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DEVELOPMENTAL CHANGES OF GLYCINE TRANSPORT IN THE DOG

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The renal clearance of amino acids was measured in canine pups between 5 days and 12 weeks of age. The reabsorption of glycine was incomplete at 5 and 21 days, indicating a physiologic aminoaciduria of immaturity. An adult pattern of 97–100% reabsorption appeared by 8 weeks of age. The uptake of glycine by isolated renal tubules from 5-day-old, 3-month-old and adult dogs was examined towards an understanding of the events underlying this aminoaciduria. The initial uptake of 0.042 mM glycine by isolated tubules from the newborn was lower than that of the adult, but after 30 min of incubation the newborn surpassed the adult. A steady state of uptake was not achieved by the newborn even after 90 min of incubation, while it was achieved in the adult after 30 min. The uptake by the 3-month-old tubules resembled the adult at the early time points and the newborn at later points. With 1.032 mM glycine, a similar relationship of uptake between adult and newborn tubules was found, except with this concentration, the uptake by both the newborn and adult tubules reached a steady state. The concentration dependence of glycine uptake showed two saturable transport systems with similar apparent K_m and V_{max} values after 30 min of incubation for all three age groups. Determination of glycine flux by compartmental analysis revealed decreased influx and efflux in the newborn, but with a greater decrease in efflux, compared to adult. These changes of influx and efflux which accompany renal tubule maturation could contribute to the increased intracellular amino acid levels and decreased reabsorption of amino acids seen in the immature dog.

Introduction

Increased aminoaciduria has been noted in the immature animal of many species, including the rat, dog and man, which resolves as the individual matures. Understanding the aminoaciduria of immaturity is important for its insights into normal neonatal physiology and abnormal aminoacidurias. Previous in vitro investigations [1–5] of the aminoaciduria in the young have focused on the rat. However, there is little known about the cellular mechanisms of amino acid uptake by the renal tubule of the immature dog, which has a similar pattern of aminoaciduria in the newborn animal [6] as the human [7].

Glycine is one of the amino acids with the highest urinary excretion during the first few weeks of life in the dog, not achieving an adult pattern until after 2 months of age [6]. Glycine is also poorly reabsorbed in the rat neonate [8], and in the human [7], which does not exhibit and adult pattern until late in infancy. Glycine transport in the kidney is also disturbed in the human dis-

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orders of iminoglycinuria, familial glycinuria and glucoglycinuria.

To appraise maturational renal transport events we have examined the developmental pattern of the fractional reabsorption of glycine and other amino acids in the dog. In addition, we have studied the development of glycine uptake using the isolated renal cortical tubules from dogs of various ages which offers the advantages of better substrate penetration, oxygenation and minimization of architectural concerns over the renal cortical slice [9]. The results of these studies form the basis of this report.

Materials and Methods

Renal clearance studies

The renal clearance of amino acids was measured in 17 mongrel dogs of either sex at various ages: 5 days, 21 days, 8 weeks and 12 weeks using methods previously described [10]. The amino acid clearance and glomerular filtration rate were measured simultaneously, the latter by the renal clearance of inulin and endogenous creatinine. The 8-week and 12-week pups were maintained on a standard commercial diet and fasted for 12 h prior to the study. The younger pups were withheld from nursing for 6 h prior to study.

20-min urine collection periods were begun not less than 30 min after starting a maintenance mannitol infusion. Blood samples obtained at the midpoint of each urine collection were collected in heparinized syringes, centrifuged immediately and the plasma was removed. 1 ml plasma was mixed with an equal volume of 3% sulfosalicylic acid for protein precipitation; the supernatant was frozen immediately after centrifugation. An additional plasma sample was refrigerated for inulin and creatinine determination.

Urine samples were acidified to pH 2.0 with 1.0 M HCl, and frozen. Inulin was determined by a modification of Davidson's method [11]. Creatinine was determined by the Jaffe reaction [12]. Individual plasma and urinary amino acids were determined by a modification of the method of Moore and Stein [13] using a Beckman Model C Amino Acid Analyzer employing a lithium citrate buffer system.

Preparation of isolated renal tubules

Mongrel dogs of either sex, from three different age groups, were used for the preparation of isolated renal tubules according to the method of Burg and Orloff [9]. Kidneys were surgically removed from dogs 5–7 days old (designated newborn), 3 months old, and more than 1 year old; the dogs were killed by an arterial injection of 2 ml of T-61 euthanasia solution (Hoechst Corp.). The kidneys were immediately perfused at 4°C via the renal artery, with a 0.375% collagenase solution (w/v) in Krebs-Ringer bicarbonate buffer containing 10 mM sodium acetate, pH 7.4. The renal cortex was excised from the medulla, and cortical slices were made using a Stadie-Riggs microtome. The slices were then homogenized gently with four strokes of a pestle in a 15 ml loose dounce homogenizer and the suspension (1 gm/3 ml of Krebs-Ringer bicarbonate buffer) was centrifuged for 1 min at $40 \times g$ in an International Equipment Co. (Needham Heights, MA), model UV centrifuge. The pellet was suspended in 3 ml of Krebs-Ringer bicarbonate buffer containing 0.375% collagenase and 0.4% hyaluronidase (w/v) for each gram of original kidney cortex.

After a 45 min digestion at 37°C in a Dubnoff Metabolic Shaking Incubator, 3-times the incubation volume of iced Krebs-Ringer bicarbonate buffer was added to the suspension and this was centrifuged at $40 \times g$ for 1 min. The supernatant was discarded and the tubules were resuspended in the same volume of iced Krebs-Ringer bicarbonate buffer. This was repeated twice. After the final wash, the tubules were resuspended in Krebs-Ringer bicarbonate buffer to a concentration of approx. 5–7 mg wet weight per ml, and filtered through a 104 μm mesh. Fetal calf serum was added to a final concentration of 5% (v/v).

Uptake studies were performed in Burg and Orloff flasks with continuous bubbling of a 95% O_2/5% CO_2 mixture as described previously [2]. Substrate uptake was initiated by the addition of [^{14}C]glycine and terminated by removing 2 ml samples into tared tubes which were placed in an ice-water bath. The tubes were then centrifuged at 4°C for 10 min at $33\,000 \times g$ and the supernatants removed for counting. The pellet surface and the test tube wall were washed once with ice-cold Krebs-Ringer bicarbonate buffer and dried by suc-

tion. After weighing the tubes, the pellets were resuspended in 1 ml distilled water and the tubes were placed in a boiling water bath for 3 min. The tubes were then centrifuged and a 0.1 ml aliquot of the water extract of the pellet and the original incubation supernatants were added to 10 ml of a scintillation cocktail (2.8 ml absolute alcohol/7 ml OCS) and counted in a liquid scintillation counter.

Distribution ratios of radioactivity, the ratio of cpm/ml of intracellular fluid to cpm/ml of incubation medium were calculated as described previously [14]. Since glycine metabolism is slow in renal tissue in vitro with 80–90% of the intracellular label as glycine [15,16], the distribution ratio can be considered to reflect a concentration gradient. The intracellular fluid volume was calculated as the difference between the total tissue fluid (wet weight minus dry weight after overnight dessication) and the volume of 'trapped' fluid. The trapped fluid volume was determined using [^{14}C]-poly(ethylene glycol) as previously described [17].

Materials

Collagenase, grade II, was obtained from Worthington Biochemical Corp., with a specific activity of 161 U/mg; hyaluronidase, Type I-S was obtained from Sigma Chemical Co. Fetal calf serum was obtained from Flow Laboratories, divided into 10-ml aliquots, and stored frozen until used to prevent bacterial growth. [U – ^{14}C]Glycine (113 mCi/mmol) and [^{14}C]poly(ethylene glycol) (21.7 mCi/g) and OCS scintillation fluid were obtained from Amersham Corporation, Arlington, IL. Additional chemicals were obtained from commercial sources, and were of the highest purity available.

Results

Renal clearance determinations

The fractional reabsorption of amino acids by canine pups is shown in Table I. The reabsorption of most amino acids was incomplete in the 5-day-old pups compared to that in older animals. In 5-day-old dogs the fractional reabsorption of glycine was the most incomplete, being 67% of the filtered load. The range of reabsorption at this age was between 67% and 100% for all amino acids studied. Examples of this spectrum of reabsorption are the data for alanine and arginine also shown in Table I. At 21 days, glycine reabsorption was 95% of the filtered load, while most other amino acids had an adult pattern of reabsorption. An adult pattern of 97–100% reabsorption of all filtered amino acids appeared by 8 weeks of age. In contrast, the human infant does not achieve adult levels of amino acid reabsorption until after 4 months of life [7].

TABLE I

FRACTIONAL REABSORPTION OF AMINO ACIDS

The fractional reabsorption of various amino acids in dogs of various ages determined by standard renal clearance techniques, as described in the text. Values shown are the means of three determinations in each pup studied at 21 days, 8 weeks and 12 weeks. Among the 5-day-old pups, measurements were made in triplicate in four pups, and as individual measurements in the remaining six pups. n = number of pups.

Amino acid	Age of pups			
	5 days ($n = 10$)	21 days ($n = 3$)	8 weeks ($n = 2$)	12 weeks ($n = 2$)
Glycine	67	95	99	99
Alanine	91	99	99	99
Arginine	99	99	100	100

Amino acid uptake by isolated renal tubules

Estimation of tissue water. The water content of the spun tissue pellet was 78.99 ± 0.01% ($n = 4$) of the wet weight of the newborn tubule preparation, and was significantly less than the 83.49 ± 0.01% ($n = 6$) ($P < 0.01$) as determined in the adult tubule preparation. The trapped medium space (intertubule water) was 20.43% of wet weight for newborn tubules and 29.66% for the adult tubules. Therefore in the newborn tubules intracellular water was 58.56 ± 0.46% ($n = 4$) of wet weight, which differed significantly from the 53.83 ± 0.40% ($n = 6$) ($P < 0.001$) as determined in the adult tubule preparation.

Time-dependent uptake. The accumulation of 0.042 mM glycine by isolated renal tubules prepared from dogs of different ages is shown in Fig. 1A. In the tubules from adult dogs the uptake of glycine was curvilinear reaching a steady-state by 30 min of incubation. However, in tubules from newborn dogs, glycine uptake was nearly linear

over the entire 60 min incubation period and had not come to a steady state by 90 min (data not shown). Initially, the distribution ratios determined for the newborn tubules were lower than those for adult tubules, but because the distribution ratio progressively rose in the newborn, the value measured at 30 min nearly equalled that of the adult and was greater than the adult after 60 min of incubation. In tubules from 3-month-old dogs, the uptake of glycine was also curvilinear, but, like the newborn, it had not come to a steady state by 90 min of incubation (data not shown). This uptake pattern by tubules from 3-month-old dogs may be reflective of a transition from an immature to a mature pattern, since uptake at early time points resembles that of the adult, while uptake at later time points more closely resembles that of the newborn.

When tubules from adult dogs were incubated with 1.032 mM glycine, as shown in Fig. 1B, a steady state is achieved by 30 min. The distribution ratio of 1.032 mM glycine after 60 min incubation was lower than that achieved with a glycine concentration of 0.042 mM (Fig. 1A) by tubules from adult dogs, indicating saturable uptake. The uptake of 1.032 mM glycine by tubules from newborn dogs, in contrast to that found with 0.042 mM glycine, did achieve a steady state by 30 min of incubation. This pattern of uptake by tubules from newborn dogs also implies the presence of a saturable system(s) for glycine uptake, and is similar to that observed previously using slices of renal cortex and isolated renal tubules from Sprague-Dawley rats [2–5].

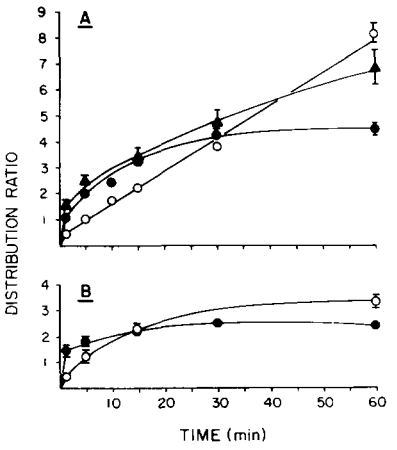

Fig. 1. Uptake of 0.042 mM (A) and 1.032 mM (B) glycine by isolated renal cortical tubules. Isolated tubules from newborn (○), 3 month old (▲) and adult (●) dogs were prepared and incubated as described in the text. Each point represents the mean ± S.E. of at least six determinations. Standard errors not shown are within the size of the point.

Concentration dependence of glycine uptake. The uptake of glycine by tubule preparations from the three age groups, over a concentration range of 0.042 to 50.032 mM, was examined to determine the apparent kinetic parameters describing glycine uptake. A 30 min incubation time was chosen as it was the only one which allowed for comparison between the three age groups. In incubations under 30 min, glycine uptake by tubules from newborn and 3-month-old dogs was not observed to be saturable. This can be seen in Fig. 1A and B, where the distribution ratio for 0.042 and 1.032 mM is the same up to 15 min of incubation. This is not the case for tubules from adult dogs which do exhibit a difference in their 15 min distribution ratios. The utility of a 30 min incubation for kinetic analysis using the isolated renal tubule preparation has been presented previously [18]. Scriver and Moyhuddin showed that 5 min and 40 min incubations of α-aminoisobutyric acid result in delineation of transport systems with similar K_m values, but different V_{max} values as would be expected [18]. This is supported in our studies by the finding that 15 and 30 min incubations of the adult tubule preparation yield the same apparent K_m value, and the same V_{max} value when corrected for incubation time.

A Lineweaver-Burk plot of the data revealed a two-limbed curve for the newborn, 3-month and adult tubule preparations, and the apparent kinetic parameters of transport derived from this plot are shown in Table II. This reveals that in all three age groups glycine transport occurs by two systems of high and low affinity for the substrate, and the values describing them are the same for all ages shown. The finding of two transport systems for glycine transport in the dog is similar to that described for other species [2,3,15]. The similarity of the apparent K_m and V_{max} values in all three age groups, however, does not explain the slower initial uptake rate in the newborn, as seen in Fig. 1. Since the V_{max} determinations at 30 min gave no insight into the apparent differences seen in the

TABLE II

OBSERVED VALUES FOR APPARENT K_m AND V_{max} OF GLYCINE TRANSPORT

Observed values were obtained from a Lineweaver-Burk plot after a 30 min incubation of isolated renal cortical tubules with [^{14}C]glycine over a concentration range of 0.042 to 50.032 mM as described in the text. Values derived from each age group are from at least three experiments in which quadruplicate determinations were made at each of the nine different glycine concentrations. K_m values are measured in mM and V_{max} values as mmol/liter intracellular fluid per 30 min.

Age	K_{m1}	V_{max1}	K_{m2}	V_{max2}
Newborn	2.24 ± 0.09	9.42 ± 0.79	26.45 ± 0.63	96.65 ± 6.83
3-month-old	1.47 ± 0.28	6.96 ± 1.03	29.11 ± 3.19	103.38 ± 10.80
Adult	1.42 ± 0.10	9.18 ± 0.96	22.88 ± 1.61	90.78 ± 5.25

initial glycine uptake, we chose to determine glycine flux by a compartmental analysis.

Determination of flux constants. If one assumes a simple two-compartment model of medium and intracellular fluid [19], the combination of a slower rate of uptake by the immature tubule at early times and the higher distribution ratios at later times can be explained by alterations of influx and efflux in the newborn. The data shown in Fig. 1B for 1.032 mM glycine were used for the analysis of a closed two-compartment system which attains a steady state [19,20]. The uptake of 0.042 mM glycine by tubules from newborn dogs could not be used for this type of analysis, because, as shown in Fig. 1A, uptake does not achieve a steady state; the higher concentration of glycine (1.032 mM) does reach a steady state in both newborn and adult tubule preparations. The calculated parameters of the two-compartment system are shown in Table III. While the newborn preparation achieves a higher steady-state distribution ratio than the adult, the net flux of glycine is higher in the adult (0.022 vs. 0.015).

There is also a difference in the fractional turnover rates for the two groups. The newborn preparation has an influx constant (λ_{IM}) which is 70.2% of the adult value and an efflux constant (λ_{MI}) which is only 49.6% of the adult value. The relative differences in influx and efflux rate constants explain the character of the uptake curves.

Inhibition studies. Although there are differences in the flux constants determined for the newborn and adult tubule preparations, the similar apparent K_m values of the three age groups

TABLE III

KINETIC PARAMETERS OF STEADY-STATE GLYCINE TRANSPORT IN NEWBORN AND ADULT DOGS

All calculations are based on 100 mg of tissue and an intracellular space of 58.56% of wet tissue weight and 53.83% of wet tissue weight for the newborn and adult renal tubule preparation, respectively. The rate constants are related by the equation $M \cdot \lambda_{IM} = ICS \cdot \lambda_{MI}$, where M is the medium concentration and ICS is the intracellular fluid substrate pool

Age group	Medium pool size (μmol)	Medium $\overset{\lambda_{IM}}{\underset{\lambda_{MI}}{\rightleftharpoons}}$ intracellular space				
		Steady-state distribution ratio	Intracellular fluid (μmol)	Fractional turnover Rates per min		Net flux (μmol/min per 100 mg final wet weight)
				λ_{IM}	λ_{MI}	
Newborn	0.026	3.248	0.1962	0.00059	0.07789	0.01528
Adult	0.026	2.483	0.1393	0.00084	0.15683	0.02218

TABLE IV

INHIBITION OF GLYCINE UPTAKE IN ISOLATED RENAL TUBULES FROM NEWBORN, 3-MONTH-OLD AND ADULT DOGS

Isolated renal cortical tubules from each age group were incubated with 0.042 mM [^{14}C]glycine in the presence and absence of a 10 mM concentration of various inhibitors. The distribution ratios of radioactivity after 30 min of incubation in the presence of the inhibitors were expressed as a percentage of the distribution ratios obtained in the absence of inhibitors. Each percentage is the mean ± S.E. for six determinations and comparisons of the percentage uptake observed with the inhibitors to that of the control were made within each group.

Inhibitor	Percentage of [^{14}C]glycine uptake		
	Newborn	3-month	Adult
None	100 ± 1.9	100 ± 7.4	100 ± 3.5
α-Aminoisobutyric acid	91.0 ± 1.1 [b]	82.7 ± 4.8 [b]	91.5 ± 2.1 [n.s.]
Lysine	86.7 ± 2.6 [b]	71.9 ± 3.4 [b]	80.9 ± 4.5 [c]
Proline	86.1 ± 3.8 [c]	81.4 ± 3.9 [a]	76.0 ± 1.6 [a]
Valine	56.2 ± 1.2 [a]	48.8 ± 3.4 [a]	61.0 ± 4.0 [a]

[a] $P < 0.001$.
[b] $P < 0.01$.
[c] $P < 0.02$.
n.s., not significant.

suggest a comparable affinity for glycine by its transport systems. This is supported by results shown in Table IV which describe the transport interactions of glycine with other amino acids using tubules from the three age groups. The degree of inhibition afforded by each amino acid is similar in all three age groups.

Discussion

The occurrence of physiological hyperaminoaciduria in the newborn of many species, including the dog, is well documented [6–8] and glycine is one of the amino acids most affected by this phenomenon. As the dog matures, this aminoaciduria abates with the attainment of an adult pattern of amino acid excretion between 3 and 8 weeks of life. It is clear, however, that this maturation of tubular transport capability does not parallel the functional and anatomical development of the canine kidney. The glomerular filtration rate per body mass increases rapidly during the first 2 weeks of life and begins to stabilize by the 4th week [21]. Adult values for glomerular filtration rate and clearance of *para*-aminohippurate per body mass are only achieved after 2 months of age [21]. After the 3rd week of life, renal growth is primarily limited to changes in tubular volume which increases by 235% during the 3rd to 10th week while glomerular filtration rate only increases 33% during this time period [22,23]. Therefore, the maturation of tubular reabsorption of amino acids occurs prior to attainment of adult levels of glomerular filtration rate and full development of tubular mass. Physiological hyperaminoaciduria is therefore likely due to age-related differences in the ability of the renal tubular epithelium to transport amino acids from lumen to the blood.

In spite of the differences in the in vivo renal handling of glycine between the adult and newborn, a number of similarities exist in mature and immature renal cortical tubule epithialia. Our data are consistent with the interpretation that all age groups studied had two saturable systems for glycine uptake with a pattern of inhibition by α-aminoisobutyrate, proline, lysine and valine that was comparable in the three groups. However, there are several changes in glycine transport which occur with maturation. Initial uptake, as measured by the distribution ratio after 5 min of incubation, was significantly lower in the newborn when compared to the adult. A marked difference in the uptake curves between the various age groups is

noted also at the later time points. A steady state of glycine was not achieved in the newborn tubules even after 90 min of incubation, while the uptake by adult tubules had reached a steady state by only 30 min of incubation. Glycine uptake by tubules from 3-month-old dogs may be reflective of a transition phase between the newborn and adult animals. The uptake pattern of 3-month-old tubules resembled that of the adult tissue initially, but then more closely resembled the newborn at the later time points. This may indicate that the decreased influx seen in newborns resolves first with tubule maturation, followed sometime later by a change in the efflux rate.

These differences in uptake patterns are reflected in the glycine flux constants. The influx constant of newborn tubules was 70% that of the adult while the efflux constant was lower, being only 50% of the adult value. These findings are similar to those described by McNamara et al. [24] to explain the temperature dependence of α-methyl-D-glucoside uptake by rat kidney cortex slices. The difference in timed uptake of α-methyl-D-glucoside at 37°C versus 20°C appeared to be related to a decrease in both influx and efflux but with a greater effect on efflux. The profile of α-methyl-D-glucoside uptake at 37°C was similar to that of 0.042 mM glycine in adult dog tubules, while the uptake at 20°C resembled that of 0.042 mM glycine in newborn dog tubules. In newborn rat renal cortical slices which take up glycine linearly for long incubation times as does the dog tubule, a direct experimental demonstration of slow glycine efflux has been reported [25,26].

In spite of differences in the initial rate of glycine uptake and the flux constants, the apparent K_m and V_{max} values studied after a 30 min incubation were similar. Determination of these values with shorter incubations would have been desirable, but saturability could not be demonstrated in tubules from newborn and 3-month-old animals prior to 30 min. The V_{max} values measured after such an incubation time, therefore, do not represent influx rates alone, but the balance between influx and efflux after 30 min. Because influx and efflux rates are both lower in the newborn animal, this results in V_{max} values after 30 min of incubation that are similar to those from adults and 3-month-old and are not reflective of the actual kinetics of glycine transport in the three age groups. However, Scriver et al. [18] have shown that determination of transport affinity constants (K_m) after long incubation periods does give the same values as those measured after short incubation times. Therefore, the similar apparent K_m values noted in the three age groups suggests that glycine interacts with its membrane carriers with equal affinities in the newborn, 3-month-old and adult dogs. The importance of this finding and, indeed, of the study of the concentration dependence of uptake is the demonstration that dual systems for transport are present at these ages and that developmental transport changes are not due to postnatal emergence of transport systems absent in the newborn.

Previous reports of glycine transport in developing rats have noted findings similar to our present studies in the developing dog. Two saturable transport systems for glycine were present in renal cortical slices from newborn Sprague-Dawley and Long-Evans rats [3], and in isolated renal tubules from newborn rats. In the newborn rat as in the dog, a steady state of uptake at low glycine concentrations was not achieved, even with long incubation periods using the above preparations [2,3]. No difference in the initial rate of substrate uptake was observed in our experiments with the developing rat tubule [3] as there was in the dog, illustrating a species difference. In the rat the aminoaciduria of immaturity correlates with slow in vitro efflux while in the immature dog, both influx and efflux appear to be altered.

Although the exact sequence of events in the renal tubule cell which leads to an aminoaciduria has not been clearly delineated, several mechanisms have been postulated. One such mechanism is that reabsorbed amino acids quickly efflux out of the cell back across the brush-border membrane. This may be the method by which maleic acid produces aminoaciduria, since markedly increased rates of efflux have been demonstrated in both the cortical slice [27] and the isolated tubule [28]. Increased efflux rates are also suggested by the low intracellular amino acid pools in the face of nearly normal influx rates [29]. However, the physiological aminoaciduria of immaturity appears to occur by a different mechanism. The intracellular amino acid pool in newborn animals

is higher than that of adults. The most constant flux alteration that is demonstrable in renal tissue from young animals is a decrease in efflux which is consistent with the raised intracellular pools [25, 26]. These raised intracellular amino acid pools in vivo, if due to decreased efflux across the basolateral membrane, might then further impair influx of filtered amino aids and result in increased urinary excretion. Evaluation of this hypothesis directly requires the development of methodology for the isolation of brush border and basolateral membranes and perfusion of isolated tubule segments that is suitable for the newborn.

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