Development of Dipeptide Transport in Rat Renal Brush Border Membranes: Studies with Glycylsarcosine

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ABSTRACT. Dipeptide-proton cotransport was studied in rat renal brush border membrane vesicles from animals of different ages, starting at 7 days after birth. In the presence of an inward-directed proton gradient, glycyl-sarcosine uptake exhibited the overshoot phenomenon in all age groups studied. The magnitude of the "overshoot" increased with age and maximum accumulation of glycyl-sarcosine was observed in 42-day-old rat renal brush border membrane vesicles. Kinetic studies with glycyl-sarcosine indicated changes only in maximal velocity without any significant change in the apparent affinity value during the postnatal development of the renal brush border membrane. Since the Na^+/H^+ exchanger located at the brush border membrane is primarily responsible for the generation of the proton gradient, the driving force for dipeptide transport, we also measured the Na^+/H^+ exchanger activity in rat renal brush border membrane vesicles from suckling and adult animals. The exchanger activity was significantly greater in the adult rats compared to the suckling rats. In addition, we have confirmed in the present study previous findings from other laboratories that the activities of the Na^+ gradient-driven glucose and amino acid transport systems in renal brush border membrane vesicles were higher in the adult than in the suckling rats. (*Pediatr Res* 22: 641-646, 1987)

Abbreviations

Gly-Sar, glycyl-sarcosine
MES, 2-(N-morpholino)ethanesulfonic acid
Tris, Tris(hydroxymethyl)aminomethane
DMA, dimethylamiloride
BBM, brush border membrane
 V_{max} , maximal velocity
 K_t , apparent affinity

The epithelial cells of the small intestine and the renal proximal tubule are involved in the absorption of various organic solutes such as glucose, amino acids, and small peptides. Glucose and amino acids are transported across the brush border membrane of these absorptive cells via Na^+ gradient-driven processes. The developmental aspects of these Na^+ -dependent transport systems in the small intestine as well as in the renal proximal tubule have been studied by several investigators using isolated brush border membrane vesicles from rats of different ages (1-5).

Transport of small peptides across the brush border membrane of intestinal and renal epithelial cells differs from the transport of glucose and amino acids in that the former is energized by a H^+ gradient rather than a Na^+ gradient (6-12). The H^+ gradient which is necessary to energize peptide transport is generated *in vivo* by the concerted actions of the basal lateral membrane Na^+/K^+ ATPase and the brush border membrane Na^+/H^+ exchanger. Studies directed toward understanding the prenatal and/or postnatal development of this unique and novel transport system in the small intestine and in the proximal tubule are limited. There are only two investigations to date on the development of peptide transport in the small intestine (13, 14) and there are no studies of a similar nature in the renal proximal tubule. The aforementioned two studies on intestinal peptide transport were carried out by measuring dipeptide influx in everted intestinal sacs (13) or in pieces of intestinal segments (14). Both studies have demonstrated that the dipeptide transport was significantly greater in the suckling animals than in the adult animals and that, kinetically, the difference in transport was primarily due to the change in the V_{max} rather than in the K_t . The present study was designed to investigate the developmental aspects of the dipeptide transport system in the rat renal proximal tubule using purified BBM vesicles from animals of different ages.

MATERIALS AND METHODS

Animals. Gravid female Sprague-Dawley rats were purchased from Harlan, Inc. (Indianapolis, IN) at 16 days of gestation and fed *ad libitum* on Purina Rat Chow (Purina, St. Louis, MO) until parturition. After birth, rat pups were distributed among mothers to maintain the litter size at 11 until the time of study. Sprague-Dawley rats, 90 days old and designated as adults (body weight 150-200 g) were from the same source. No distinction was made with regard to the sex of the animals. Animals of all age groups were killed by anesthetization with ether.

Preparation of brush border membrane vesicles. Whole kidneys were used for the preparation of BBM vesicles (5). BBM vesicles were isolated by a Mg^{2+} -precipitation method in the presence of EGTA (11). Kidneys were homogenized in 10 volumes (w/v) of ice-cold 12 mM Tris/NaOH buffer, pH 7.5 containing 5 mM EGTA and 300 mM mannitol for 90 s using an Ultra-Turrax with powerstat set at 50. The homogenate was allowed to stand at 4°C for 15 min and then an equal volume of ice-cold distilled water was added. To this homogenate, 1 M MgCl_2 was added to give a final concentration of 10 mM and the mixture was stirred for 1 min and allowed to stand for 15 min at 4°C. It was then centrifuged at $4000 \times g$ for 10 min. The supernatant was centrifuged at $42,000 \times g$ for 30 min. The pellets were suspended in a preloading buffer, consisting of 100 mM K_2SO_4 , 50 mM HEPES, and 75 mM Tris, pH 8.4 for Gly-Sar uptake experiments (10) or 300 mM mannitol, 10 mM HEPES and 20 mM Tris, pH 7.5 for D-glucose and L-alanine uptake experiments. The suspension was

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again centrifuged at $42,000 \times g$ for 30 min and the pellets were suspended in respective preloading buffers using a syringe with 25-gauge needle. The centrifugation was repeated once more and the final pellets were suspended in small volumes of the same buffers. The protein concentration was adjusted to 5 mg/ml and used for further studies.

To measure the $\text{Na}^+\text{-H}^+$ exchanger activity, BBM vesicles were isolated according to the method described by Grillo and Aronson (15) except that the preloading buffer was 50 mM MES, 16.5 mM Tris, pH 5.5 containing 150 mM KCl. Alkaline phosphatase and dipeptidylpeptidase IV were assayed as described by Bessey *et al.* (16) and Hama *et al.* (17), respectively. Ouabain-sensitive K^+ -phosphatase was measured using the method of Gustin and Goodman (18).

Uptake studies. The uptake measurements were carried out by a rapid filtration technique (19) using Millipore filters (type DAWP, pore size $0.65 \mu\text{m}$). The specific conditions for each experiment are given in the respective figure legends. In general, uptake was initiated by adding $40 \mu\text{l}$ of the brush border membrane suspension containing 0.2 mg of membrane protein to $160 \mu\text{l}$ of uptake buffer. The mixture was shaken gently at 25°C in a gyratory water bath shaker. At the end of the incubation period, uptake was stopped by adding 3 ml of ice-cold stop buffer and then it was rapidly filtered through a Millipore filter. The filter was washed three times with 5 ml of the stop buffer and then transferred to a counting vial. The radioactivity associated with the filter was measured by liquid scintillation spectrometry.

Determination of kinetic constants. The kinetic constants (K_t and V_{max}) for the Gly-Sar transport system in renal BBM vesicles prepared from different age group rats were calculated by computer analysis of the experimental data. The individual, nonweighted values of initial uptake (v) at each concentration of the substrate (s) were fit into the Michaelis-Menten equation describing a single transport system,

$$v = \frac{V_{\text{max}} \cdot s}{K_t + s}$$

and the kinetic constants, K_t and V_{max} , were determined using a nonlinear regression analysis.

Materials. [Glycyl- $\text{U-}^{14}\text{C}$]Glycylsarcosine (112 mCi/mmol) and carrier-free $^{22}\text{NaCl}$ (200 $\mu\text{Ci/ml}$) were obtained from Radiochemical Center, Amersham Corp., Arlington Heights, IL. D-[1- $^3\text{H(N)}$]-glucose (15.5 Ci/mmol) and L-[$^{14}\text{C(U)}$]alanine (170 mCi/mmol) were purchased from New England Nuclear. Dimethylamiloride was a generous gift from Edward J. Cragoe, Jr., Merck, Sharp & Dohme Research Laboratories, West Point, PA. All other chemicals used were of analytical grade.

RESULTS

Purity of BBM vesicles. Table 1 shows the specific activities of marker enzymes in kidney homogenate and in purified BBM from rats belonging to different age groups. The BBM marker enzymes, alkaline phosphatase and dipeptidylpeptidase IV, were enriched 7- to 11-fold over the initial homogenate in all age groups. The basal-lateral membrane marker enzyme, ouabain-

sensitive K^+ -phosphatase, was enriched less than 2.5-fold in all the BBM vesicle preparations. The purity of BBM vesicles obtained in different age group rats were similar to the earlier studies reported (1, 2, 5). Even though the specific activities of alkaline phosphatase and dipeptidylpeptidase IV in the homogenate as well as in the BBM gradually increased with age, the enrichment of these enzymes in the BBM was comparable in all age groups.

Purity of radiolabeled glycylsarcosine. The radiopurity of [glycyl- $\text{U-}^{14}\text{C}$]glycylsarcosine was determined by thin layer chromatography using silica gel sheets (20). The solvent system was methanol:acetonitrile:water (9:0.5:0.5, v/v). As shown in Figure 1, the radioactivity of the sample moved as a single peak, corresponding to that of unlabelled glycylsarcosine and the radioactivity associated with free glycine, if any, was negligible.

Proton gradient-dependent glycylsarcosine uptake. The time-dependent uptake of 0.03 mM Gly-Sar by renal BBM vesicles from 7-, 14-, 21-, 28-, and 42-day-old and adult rats in the presence of an inward-directed H^+ gradient ($[\text{pH}]_i = 8.4$; $[\text{pH}]_o = 6.6$) is shown in Figure 2. The time course of Gly-Sar uptake exhibited the overshoot phenomenon in all age groups studied. Therefore, the BBM vesicles from kidneys of all age group rats were capable of active accumulation of the dipeptide against a concentration gradient. In adult rats, the intravesicular concentration of Gly-Sar after 15 s incubation was approximately three times that of the equilibrium value (60-min incubation). This equilibrium uptake however did not change significantly in different age group animals, indicating that there was no age-dependency for the intravesicular volume of the BBM vesicles. But, the uptake rate measured with a 15-s incubation increased

Fig. 1. Radiopurity of [glycyl- $\text{U-}^{14}\text{C}$]glycylsarcosine. Thin layer chromatography was performed using silica gel sheets. The solvent system was methanol:acetonitrile:water (9:0.5:0.5, v/v). After completion of the run, the sheet was air-dried, cut into 2-mm strips and the radioactivity associated with each strip counted. The reference spots corresponding to glycine and glycylsarcosine were detected with ninhydrin.

Table 1. Specific activities of marker enzymes in BBM and homogenates of different age group rat kidneys*

No. days	Alkaline phosphatase ($\mu\text{mol/mg/min}$)			Dipeptidylpeptidase IV ($\mu\text{mol/mg/min}$)			Ouabain-sensitive K^+ -phosphatase ($\mu\text{mol/mg/h}$)		
	Homogenate	BBM	Enrichment	Homogenate	BBM	Enrichment	Homogenate	BBM	Enrichment
7	0.056 ± 0.002	0.604 ± 0.010	10.8 ± 0.2	0.120 ± 0.001	1.120 ± 0.042	9.3 ± 0.5	0.282 ± 0.036	0.438 ± 0.066	1.6 ± 0.5
14	0.052 ± 0.038	0.380 ± 0.044	7.3 ± 0.2	0.140 ± 0.003	1.027 ± 0.011	7.3 ± 0.1	0.198 ± 0.022	0.492 ± 0.018	2.5 ± 0.5
21	0.086 ± 0.012	0.878 ± 0.007	10.2 ± 1.4	0.195 ± 0.015	1.463 ± 0.065	7.5 ± 0.3	0.282 ± 0.008	0.156 ± 0.012	0.6 ± 0.1
28	0.128 ± 0.011	1.460 ± 0.220	11.4 ± 0.7	0.177 ± 0.014	1.400 ± 0.110	7.9 ± 0.1	0.351 ± 0.060	0.690 ± 0.150	2.0 ± 0.1
42	0.193 ± 0.002	2.016 ± 0.087	10.4 ± 0.5	0.194 ± 0.014	1.530 ± 0.120	7.9 ± 0.3	0.270 ± 0.050	0.522 ± 0.025	1.9 ± 0.1
Adult	0.234 ± 0.009	2.160 ± 0.008	9.2 ± 0.4	0.226 ± 0.002	1.633 ± 0.020	7.2 ± 0.1	0.273 ± 0.026	0.616 ± 0.294	2.3 ± 0.9

* Values are means $\pm$ SEM for each group from at least four different membrane preparations.

Fig. 2. Proton gradient-dependent Gly-Sar uptake in renal BBM vesicles from rats of various ages. Vesicles were prepared from the kidneys of 7 (Δ), 14 ($\blacktriangle$), 21 ($\square$), 28 ($\blacksquare$), and 42 ($\bullet$) day old and adult ($\circ$) animals. Brush border membrane vesicles were suspended in a buffer, pH 8.4, containing 50 mM HEPES, 75 mM Tris, and 100 mM K_2SO_4 . Uptake of Gly-Sar (30 μ M) was measured by mixing 40 μ l of BBM (0.2 mg protein) and 160 μ l of the uptake buffer (50 mM HEPES, 50 mM MES, 25 mM Tris, and 300 mM mannitol, pH 5.8) containing the labeled dipeptide. Under these conditions, the intravesicular pH was 8.4 and extravesicular pH was 6.6. Incubation was carried out at 25°C. The results are expressed as means $\pm$ SEM from at least three experiments, each done in duplicates or triplicates.

Fig. 3. Age-dependent changes in the 15 s uptake rate ($\circ$) and equilibrium uptake ($\bullet$) of Gly-Sar in renal BBM vesicles.

with age (Fig. 3) and the maximal uptake rate was observed in 42-day-old rat BBM vesicles.

Binding vs transport. To differentiate between transport of the dipeptide into the intravesicular space and nonspecific binding to the membranes, the effect of osmolality of the incubation medium on the equilibrium uptake (90-min incubation) of Gly-Sar was studied in membrane vesicles prepared from 14-day-old and adult rats. In both cases, equilibrium uptake of the dipeptide

increased when the osmolality of the incubation was decreased (Fig. 4). The y-intercept of the $1/\text{osmolality}$ versus equilibrium uptake plot gives an approximate value for the non-specific binding. Under the experimental conditions employed for uptake measurements, the values calculated from Figure 4 for the non-specific binding of Gly-Sar in membrane vesicles from 14-day-old and adult rats were similar (33 and 25%, respectively).

Kinetics of Gly-Sar uptake. The kinetic aspects of Gly-Sar uptake in renal BBM vesicles from rats of various age groups were studied by measuring the uptake rates over a Gly-Sar concentration range of 0.03–0.60 mM. To keep the experimental variations to a minimum, a 10-s incubation was employed for uptake measurements, even though uptake was not linear over this period. It has to be mentioned that the calculated kinetic constants therefore represent only approximate values. Nonetheless, some general conclusions can be drawn from the data. In all age groups, the uptake was saturable and the data conformed to simple Michaelis-Menten kinetics representing a single transport system over the concentration range employed. Figure 5 shows the Eadie-Hofstee plots (initial velocity/substrate concentration versus initial velocity) of the kinetic data from 14-day,

Fig. 4. Effect of osmolality on the equilibrium uptake of Gly-Sar in renal BBM vesicles prepared from 14-day-old ($\bullet$) and adult ($\circ$) rats.

Fig. 5. Eadie-Hofstee plot of the concentration-dependence of Gly-Sar uptake in renal BBM vesicles from 14- ($\bullet$), 28-day ($\square$), and adult ($\circ$) rats. v , nmol/mg of protein/10 s; S , substrate concentration in mM.

28-day-old rats and adult rats. The plots were linear in all cases ($r^2 > 0.96$). The y-intercept varied significantly among the age groups, but there was no change in the slope. This indicates that the age-dependent alterations in Gly-Sar uptake were primarily due to changes in the maximal velocity rather than the affinity constant. Table 2 summarizes the kinetic constants, the V_{max} , and the K_t , determined for 7-, 14-, 21-, 28-, 42-day-old rats and adult rats. The increase in V_{max} without any change in K_t among the developing rats observed in this study for the dipeptide, Gly-Sar, is similar to the previous reports on the Na-dependent L-proline uptake (5).

Na^+H^+ exchanger activity. It has been well established in recent years that small peptides are cotransported with H^+ across the small intestinal and renal brush border membranes (6–12). Under physiological conditions, the inward-directed H^+ gradient which is the driving force for peptide transport is generated by the action of the Na^+H^+ exchanger in the BBM (6, 7). Therefore, the developmental pattern of this exchanger activity would be highly relevant to the development of peptide transport in these membranes. We measured the Na^+H^+ exchanger activity by

Table 2. Kinetic parameters of Gly-Sar transport in renal BBM vesicles from different age group rats*

No. days	K_t (mM)	V_{max} (nmol/mg/10 s)
7	0.543 ± 0.258	0.592 ± 0.350
14	0.391 ± 0.075	0.785 ± 0.077
21	0.343 ± 0.035	0.915 ± 0.053
28	0.333 ± 0.028	0.928 ± 0.038
42	0.293 ± 0.040	1.340 ± 0.083
90	0.320 ± 0.025	1.314 ± 0.050

* The values represent the mean $\pm$ SEM for two to three experiments, each done in triplicate.

Fig. 6. Uptake of Na^+ in the presence of an outward-directed H^+ gradient in renal BBM vesicles prepared from 14-day-old and adult rats. BBM vesicles were suspended in a buffer, pH 5.5, containing 50 mM MES, 16.5 mM Tris, and 150 mM KCl. Uptake of Na^+ (0.5 mM) was measured by mixing 40 μ l of brush border membrane suspension (0.2 mg protein) with 160 μ l of uptake buffer (36 mM HEPES, 33 mM Tris, and 150 mM KCl, pH 7.5) containing 0.625 mM NaCl and tracer amounts of ^{22}Na . DMA-sensitive Na^+ uptake was calculated by subtracting the uptake measured in the presence of 0.5 mM DMA from the total uptake measured in the absence of DMA.

Fig. 7. Uptake of D-glucose (A) and L-alanine (B) in renal BBM vesicles from 14-day-old (●) and adult (○) rats. BBM vesicles were suspended in a buffer, pH 7.5, containing 10 mM HEPES, 20 mM Tris, and 300 mM mannitol. Uptake of 10 μ M D-glucose or 10 μ M L-alanine was measured in the presence of an inward-directed Na^+ gradient ($[Na^+]_o = 100$ mM; $[Na^+]_i = \text{zero}$).

determining the dimethylamiloride-sensitive uptake of Na^+ into the brush border membrane vesicles in the presence of an outward-directed H^+ gradient ($[pH]_i = 5.5$; $[pH]_o = 7.2$). Figure 6 compares the total Na^+ uptake as well as the uptake that occurred via the Na^+H^+ exchanger in membrane vesicles prepared from suckling rats (14 day old) and adult rats. The exchanger activity was significantly greater in the adult rats than in the suckling rats.

Development of Na^+ -dependent transport systems. The data presented herein on the H^+ -dependent Gly-Sar transport in developing rat renal BBM vesicles are similar to earlier reports on the Na^+ -dependent glucose and amino acid transport systems. In order to validate our results on the age-dependent development of Gly-Sar transport, we have measured the Na^+ -dependent transport of D-glucose and L-alanine in renal brush border membrane vesicles from suckling and adult rats. As can be seen in Figure 7, the activities of these transport systems are greater in the adult than in the suckling animals. These data confirm earlier findings from other laboratories (1, 2, 5).

DISCUSSION

Mammalian kidney undergoes morphological as well as functional changes during maturation (21). The immature mammalian kidney is unable to reabsorb filtered amino acids completely, and a generalized amino aciduria is seen in young animals (22) and in man (23). The incomplete development of the amino acid transport systems at this stage may be at least partly responsible for the presence of more than normal levels of amino acids in urine. These observations provided impetus for further detailed studies on the developmental pattern of various solute transport systems in the mammalian kidney. However, all the investigations reported so far on this aspect of renal organic solute transport involve Na^+ -dependent glucose and amino acid transport systems. In recent years, it has been established that di- and tripeptides are transported across the renal and intestinal BBM by a mechanism distinct from that of glucose and amino acid transport. There is strong evidence that an inward-directed H^+ gradient rather than a Na^+ -gradient serves as the energy source for the uphill transport of these peptides (6–12), even though claims contradictory to this statement have been made (24). The present study describes for the first time the postnatal development of this novel and unique transport system, peptide- H^+ cotransport, in renal BBM. Dipeptide uptake was measured

using Gly-Sar as the substrate because this dipeptide has been shown to be highly resistant to hydrolysis by purified renal BBM (10).

The data reported herein can be summarized as follows. 1) The presence of an inward-directed H^+ gradient across the BBM causes a transient accumulation of Gly-Sar inside the vesicles against a concentration gradient in all age group rats. 2) There was an age-dependent increase in the magnitude of the overshoot. 3) The equilibrium uptake of Gly-Sar was constant in all membrane preparations, indicating that the intravesicular volume remained the same despite the differences in the ages of the animals. 4) The activity of the Na^+-H^+ exchanger in the renal BBM vesicles, the transport system responsible for the generation of the driving force for peptide transport, was also significantly higher in the adult rats than in the suckling rats. 5) The kinetic analyses revealed that the differences in the initial rates of Gly-Sar uptake among the membrane vesicles prepared from different age group rats were primarily due to changes in the maximal velocity rather than alterations in the affinity of the transport system for its substrate.

Another interesting observation in the present investigation was the age-dependent increase in the specific activities of alkaline phosphatase and dipeptidylpeptidase IV in the kidney homogenate as well as in the purified kidney BBM. The developmental pattern of dipeptidylpeptidase IV is even more interesting, particularly in relation to the more or less parallel developmental pattern of peptide transport, because this enzyme plays an important role in the renal reclamation of peptide nitrogen from larger polypeptides and proteins (25, 26). This enzyme hydrolyzes polypeptides and proteins present in the glomerular filtrate and generates peptide substrates (di- and tripeptides) for the renal peptide transport system.

The possible reasons for the age-dependent increase in Gly-Sar transport in renal BBM vesicles are: a) there may be an age-dependent incorporation of the dipeptide transport proteins into the BBM, b) the developing membrane may dissipate the H^+ gradient at a much faster rate than the adult membrane, thus resulting in the age-dependent changes in the H^+ gradient-dependent Gly-Sar transport rates, and c) the composition of the structural components of the BBM may change during the postnatal development which could lead to alterations in the physical properties of the membrane and consequently in the catalytic activities of the transport proteins. There is evidence that BBM isolated from small intestine and kidney of young animals exhibit greater Na^+ permeability than the membranes from adult animals (3, 5). If H^+ permeability of these membranes also undergoes similar age-dependent alterations, the developing membrane may dissipate the H^+ gradient faster than the adult membrane. This would at least partly explain the greater activities of the Na^+-H^+ exchanger and the dipeptide transport system in membrane vesicles from adult animals than from young animals, because these activities were measured in the presence of a H^+ gradient as the driving force. Dissipation of this driving force at a faster rate would result in reduced activity of these transport systems. The age-dependent changes in the physical state of the BBM may play a role in determining the permeability characteristics of this membrane. Considerable evidence exists that membrane fluidity is closely related to the functions of many transport systems in intestine and kidney (27–29). The fluidity of the membrane is mainly determined by the lipid composition (30, 31). It has been recently reported that the fluidity of intestinal BBM vesicles from rat and rabbit changes during postnatal development (32, 33). Furthermore, Schwartz *et al.* (32) and Heubi and Fellows (34) have shown that age-related changes in the composition and fluidity of the intestinal BBM may be responsible for the accompanying changes in ileal bile salt uptake. Medow and Segal (35) have recently demonstrated an age-dependent decrease in the membrane fluidity of rat kidney BBM. They have shown further that a direct relationship exists between the decrease in membrane fluidity and the increase in Na^+ gradient-

dependent proline uptake in BBM vesicles prepared from rats of different age groups. Therefore, it is possible that the differences observed in the H^+ gradient-dependent Gly-Sar accumulation in developing kidney BBM vesicles may also be related to the age-dependent changes in the fluidity of these membranes.

The present findings that the activity of the dipeptide transport system in rat renal BBM vesicles increases with the age of the animal (adults > weanlings > sucklings) are in direct contrast with the available data on the age-related changes in the dipeptide transport system of the small intestine (sucklings > weanlings > adults) (13, 14). The physiological significance of the different developmental patterns of the intestinal and renal dipeptide transport systems is not immediately clear. The greater activity of the intestinal dipeptide transport system found in immature animals may be related to the incomplete development of the protein digestive processes in these animals (13). The dipeptide transport system may thus play a greater role than the amino acid transport systems in the maintenance of protein nutrition of the young animals. The function of the renal dipeptide transport system is to salvage the peptide nitrogen filtered at the glomerulus, and the activity of this transport system may be related to the concentration of the peptide substrates in the glomerular filtrate. With the exception of hormonal peptides, circulating peptides primarily arise from protein catabolism. It is likely that the concentration of peptides in the circulation is lower in young animals than in adults due to the age- and growth-dependent changes in the anabolic and catabolic states of the animal. The greater activity of the renal dipeptide transport system found in adult animals may thus have physiological significance. However, it has to be stressed that these suggestions are purely speculative at present and need proper experimental verification.

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