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ISOLATION AND CHARACTERIZATION OF THE BRUSH BORDER FRACTION FROM NEWBORN RAT RENAL PROXIMAL TUBULE CELLS

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SUMMARY

A renal brush border fraction was isolated from newborn Sprague-Dawley rats, and its morphological and enzymatic characteristics were studied in comparison to that from the adult Definite microvillar structures are seen by electron microscopy, and brush border preparations from the newborn are enriched in known marker enzymes Though morphological development is more advanced and enzyme specific activities are greater in the adult, polyacrylamide gel electrophoresis of membrane proteins reveals no significant change in pattern with increasing age These studies suggest that the brush border of the proximal tubule cell is present at birth as a significantly developed structure

INTRODUCTION

In recent years the importance of the apical microvilli or brush border of luminal epithelial cells in the absorption of various substances has been continually emphasized The isolation of this organelle has been carried out by several investigators including Thuneberg and Rostgaard [1] and Berger and Sacktor [2] in rabbit kidney as well as Wilfong and Neville [3] and Kinne et al [4, 5] in rat kidney In each case the brush border fraction has been characterized morphologically in electron microscopic studies and biochemically through the localization of various enzymes in the microvilli. Thus isolated, the brush border has served as a valuable tool in the study of the uptake of sugars [6–11] as a first step in their transport into the cell and has great potential for delineating various aspects of membrane function In view of our interest in the development of transport systems in the proximal tubule of rat renal cortex and our reported findings of differential uptake of various substrates in newborn and adult whole cortical slices [12–14], we have isolated a kidney tubule brush border fraction from the newborn rat and have defined some of its morphological and enzymatic characteristics as compared to a similar preparation from the adult.

MATERIALS AND METHODS

Adult male and pregnant female Sprague-Dawley rats were obtained from West Jersey Biological Laboratories, Wenonah, New Jersey, housed in our own animal quarters, and fed ad libitum before use In the isolation of brush border from the newborn, rats from 4–6 litters, numbering approx 10 animals each, were decapitated and their kidneys removed The animals were less than 36 h of age and were not separated according to sex The entire kidney was utilized in the preparation of the membranes, except in one case in which an effort was made to remove the cortex only Male animals were chosen whenever possible for the preparation of membranes from older rats The animals were stunned and decapitated, the kidneys removed and decapsulated, and cortical slices prepared with a Stadie-Riggs microtome

The isolation procedure was the modified method of Kinne and Kinne-Saffran [4, 5], employing differential centrifugation but carried out in buffer comprising 0 25 M sucrose, 0 005 M EDTA, 726 units/ml sodium penicillin G, 64 μg ml streptomycin sulfate, and 140 mM NaCl, pH 7 4, at 4 °C An aliquot of whole newborn kidney or adult male cortex homogenate was diluted 10- to 20-fold in distilled water before the isolation procedure was begun The only departure from the isolation method was the substitution of a 20-min centrifugation at 31 500 × g in place of the third 17 000 × g centrifugation step in the Kinne protocol The final pellet was suspended in distilled water

In some cases the final pellet was suspended in a solution of 4 mM $NaHCO_3$ and 1 mM $MgCl_2$, pH 8 1, in preparation for a final purification step on a continuous sucrose gradient as utilized by Wilfong and Neville [3] Sucrose gradients were prepared in the above solution, and centrifugation was carried out in a Beckman-Spinco Model L preparative centrifuge equipped with an SW-25 1 rotor Centrifugation for 1 h at 14 400 × g was required in order to achieve any appreciable separation, after which a broad zone of particulate matter was noted in the upper portion of the gradient and a pellet at the bottom The sucrose was carefully decanted and the pellet suspended in distilled water Protein concentrations were determined by the method of Lowry et al [15]

Suspensions of isolated brush border membranes from newborn and adult animals were examined by electron microscopy Negatively stained wet preparations were made by placing a drop of the membrane suspension on a carbon-coated Formvar grid, adding one drop of a 4 % phosphotungstic acid, pH 7 0, and examing the preparations in a Philips 300 electron microscope Membrane suspensions and cortical slices were subsequently fixed in 1 % phosphate-buffered osmic acid, dehydrated in graded ethanol, and embedded in epoxy resin Thin sections were then stained with lead citrate and uranyl acetate and examined in the microscope

All enzyme studies were carried out on both whole homogenate and isolated brush border fractions prepared with and without the final sucrose gradient centrifugation step Assays for alkaline phosphatase [16], maltase [17], $(Na^+ - K^+)$-ATPase [18], β-glucuronidase [19], succinate dehydrogenase [20] and DNA [21] were carried out as previously described, without modification, using suitable aliquots of membrane suspension γ-Glutamyltranspeptidase was assayed using the microassay of Neville and Glossmann [22] with γ-glutamyl-p-nitroanilide as substrate and glycylglycine as acceptor in 0 05 M Tris HCl buffer, pH 8 2 Leucine aminopeptidase

activity was determined using 0 05 M L-leucine-*p*-nitronanilide in methanol as substrate 0 02 ml of substrate, 0 02 ml of 0 04 M $MgCl_2$, 0 14 ml of 0 05 M Na_2PO_4 buffer at pH 7 2, and 0 02 ml of membrane suspension were incubated for 10 min at 37 °C The reaction was stopped with 0 1 ml of 1 5 M acetic acid and the yellow color read at 405 nm on a Beckman 151 Spectro-Colorimeter Glucose-6-phosphatase activity was measured in an assay modified from that of Hubscher and West [23] 0 1 ml of 0 2 M glucose 6-phosphate, 0 5 ml of 0 05 M maleate buffer containing 4 mM EDTA, pH 6 0, and 0 1 ml of membrane suspension were incubated for 15 min at 37 °C The reaction was stopped by adding 1 0 ml of 20 % trichloroacetic acid and, after centrifugation, 1 0 ml of the supernatant was used for determination of inorganic phosphate as previously described [18]

Polyacrylamide gel electrophoresis of the proteins of the brush border fraction was carried out in a Hoeffer electrophoresis unit according to the method of Neville and Glossman [24], employing a discontinuous multiphasic buffer system with running pH of 9 5 Lower gels measuring 10 cm in length were cast in glass tubes from a stock solution of 11 % acrylamide and 0 1 % methylenebisacrylamide, and upper gels measuring 1 5 cm in length were cast from a solution of 3 % acrylamide and 0 027 % methylenebisacrylamide The gels were polymerized at room temperature with 0 05 % (w/v) ammonium persulfate and 0 15 % (v/v) *N,N,N',N'*-tetramethylethylenediamine Aliquots of membrane suspension were prepared as above omitting the final sucrose gradient step and adding sodium dodecyl sulfate to a concentration of 1 % (w/v) and dithiothreitol to a final concentration of 40 mM (w/v) The samples were heated for 10 min at 37 °C and a crystal of sucrose dissolved in each 0 05 to 0 1 ml of each sample was layered onto a gel to achieve a protein load of about 0 05 mg per gel The gels were run for 2 h at a current of 2 mA per tube in parallel with a standard gel containing β-galactosidase, ovalbumin, bovine serum albumin and cytochrome *c* of known molecular weights Gels were fixed and stained with Coomassie blue G according to the method of Fairbanks et al [25]

RESULTS

Electron microscopy

Electron micrographs of negatively stained wet preparations of the brush border fraction from newborn and adult rats after the final sucrose gradient step both show collections of long tubular microvilli (Figs 1A and 1B) They are seemingly fewer in number in the newborn, and scanning of the preparation reveals a somewhat greater number of mitochondria present than in the adult Embedded preparations reveal that in the adult sizeable portions of connected microvilli have been preserved intact (Fig 1D), while in the newborn small membrane fragments, some in vesicular form, with fewer single microvillar structures make up the preparation (Fig 1C)

Specific activities of enzymes in homogenates and brush border fractions

Specific activities of enzymes tested in whole rat kidney homogenates and brush border preparations are shown in Table I The specific activities of all enzymes tested were significantly higher in homogenates from the adult than in homogenates from the newborn with the exception of β-glucuronidase, in which the specific activity in the homogenate from the newborn is higher than in that from the adult, and Mg^{2+}-

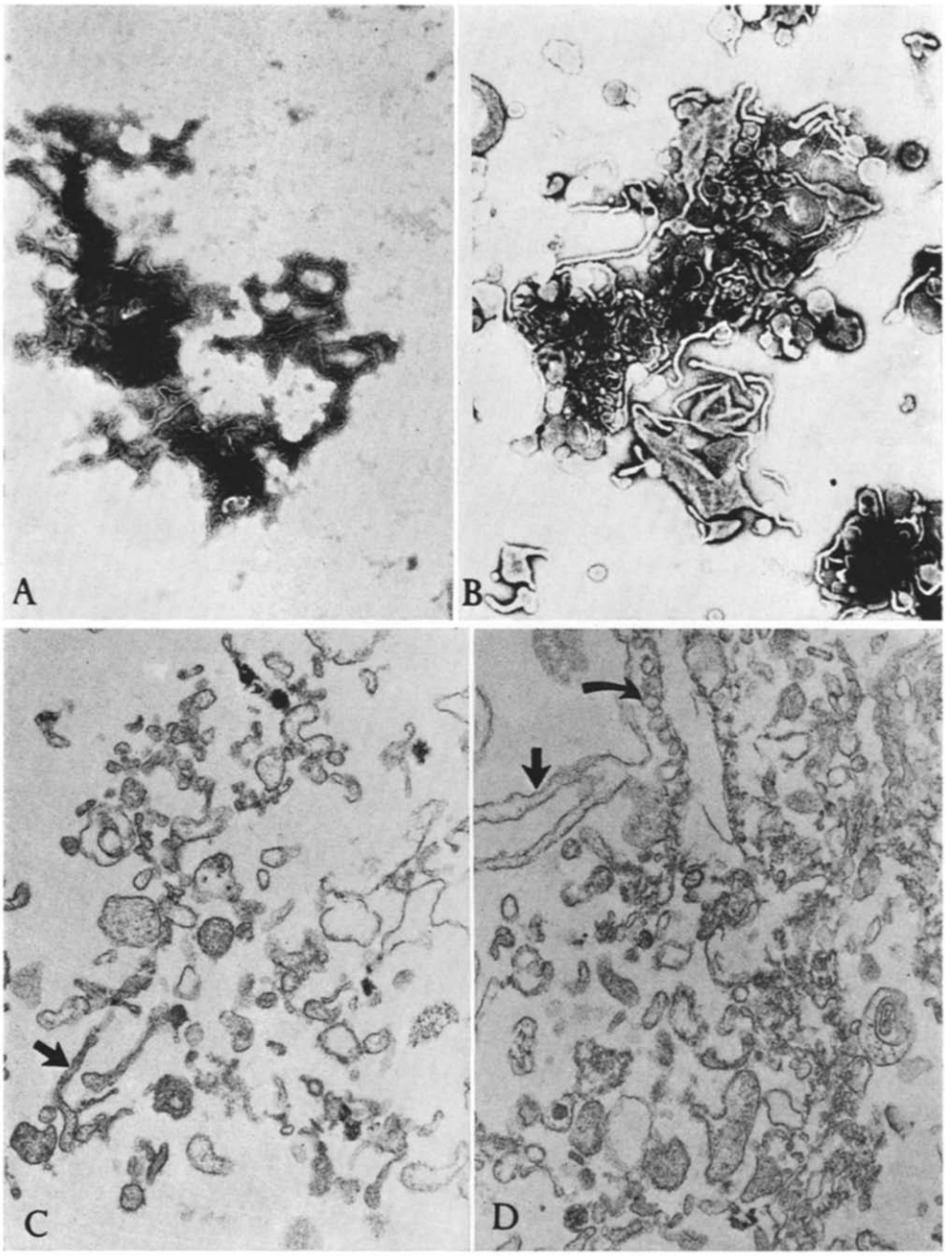

Fig 1 Electron micrographs of negatively stained wet preparations of newborn (A) and adult (B) brush border membranes (approx 16 100) (C) Preparation of newborn brush border membranes embedded in epoxy resin Arrow shows microvillus (approx 30 450) (D) Preparation of adult brush border embedded in epoxy resin Straight arrow shows longitudinal section of microvillus, and curved arrow shows cross-section of same structure (approx 30 450)

TABLE I

SPECIFIC ACTIVITIES OF ENZYMES IN WHOLE HOMOGENATES AND BRUSH BORDER PREPARATIONS FROM NEWBORN AND ADULT RAT KIDNEYS

Values in parentheses are for preparations not subjected to sucrose gradient centrifugation $(Na^+ + K^+)$-ATPase values represent the difference between $(Na^+ + K^+)$-ATPase + Mg^{2+}-ATPase and Mg^{2+}-ATPase P = Statistical significance of increase in specific activity from newborn to adult homogenate N = Number of determinates BB/H = Ratio of activity in brush border to activity in homogenate

	Newborn					Adult					
	Brush border	N	Homogenate	N	Ratio BB/H	Brush border	N	Homogenate	N	Ratio BB/H	P
Leucine aminopeptidase (μmol/mg protein per h)	2 10±0 10 (0 79±0 13)	8 5	0 28±0 02	13	7 50 (2 82)	4 70± 0 13 (1 60±0 18)	7 5	0 39±0 03	11	10 44 (4 10)	< 0 01
γ-glutamyltranspeptidase (μmol/mg per min)	1 65±0 10	9	0 50±0 03	6	3 30	4 10±0 22	11	0 71±0 04	5	5 77	< 0 01
Alkaline phosphatase (μmol/mg per h)	114 38±5 66 (81 41±1 05)	5 3	26 38±1 86	8	4 34 (3 08)	376 80±12 99 (313 81± 6 49)	6	80 86±5 94	6	4 66 (3 88)	< 0 001
Maltase (μmol/mg per h)	(11 46)	1	2 01	1	5 70	(108 96)	1	16 37	2	6 66	< 0 001
Succinate dehydrogenase (μmol/mg per h)	0 (12 60±0)	4 2	8 74±0 46	3	0 (1 44)	0 (11 94±0 80)	3 11	22 92±1 03	12	0 (0 52)	0 001
β-Glucuronidase (mmol/mg per h)	0 39±0 02	8	0 26±0 02	9	1 50	0 10±0 01	7	0 14±0 01	7	0 71	< 0 01
Glucose-6-phosphatase (μmol P_i liberated/mg per h)	8 62±0 73	6	3 71±0 29	8	2 32	14 57±1 30	8	6 27±0 47	10	2 32	< 0 001
$(Na^+ + K^+)$-ATPase (mmol P_i liberated/mg per h)	1 12		2 42		0 46	6 99		11 28		0 62	–
$(Na^+ + K^+)$-ATPase plus Mg^{2+}-ATPase	35 62±2 39	6	13 60±1 71	7	2 62	50 08±3 96	6	22 98±3 57	5	2 18	< 0 05
Mg^{2+}-ATPase	34 50±0 83	7	11 18±1 21	7	3 09	43 09±3 67	6	11 70±1 25	5	3 68	NS

ATPase, in which the activities are equal Values shown for the brush border fractions are the averages of activities determined in preparations isolated with and without the final sucrose gradient centrifugation step except in the cases of leucine aminopeptidase, alkaline phosphatase and succinate dehydrogenase in which this step substantially affected enzyme activity and activities measured in both preparations are given

The relative specific activities of the known brush border marker enzymes (leucine aminopeptidase [26, 27], γ-glutamyltranspeptidase [28], alkaline phosphatase [29, 30] and maltase [31]) are greater than one in apical membrane preparations from both the newborn and the adult kidney Relative specific activities greater than one in the newborn suggest that development of the apical portion of the tubule cell has already progressed substantially by birth Small but significant increases in the specific activities of leucine aminopeptidase and γ-glutamyltranspeptidase are seen in comparing newborn and adult homogenates, but there is a correspondingly greater increase in the specific activities of these enzymes in the brush border fractions The greater relative specific activities in the adult indicate that most of the development of these enzymes in the brush border occurs in the postnatal period However, in the case of alkaline phosphatase and maltase, the magnitude of the increase in specific activities in the homogenates is approximately equal to that in the brush border fractions The relative specific activities of these enzymes are the same in newborn and adult preparations, indicating no further concentration of enzyme activity in the brush border with increasing age

Determinations of enzyme activities known to be localized in other organelles were undertaken to quantitate contamination of the brush border preparation Table I shows that the relative specific activities of succinate dehydrogenase, a marker enzyme for mitochondria [32], β-glucuronidase, that for lysosomes [32], while both less than one in preparations from adult kidney, are greater than one in preparations from the newborn, implying somewhat greater contamination of the newborn preparation with these organelles The relative specific activity of glucose-6-phosphatase is greater than unity in preparations from kidneys of both newborn and adult animals, suggesting equal contamination of both preparations with microsomes [32] The activity of $(Na^+ + K^+)$-ATPase, known to be localized in the basal portion of the tubule cell [33], is determined by subtracting the activity of Mg^{2+}-ATPase, generalized membrane marker enzyme, from the total ATPase activity $(Na^+ + K^+)$-ATPase $- Mg^{2+}$-ATPase $(Na^+ + K^+)$-ATPase is negligible in the newborn kidney homogenate and brush border fraction, but its relative specific activity in the adult is less than one, indicating that antiluminal membranes have been substantially removed from the brush border preparation

Electrophoretic analysis

Polyacrylamide gel electrophoresis of the rat kidney brush border preparations reveals the presence of at least 30–35 different protein bands (Fig 2) Nearly all of the bands migrate between the origin and the range of ovalbumin, indicating molecular weights of 40 000 or greater There is no appreciable change in the pattern with age Newborn brush borders prepared from whole kidney and those made from outer cortex only give identical patterns Densitometry proved of no help in further elucidating the complex patterns, most probably because of the close apposition of many of

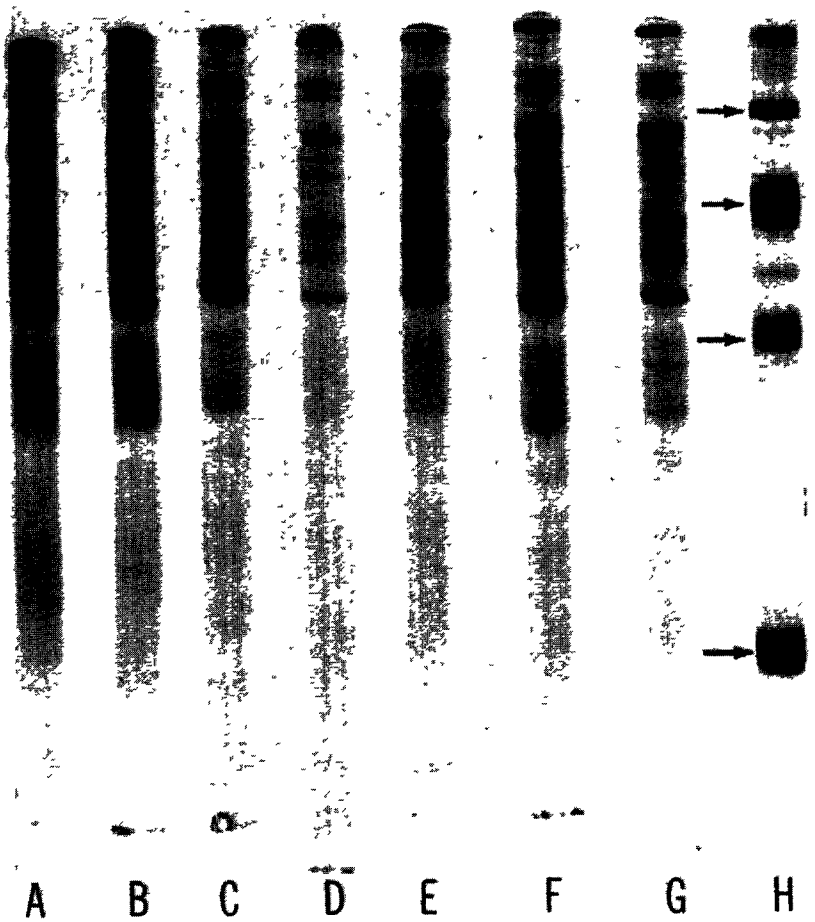

Fig 2 Polyacrylamide gel electrophoresis of brush border membrane proteins isolated from Sprague-Dawley rats of various ages (A) Newborn brush border prepared from cortex only (B) Newborn prepared from whole kidney (C) 9 days (D) 2 weeks (E) 3 weeks (F) 4 weeks (G) Adult (H) Standards of known molecular weight Arrows from top to bottom show β-galactosidase (130 000), bovine serum albumin (68 000), ovalbumin (45 000) and cytochrome *c* (13 000)

the bands and impurity of the preparation, therefore, it was not possible to examine the relative intensities of different bands at different ages in an effort to trace the development of particular proteins

DISCUSSION

Our studies confirm the report of Suzuki [34] that the apical brush border of the proximal renal tubular cell in rat is present at the time of birth It is already a differentiated structure in the newborn animal, and further maturation to adulthood appears to take the form of an increase in the specific activities of some constituent enzymes rather than in a radical alteration in its protein composition This quantitative rather than qualitative maturation process is reflected in the morphologic develop-

ment seen both in our electron microscopic studies and in those of Suzuki which show lengthening of the microvilli already present at birth

The isolation of the brush border fraction from the newborn rat kidney presents difficulties not encountered in that from the adult The yield of brush border membranes from the newborn is small, and at least 40–60 animals must be used to obtain 1–2 mg of membrane protein Secondly, there is no clear separation of cortex from medulla in the newborn kidney, necessitating use of the entire organ for preparation of the brush borders Finally, the isolation procedure, originally described for the adult [4, 5], involves several differential centrifugation steps in which separation of an upper fluffy pellet containing the membrane fragments from a lower dark pellet contaminating organelles plays a major role In the newborn, separation of a bilayered pellet proves far more difficult, contributing to the greater degree of contamination seen when this method is applied to the immature kidney

The use of the continuous sucrose gradient centrifugation as a final step seems to do little to improve the quality of the membrane preparations and is probably unnecessary It was hoped that suspension in the hypotonic $NaHCO_3$ and $MgCl_2$ solution would facilitate the opening of any formed membrane vesicles, allowing contaminating organelles to escape and be removed to the upper portion of the gradient Unfortunately, there is little evidence that this is the case If aliquots of membranes from the pellet and the broad upper zone of the sucrose gradient are assayed for alkaline phosphatase and glucose-6-phosphatase, marker enzymes for brush borders and microsomes, respectively, there is no significant difference in their specific activities, indicating no increase in purity It is probable, especially in the case of the newborn, that the brush border fragments are not uniform in size and that a large portion of them are too near in density to fragments of contaminating organelles like microsomes, lysosomes and mitochondria to be pelleted at the bottom of the tube Moreover, though comparison of the specific activities of leucine aminopeptidase alkaline phosphatase and succinate dehydrogenase in the brush borders after preparation with and without the final sucrose gradient step might suggest that additional purification has been achieved, the presence of $NaHCO_3$ and $MgCl_2$ in the gradient affects the activities of these enzymes Replacing Mg^{2+} chelated by EDTA in the early steps of the preparation partially restores alkaline phosphatase activity and enhances that of leucine aminopeptidase [35], and both Mg^{2+} and the alkaline pH of the sucrose gradient inactivate succinate dehydrogenase irreversibly [36]

Our data concerning the various adenosine triphosphatases deserve further comment The specific activity of Mg^{2+}-ATPase is the same in both newborn and adult homogenates, and the significant difference in the total ATPase activity (Na^+ K^+)-ATPase$-Mg^{2+}$-ATPase) between the newborn and adult is the result of development of substantial (Na^+ K^+)-ATPase activity from negligible levels in the newborn, a finding in accord with that of Davis and Dixon [37] and Beyth and Gutman [38] in homogenates of a microsomal fraction from rabbit kidney Since it has been shown that (Na^+ K^+)-ATPase activity is localized in the basal infoldings of the antiluminal membrane [33], our data would indicate appreciable differentiation of the brush border marker enzymes in the newborn, but a lesser degree of enzymatic differentiation of the basal membrane Though Suzuko does not draw the conclusion that a greater structural development of the apical brush border has occurred by birth as compared to the basal infoldings examination of his electron micrographs

suggest that this may be the case Therefore, it may be that an imbalance between apical and basal membrane transport systems exists in the neonatal period of kidney development

Studies in our laboratory have emphasized the differences in the transport of sugars and amino acids into newborn and adult Sprague-Dawley rat cortical slices [12, 14] There appears to be little evidence for active transport processes involving the entry of sugars into neonatal cortical cells, while in the case of amino acids active influx is comparable to the adult but efflux is slowed Since the brush border seems to be already reasonably differentiated morphologically and enzymatically at birth, the immaturity of transport systems involving sugar entry and amino acid effiux may be related to maturation of basal membrane function One might speculate that the $(Na^+ + K^+)$-ATPase may be involved in the increasing ability of the tubule cell to accumulate sugars and for its decreasing ability to form intracellular-extracellular amino acid gradients with aging

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