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THE ONTOGENY OF AMINO ACID TRANSPORT IN RAT KIDNEY

I. EFFECT ON DISTRIBUTION RATIOS AND INTRACELLULAR METABOLISM OF PROLINE AND GLYCINE*

KURT E. BAERLOCHER, CHARLES R. SCRIVER AND FAZL MOHYUDDIN

The deBelle Laboratory for Biochemical Genetics, McGill University-Montreal Children's Hospital Research Institute, 2300 Tupper Street, Montreal 108, Quebec (Canada)

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SUMMARY

Newborn Long–Evans rat pups have reduced tubular reabsorption of proline, hydroxyproline and glycine *in vivo*. Reabsorption of iminoacids improves one week after birth; glycine transport improves in the third week.

Kidney *in vivo* accumulates proline and glycine from plasma and urine against a large chemical gradient. Newborn kidney cortex slices *in vitro* take up L-proline, glycine and α-aminoisobutyric acid less efficiently than mature tissue. The age-dependent differences are most apparent after short incubation (<15 min) and at specific concentrations of the substrates. Net uptake of amino acids, after long incubation, is usually greater in newborn kidney than in mature tissue.

After uptake proline and glycine incorporation is greater, whereas oxidation and metabolic conversion is less in newborn tissue than in mature kidney. Newborn and adult kidney slices exhibit similar pH optima for L-proline and α-aminoisobutyric acid uptake. Temperature elevation increases their steady-state uptake more in newborn than in adult kidney.

INTRODUCTION

The migration of proline, hydroxyproline and glycine into mature mammalian kidney cells has been studied intensively[1–10]. At concentrations normally encountered under physiological circumstances, proline and hydroxyproline (the imino acids) are taken up together by a system with high affinity and low capacity, which is capable of excluding the entry of other external amino acids but which permits exchange with internal glycine. Glycine, in its turn, is taken up by a high-affinity, low-capacity system which excludes the entry of imino acids from the external pool, but will allow counterflow with their internal pool. At extracellular concentrations above the physiological range (0.1–0.4 mM), these three amino acids can enter kidney together on a shared system which has lower affinity but greater capacity for the three substrates. Ontogeny should be an informative probe of these uptake systems in mammalian

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kidney[11] since hyperexcretion in urine of proline, hydroxyproline and glycine characterizes the early postnatal period of man[12,13] and other mammals[11]. In this paper, we describe the development of renal tubular transport processes for these substances in the young rat *in vivo*. Parallel studies were performed to describe uptake, oxidation and incorporation of these compounds in *vitro*. The non-metabolizable compound α-aminoisobutyric acid which interacts in a defined way with the imino acids and glycine during uptake, was used as a control amino acid.

METHODS AND MATERIALS

In vivo

Long-Evans hooded rats (Quebec Breeding Farm) were fed *ad libitum* on Purina rat chow and water. Adult rats (150 g) and pups 7, 14 and 21 days of age were used. Urine was obtained from adult animals, caged over collection funnels and from pups by spontaneous voiding or by bladder puncture. Heparinized blood was obtained from adult animals by retroorbital puncture or from the heart. 1 vol. of plasma was deproteinized immediately with 5 vol. of sulfosalicylic acid (3 %, w/v). All samples were frozen immediately at —20° until analysis.

Amino acids were measured by elution chromatography on ion exchange resin columns using a modified Beckman–Spinco amino acid analyzer[14]. Total nitrogen was determined by a micro-Kjehldahl method.

In vitro

Cortex slices were obtained from adult kidney and incubated as described previously[1]. The kidneys of young rats were removed, weighed and divided along their long axis into three portions after removal of the capsule. One or two slices (2–7 mg) were obtained under light pressure with a chilled Stadie–Riggs microtome, and kept briefly on cold, moist filter paper until transfer to Warburg flasks prepared for measurement of uptake and oxidation of substrate, as described previously[1,11]. One slice was incubated in 2 ml Tris–Ringer–glucose buffer (300 mosM, pH 7.4, at 37° under 100 % O_2). After incubation, slices were removed, rinsed in saline, blotted, weighed and boiled for 5 min in 1 ml distilled water; 0.8 ml of the boiled supernatant was removed, placed in a scintillation vial and dried over P_2O_5 in a vacuum dessicator. The residue was dissolved in hyamine, shaken vigorously and diluted with 5 ml scintillation fluid (4.0 g PPO and 0.1 g POPOP dissolved in 1 l toluene). Filter papers soaked in KOH were used to trap CO_2; they were then dried and placed in scintillation fluid. The efficiency of CO_2 collection by this method is 59 ± 4 % in our hands. Radioactivity was measured with a Nuclear Chicago Unilux II Scintillation counter operating at 50 % efficiency.

Total tissue water was determined in slices by drying to constant weight at 100° for 48 h. Extracellular fluid was determined *in vitro* by the ^{14}C-labelled inulin method[15]; the intracellular fluid volume was estimated by difference.

Metabolic conversion of labelled substrate to soluble products was evaluated as described previously[1]. Incorporation into insoluble precipitable material was measured by estimating radioactivity in the boiled slice dissolved for three days in hyamine; a correction was made for the soluble counts trapped in fluid spaces. This method yields values equivalent to "trichloroacetic acid-precipitable" material and is taken

to represent protein. Homogenates of kidney cortex slices were prepared in isotonic saline and incubated in buffer to study substrate oxidation to CO_2 as described above. Protein content of homogenates was measured by the method of LOWRY *et al.*[16].

Materials

[*Carboxy*-^{14}C]Inulin (specific activity 2.47 mC/g), α-amino[1-^{14}C]isobutyric acid (specific activity 8.59 mC/mmole), uniformly ^{14}C-labelled L-proline- (specific activity 80 mC/mmole) and uniformly ^{14}C-labelled glycine (specific activity 120 mC/mmole) were obtained from the New England Nuclear Corporation. Their radiochemical purity was examined by partition chromatography in a butanol, acetic acid, water mixture (12:3:5, by vol.) and by high voltage electrophoresis (90 V/cm) in acetic acid–formic acid buffer at pH 2.0. No detectable impurity was observed. Unlabelled amino acids were obtained from Mann Research Laboratories, New York or Nutritional Biochemical Corporation, Cleveland, Ohio.

Calculations

Amino acid uptake is expressed as the isotope distribution ratio (conc. in intracellular fluid: conc. in extracellular fluid). A chemical distribution ratio was also calculated by correcting for the conversion of soluble label in the slice to forms other than the initial labelled substrate. Ratios and rates of uptake per incubation time were also corrected in the appropriate situation for loss of ^{14}C from the slice as $^{14}CO_2$. Total entry of substrate into the slice was expressed as the sum of counts/min in the water extract of the boiled slice *plus* counts/min in $^{14}CO_2$. Methods for calculating K_m and v_{max} for multiple transport systems operating simultaneously have been described previously[1,17].

RESULTS

Concentration of amino acids in plasma, urine and kidney in vivo

Rat pups excrete more proline, hydroxyproline and glycine in their urine than the mature animal (Fig. 1). Iminoaciduria diminishes after the first week of life, while the decline in glycinuria is delayed until the third postnatal week. The changes

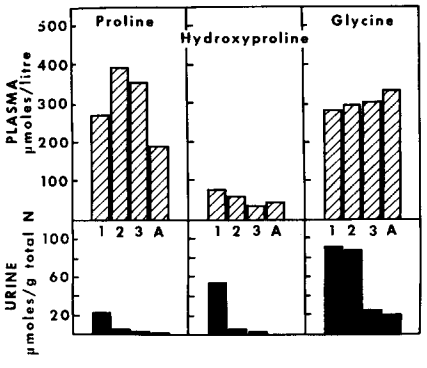

Fig. 1. Amino acid concentrations in plasma and urine of unweaned Long–Evans rat pups at 1, 2 and 3 weeks after birth and at maturity (150 g). Data are the mean of 20 rats, from at least four litters of different dams.

TABLE I

AMINO ACID CONTENT OF RAT KIDNEY DURING ONTOGENY

ECF, extracellular fluid; ICF, intracellular fluid.

Age of animal	*Total kidney wt. (mg)*	*Tissue water; % wet wt.*			*Proline content in cortex slice* (μmoles/g wet wt.)*	*Intracellular fluid plasma ratio***	*Glycine content in cortex slice* (μmoles/g wet wt.)*	*Intracellular fluid plasma ratio***
		Total water	*ECF*	*ICF*				
1	145 ± 20	78.8	23.2	55.6	0.72	4.6	5.3	32
2	248 ± 30	76.8	26.0	50.8	0.90	3.9	6.5	42
3	380 ± 50	77.4	24.6	52.8	1.08	5.2	5.3	32
Adult	1200	76.0	25.0	51.0	0.71	6.8	4.8	27

* Mean value of 4 separate determinations, performed on homogenates of kidney cortex pooled from 4 animals in same litter.

** Derived by calculating the amino acid concentration in total tissue water and substracting amount in extracellular fluid, assuming the latter is equivalent to the plasma concentration (given in Fig. 1); the difference is the concentration in intracellular fluid. The distribution ratio is μmoles/ml intracellular fluid: μmoles/ml plasma. The same 16 animals were used to obtain data on amino acid concentration in kidney and plasma.

in urine excretion were not accompanied by a corresponding alteration in plasma amino acid concentrations. These findings imply improvement in the net tubular absorption of amino acids during postnatal maturation.

Kidney weight increases about 3-fold during the first three weeks of life (Table I) but remains about 1% of the total body weight throughout this period. The total tissue water and intracellular water fractions of kidney wet weight decline slightly with aging. The tissue content of proline and glycine is slightly higher in the pup than in the adult and is sufficient for intracellular uptake at all ages to take place, against a true chemical gradient *in vivo* (Table I).

Amino acid accumulation by kidney in vitro

The transfer of low concentrations of ^{14}C-labelled amino acid into the intracellular compartment of cortex slices is less in pups than in the adult (upper half; Fig. 2). There is no difference between young and adult kidney at high concentrations of external amino acid. Intracellular label is incorporated at a greater rate into insoluble material in the young animal compared to the adult. On the other hand the apparent rate of substrate oxidation is less in the young animal.

At low external concentrations, 15-min uptake of L-proline and glycine is less in the pup compared to the adult (Table II). At high concentrations the difference between young and mature kidney was less evident. Net uptake of α-aminoisobutyric acid was less after 15 min at both high and low external concentrations, in the pup compared to the adult.

At or near the steady-state (120 min incubation) net uptake of L-proline and glycine at low and high concentrations and of α-aminoisobutyric acid at low concentration is greater in kidney of young animals. These findings involve consideration of the influx and efflux of amino acids and are examined in more detail in the following paper[18].

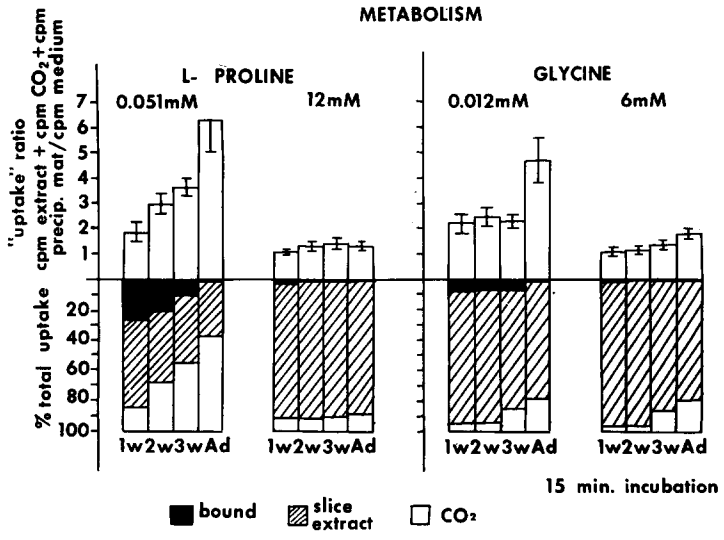

Fig. 2. Distribution of L-proline and glycine in kidney cortex slices of unweaned rat pups at 1, 2 and 3 weeks (w) after birth and at maturity (Ad). The 15-min "uptake ratio" (upper half of graph) is the sum of counts from ^{14}C in the soluble and insoluble intracellular pools and in $^{14}CO_2$ in relation to the medium counts. The relative distribution of ^{14}C between insoluble (solid) and soluble (hatched) material, and CO_2, is also shown (bottom half of the graph). Data are the mean ± 1 S.D. of at least 6 observations. Cpm = counts/min.

TABLE II

SOLUBLE ISOTOPE DISTRIBUTION RATIOS FOR L-PROLINE, GLYCINE AND α-AMINOISOBUTYRIC ACID ACHIEVED AFTER INCUBATION OF RAT KIDNEY CORTEX SLICES AT LOW AND HIGH INITIAL CONCENTRATIONS OF SUBSTRATE

Slices of kidney cortex were incubated with substrate in gassed Tris–electrolyte–glucose buffer, pH 7.4 at 37°. 15-min distribution ratios are >50% of steady-state ratios; 120-min ratios are >90% of steady-state ratio for given age.

Amino acid	*Concn. (mM)*	*Incubation time (min)*	*Age of animal*			
			1 week	*2 weeks*	*3 weeks*	*Adult*
L-Proline	0.051	15	0.95	1.38	1.60	2.23
	12.0	15	0.96	1.25	1.45	1.55
	0.051	120	4.75	4.30	3.27	2.67
	12.0	120	2.85	2.70	2.45	2.0
Glycine	0.012	15	1.96	2.17	1.85	3.63
	6.0	15	1.0	1.05	1.15	1.37
	0.027	120	13.1	13.4	11.9	8.7
	6.0	120	4.5	4.0	4.0	2.8
α-Aminoisobutyric acid	0.12	15	0.75	0.95	1.20	1.95
	20.0	15	0.37	0.52	0.57	0.75
	0.12	120	6.3	5.6	3.4	3.8
	20.0	120	1.25	1.30	1.35	1.70

Oxidation of substrate by kidney

Cortex slices from animals of increasing maturity apparently oxidize low and high concentrations of L-proline with increasing efficiency (Fig. 3). However, the pattern of oxidation is quite different in cortex homogenates (Fig. 3). The latter oxidize L-proline at similar rates in the adult and the 1-week kidney, while homogenates from 2- and 3-week-old animals oxidize L-proline with greater efficiency than at other ages. These findings imply that L-proline does not enter kidney cells efficiently across the plasma membrane at low concentration in the postnatal period.

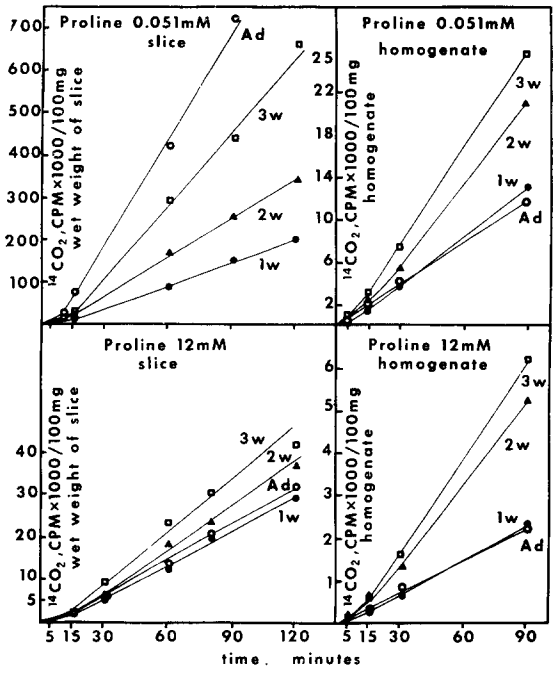

Fig. 3. Time course for the formation of $^{14}CO_2$ from L-proline by intact kidney cortex slices and by cortex homogenates. Incubations were performed in gassed pH 7.4 Tris–electrolyte–glucose buffer, at 37°. Abbreviations: see Fig. 2.

Conversion of substrate to soluble metabolites in kidney

L-Proline is converted to glutamate, ornithine and other metabolites, while glycine is metabolized to serine and glutathione by kidney cortex slices *in vitro*; the amounts converted increase with age (Table III). However, the amount of substrate retained in the initial form under steady-state conditions is sufficient to yield an *in vitro* chemical distribution ratio greater than 1.0 at all ages. This evidence for concentrative uptake *in vitro* in newborn kidney was confirmed with non-metabolizable α-aminoisobutyric acid.

Incorporation of substrate in kidney

The fraction of substrate incorporated into insoluble material is greater in the young animal than in the adult (Fig. 2). The intracellular ^{14}C label derived from L-proline and found in the insoluble fraction after 15 min incubation is 27% at one

TABLE III

EVIDENCE FOR CONCENTRATIVE UPTAKE OF METABOLIZABLE SUBSTRATES BY RAT KIDNEY CORTEX SLICES *in vitro*

Incubations were performed as described for Table II and under METHODS. Duration was 90 min. Amount of isotope remaining as substrate after *in vitro* incubation was determined by electrophoretic separation and isotope scanning (see METHODS). Chemical distribution ratio obtained by multiplying isotopic distribution ratio by the percent of isotope remaining as original substrate.

Amino acid	*Concn. (mM)*	*Age of animal (weeks)*	*Isotope distribution ratio*	*% Soluble label remaining as initial substrate*	*Chemical distribution ratio*
L-Proline	0.051	1	3.9	73	2.8
		2	3.3	66	2.2
		3	2.6	54	1.4
		Adult	2.8	41	1.15
	12.0	1	2.7	92	2.5
		2	2.9	81	2.3
		3	2.8	80	2.2
		Adult	2.0	52	1.04
Glycine	0.027	1	9.2	66	6.1
		2	9.3	76	7.1
		3	7.6	58	4.4
		Adult	7.5	72	5.4
	6.0	2	3.2	96	3.1
		Adult	2.7	85	2.4

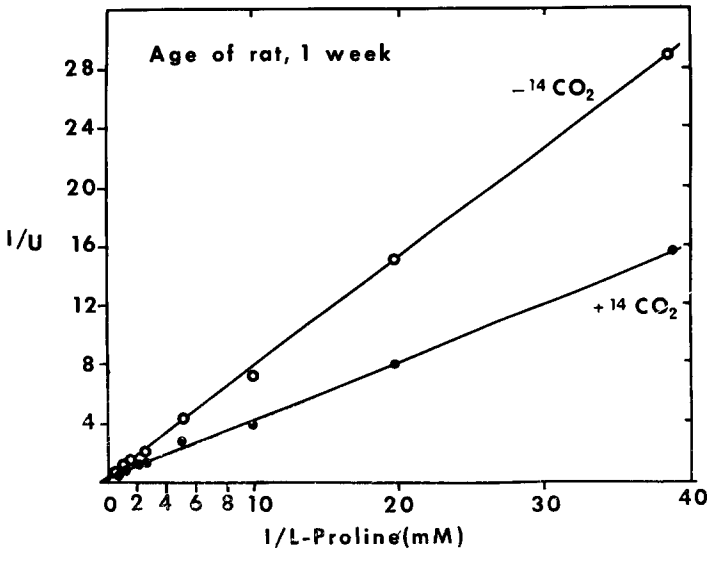

Fig. 4. Lineweaver–Burk plot ($1/u$ *vs.* $1/[S]$) of proline uptake (mmoles/ml intracellular fluid per 120 min) by kidney cortex slices from 1-week-old animals. Concentration-dependent uptake is depicted when loss of label from the slice as $^{14}CO_2$ is considered (bottom) or ignored (top). Estimate of the K_m is not influenced by oxidation in newborn kidney, and only one mode of uptake is apparent.

week of age but less than 2% in mature kidney. The amount of glycine incorporation is always less relative to L-proline, but the age-dependent relationship is similar. Labelled α-aminoisobutyric acid did not appear in the insoluble material, indicating that "incorporation" is not an artefact of absorption or nonspecific binding.

Influence of substrate metabolism on concentration-dependent uptake

Oxidation of substrate has no influence on measurement of the Michaelis constant for L-proline uptake by newborn kidney *in vitro* (Fig. 4). The same is true in adult kidney[1]. Only one mode of uptake is observed in newborn kidney over the concentration range, 0.026–2.5 mM, in contrast to adult tissue where more than one type of accumulation is apparent[1].

pH optimum for transport

Uptakes of L-proline, and α-aminoisobutyric acid at low concentrations, and intracellular oxidation of the former, are maximal between pH 7.4 and pH 8.2 in young and mature kidney (Fig. 5). Oxidation and uptake decrease at about pH 6.8 for reasons not known to us.

Temperature sensitivity

Net uptake of α-aminoisobutyric acid and L-proline is stimulated less by elevation of temperature from 20–37° in the adult compared to the one week-old animal (Table IV). This response may reflect greater efflux in adult kidney at 37° (see following paper, ref. 18).

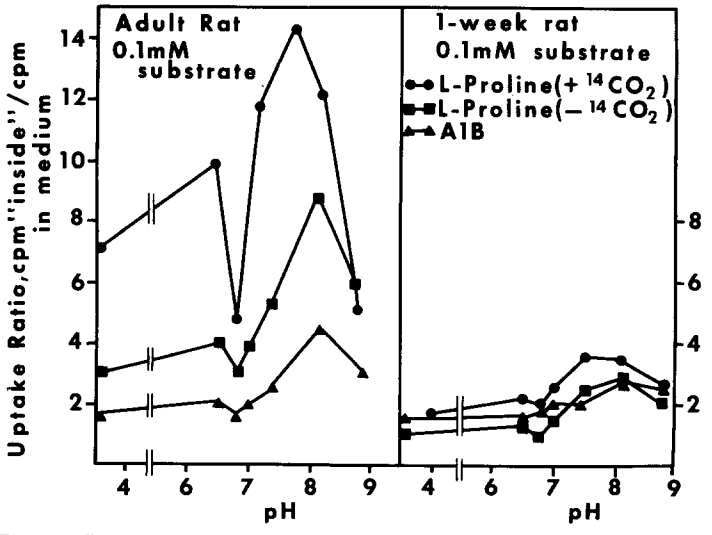

Fig. 5. Effect of pH upon 15-min uptake of L-proline and α-aminoisobutyric acid (AIB) by newborn and adult kidney. The substrate concentration chosen assigns at least 60% of L-proline uptake to its low-K_m uptake when present[1]. The pH preference of L-proline transport is the same in newborn and adult kidney slices. Uptake of 0.1 mM α-aminoisobutyric acid is divided equally between two systems in adult kidney[17]. α-Aminoisobutyric acid transport also has the same pH preference in newborn and adult kidney slices. The difference in net uptake by newborn and adult kidney at the optimal pH range (pH 7.4 to pH 8.1) reflect a deficiency of low-K_m proline transport and high-K_m α-aminoisobutyric acid transport in the newborn. The explanation for decreased uptake at pH 6.8 is unknown. Cpm = counts/min.

TABLE IV

EFFECT OF TEMPERATURE UPON ISOTOPE DISTRIBUTION RATIO IN RAT KIDNEY CORTEX SLICES FROM ANIMALS OF DIFFERENT AGES

In adult kidney the uptake of substrate is distributed between "low-K_m" and "high-K_m" systems, respectively, at the concentrations given as follows: 50:50 (0.1 mM α-aminoisobutyric acid); 27:73 (8 mM α-aminoisobutyric acid)[17]; 60:40 (0.1 mM L-proline) and 11:89 (4 mM L-proline)[1].

Substrate	*Concn. (mM)*	*Age of animal*	*Distribution ratio***		
			20°	*37°*	*37°/20°*
α-Aminoisobutyric acid	0.1	1 week	0.2	1.3	6.5
	0.1	Adult	1.2	3.7	3.1
	8.0	1 week	0.15	0.9	6.0
	8.0	Adult	1.0	1.7	1.7
L-Proline ($+^{14}CO_2$)*	0.1	1 week	2.2	3.9	1.8
	0.1	Adult	5.4	6.0	1.1
	4.0	1 week	1.2	1.9	1.6
	4.0	Adult	3.8	3.3	0.94
L-Proline ($-^{14}CO_2$)*	0.1	1 week	2.0	2.6	1.3
	0.1	Adult	2.9	2.7	0.94
	4.0	1 week	1.2	1.4	1.17
	4.0	Adult	2.6	1.9	0.73

* Indicates whether or not counts appearing in CO_2 after uptake were included in calculation of uptake ratio.

** Refers to ratio of soluble counts/min (including $^{14}CO_2$ counts) per ml intracellular fluid: counts/min per ml initial medium. Incubation for 30 min in gassed Tris–electrolyte–glucose buffer, pH 7.4, at specified temperature.

DISCUSSION

Functional maturation of the nephron involves an increase in the volume and mass of the tubule relative to the glomerulus, particularly in its proximal convolutions, and a corresponding increase in the surface area of the luminal border of epithelial cells, through development of the brush border[19–22]. What has not been made clear, is whether ontogeny of transport also involves any change in specific membrane activity, as well as the apparent change in total membrane activity. The transport systems for the iminoacids and glycine reveal some important aspects of transport ontogeny in kidney.

WEBBER[23] was the first to report that 2-week-old Wistar rat pups excrete more amino acids relative to their plasma level than rats at 4, 8 and 12 weeks after birth. We confirm this in the present study of Long–Evans rat pups. In addition, we observed an important dichotomy in the disappearance of the iminoacids and of glycine from rat urine in the first three weeks after birth. Since the plasma levels of these substances did not change significantly with age, the prior disappearance of iminoacids from rat urine at one week of age suggests that a specific membrane activity for proline reabsorption *in vivo* may appear before a corresponding activity emerges for glycine absorption at three weeks of age. SEGAL's group[24] found hyperaminoaciduria

in the postnatal Sprague–Dawley rat, but the age at which this finding disappeared was not investigated. We have observed that the iminoaciduria of human infants disappears at three months while their hyperglycinuria persists for 6 months after birth.

The gradient for proline uptake at physiological concentrations (about 0.2 mM) *in vivo* is slightly greater than that achieved by the slice under steady-state conditions *in vitro*, while glycine uptake *in vivo* occurs against a much higher gradient than *in vitro*. Since water spaces in kidney are for all practical purposes nearly identical *in vivo* and *in vitro* (F. CHINARD, personal communication, 1971), these differences are not an artefact of distribution spaces. The discrepancy between uptake ratios observed *in vivo* and *in vitro*, and between glycine and proline accumulation *in vivo* can be explained if mammalian kidney extracts these amino acids from renal arterial plasma *in vivo*. This has been shown to be the case[25,26], moreover more glycine than proline is extracted from plasma. These findings indicate that initial uptake *in vivo* at low concentrations of proline and glycine across the luminal border of renal tubular cells will occur against a chemical gradient and that exit into plasma across the antiluminal border will be down a gradient.

WEBBER AND CAIRNS[27], and our group, found short-term amino acid uptake ratios to be lower in newborn kidney of Wistar and Long–Evans rats respectively. SEGAL[24] did not observe this in the Sprague–Dawley strain. On the other hand, steady-state uptake ratios are greater in the newborn than in adult kidney in all three strains. Reduced efflux could account for this finding[18,24,28].

The initial entry of proline at low external concentration is impaired in newborn slices, relative to uptake at high concentrations. The major portion of proline uptake at the higher concentrations in mature kidney occurs on a high-K_m system[1,4,5], and uptake, at and below physiological concentrations, normally occurs on a low-K_m system. Therefore absence of the latter system could account for the pattern of proline transport in the newborn both *in vivo* and *in vitro*. A similar interpretation concerning low-K_m and high-K_m transport systems for glycine[1,3] can account for the postnatal hyperglycinuria *in vivo* and its impaired uptake at low concentrations *in vitro*.

There was also a difference between newborn and adult kidney in their respective ability to accumulate α-aminoisobutyric acid. In this case, uptake was more avid at low concentrations than at high concentrations. Again newborn kidney retained α-aminoisobutyric acid more effectively under steady-state conditions. A high-K_m mediation which serves α-aminoisobutyric acid uptake in adult kidney[17] may be less active in newborn kidney.

Increased protein biosynthesis is required for growth during early postnatal life, and as expected, incorporation of L-proline and glycine into insoluble material, presumably protein, is more active in newborn rat kidney than in the adult. The higher steady-state uptake ratio during this age period relative to the adult, appears to favor retention of an adequate free intracellular amino acid pool, even though the initial influx rates which provide substrate are relatively poor in the newborn.

The dependence of amino acid net uptake on temperature and pH is not different in newborn and adult kidney. If low-K_m transport systems for proline and glycine are lacking in one-week kidney, the present findings indicate that the high and low-K_m transport systems are not different in their pH responsiveness. Temperature elevation does not augment the distribution ratio in adult tissue as effectively as in newborn kidney at high and low concentrations of substrate. If amino acid efflux is

temperature sensitive as it is for cysteine[29], the effect of temperature in our study can be explained.

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