) is designated at 100% uptake. Each point represents the mean ± S.E. of three experiments each carried out in triplicate.

Test amino acid	Relative rate of β-alanine uptake (%)
Control	100
β-Alanine	11 ± 8
Taurine	8 ± 4
L-Alanine	85 ± 13
L-Arginine	69 ± 11
L-Glutamate	108 ± 6
L-Proline	69 ± 18
Glycine	84 ± 18

Amino acid specificity of the Na^+ gradient-dependent uptake of β-alanine

Table I shows the effects of different amino acids on the initial (30 s) rate of the net Na^+ gradient-dependent uptake of β-alanine. The uptake of 25 μM β-[^{3}H]alanine was inhibited about 90% by 1 mM unlabeled β-alanine and by the β-alanine sulfonic acid analog, taurine. None of the α-amino acids, including L-α-alanine and representatives from each of the other specific α-amino acid transport systems previously reported in the membrane [9], inhibited the uptake of β-alanine appreciably. These competition experiments indicate that the brush border membrane has a distinct transport system for β-amino acids. The small (15—30%) inhibition by the α-amino acids may represent partial dissipation of the Na^+ electrochemical gradient because of their cotransport with Na^+, as found with D-glucose-amino acid interactions [1].

Discussion

The uptake of β-alanine by renal proximal tubule brush border membranes represents transport into membrane vesicles. This conclusion is based on the findings that: (a) accumulation of β-alanine, at equilibrium, decreases with increasing medium osmolarity and extrapolation to infinite medium osmolarity, i.e. zero intravesicular space, indicates little uptake; (b) preloading the membranes with β-alanine accelerated exchange diffusion of the amino acid, i.e. counter transport; (c) a Na^+ electrochemical gradient-dependent "overshoot" of β-alanine uptake was demonstrated; and (d) at equilibrium β-alanine as well as other amino acids and Na^+ [1,3] were taken up into the same intravesicular space.

At a given concentration of β-alanine, the rate of uptake was the sum of contributions from Na^+ electrochemical gradient-dependent and -independent transport systems. The Na^+-independent uptake process did not saturate at concentrations as high as 200 μM. In contrast, the Na^+-dependent transport system was fully saturated at about 100 μM β-alanine. The concentration of β-alanine

in the glomerular filtrate of man is about 10 μM [11]. At this concentration the rate of the Na^+ gradient-dependent uptake of β-alanine by brush border membrane vesicles was approx. 4-fold that in the absence of the Na^+ gradient (Fig. 5). Therefore, it is suggested that at physiological concentrations of β-alanine the asymmetry of Na^+ across the luminal membrane of the proximal tubule cell may largely account for the transport of the β-amino acid. This finding is consistent with the observations that removal of Na^+ from the incubation media decreased the uptakes of β-alanine and taurine in renal cortex slices [29,30].

The Na^+ gradient-dependent uptake of β-alanine in brush border membrane vesicles followed Michaelis-Menten kinetics when the amino acid concentration was in the physiological range, 1—50 μM (Fig. 5, inset). In contrast, heterogeneity characterized by high and low affinity sites were reported for the uptake of the β-alanine analog, taurine, in renal cortex slices [15,30]. It is difficult to compare the findings obtained here with those previously reported, however, because (a) the taurine concentration ranged from 1 μM to 60 mM in the studies with slices; (b) uptake in slices was measured after 2 h, whereas 15 s was used here; (c) there was no Na^+ gradient in the experiments with slices; and (d) uptake of the β-amino acid by slices is indicative primarily of transport at the basal-lateral rather than the brush border membrane. As shown in Fig. 6, when the concentration of β-alanine was varied from 1 to 50 μM, increasing the concentration of NaCl in the extravesicular medium increased the affinity for the β-amino acid. The precise values of K_m at each NaCl concentration may be problematic, however. The present study indicates that the flux of β-alanine is coupled to the flux of Na^+; hence, varying the concentration of β-alanine and its rate of uptake presumably also alters Na^+ flux which, in turn, affects the Na^+ electrochemical gradient. Thus, two determinants of the rate of uptake are varied. This experimental difficulty was recently pointed out for the Na^+ gradient-dependent D-glucose uptake in intestinal membranes [31].

The demonstration of competition between the Na^+ gradient-dependent uptake of β-[^{3}H]alanine and unlabeled β-alanine and taurine in brush border membranes but no appreciable inhibition with α-amino acids (Table I) suggests that the luminal membrane has a transport system for β-amino acid which is distinct from previously described α-amino acid systems, i.e. neutral, acidic, basic, and imino acids [9]. This is consistent with in vivo excretion and in vitro cortex slice accumulation studies [11,14,29,32,33] which indicated that in the kidney β-amino acids share a common transport system which is different from that for α-amino acids. It remains to be determined, however, whether the transport process that mediate the exit of amino acids from the tubule across the basal-lateral segment of the plasma membrane can recognize the different classes of amino acids. In addition to the kidney, transport system specific for β-amino acids were reported in Ehrlich ascites tumor cells [34] and intestine [35].

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