Biochemical and morphological changes in the developing kidney.

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Original Paper

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Biochemical and Morphological Changes in the Developing Kidney

Abstract

We have studied microsomal phospholipid, cholesterol and protein concentration in rat renal papilla, medulla and cortex during postnatal development, and the relationship between these membrane biochemical parameters and morphological changes. We also determined DNA concentration in each kidney zone. No changes were observed either in papillary microsomal phospholipids, proteins and cholesterol or in DNA concentration from 10- to 70-day-old rats. Medullary microsomal proteins and cholesterol did not change but a significant increase was observed in the microsomal phospholipid concentration during development; in this case, medullary DNA was significantly lower at 70 than at 10 days. In contrast, all biochemical parameters in renal cortex were significantly higher during development except for DNA concentration which suffered a great decrease. These biochemical findings agree with the morphological changes observed. Our results demonstrate that the developmental pattern is different in each zone of the kidney and confirm the fact that the papilla, in newborn rats, is almost fully developed whereas the renal cortex and medulla are immature.

Key Words
Development
Kidney
Membranes

Introduction

During nephrogenesis, morphological and functional differentiation of nephrons happens centrifugally. Thus, in the developing kidney, those nephrons located in the outermost region of the cortex are the least differentiated morphologically [1]. In contrast, nephrons located in the inner cortex have a vascular pole and a greater proportion of

them is blood perfused [2]. During fetal and newborn life, kidney growth is accomplished by cell proliferation [3]. After birth, nephrogenesis declines and the kidney medulla expands as collecting ducts and loops of Henle lengthen [4].

It has been considered that, at the end of the first week of life, rat nephrogenesis is completed [5]. However, at this stage the kidney does not function as efficiently as in adult life and the maturation degree of the kidney zones is very dissimilar [6]. It has been reported that high protein intake during development induces acceleration of the renal cortical growth by cell proliferation and hypertrophy [7]. Additionally, increase in size of tubular cells followed by greater cell division has been also reported in compensatory renal growth after nephrectomy [8]. However, no studies have been performed on kidney growth and maturation under physiological conditions.

Kidney growth is accomplished by cell proliferation and it is clear that the formation of new cells must be accompanied by that of new membranes. Moreover, membrane generation could be necessary for the cellular differentiation required to ensure full functioning of kidney cells. The morphological construction of the cells requires the presence of the biochemical entities from which they are made.

Based on the above, the aim of this study is to determine how the different kidney zones develop under nonstimulated conditions and how the biochemical entities involved in cell membrane structure accompany the histological kidney maturation. Since eukaryotic cell membranes are made by a phospholipid bilayer, proteins and cholesterol [9], we determined their concentration as a biochemical parameter of new membrane formation and its relationship with cell number expressed as DNA concentration. These determinations have been correlated with histological morphometric analysis and mitotic index determination.

Materials and Methods

Silica gel G plates and standard phospholipids were obtained from Sigma (St. Louis, Mo., USA). All reagents were pure quality compounds.

Animals and Sample Preparations

Wistar rats of 10, 20 and 30 postnatal days, with an average weight of 20, 30 and 70 g respectively, were used. After parturition, litter size was reduced to 8 pups/mother to ensure uniform nutrition. Adult rats of 70 days were used as controls. The animals were decapitated and both kidneys were removed and placed in ice-cold Krebs solution containing 5.5 mmol/l glucose. Each kidney was cut in half through the pelvis along its longitudinal axis, and the papilla, inner and outer medulla and cortex were dissected. For each experiment, tissue from 8 rats was pooled.

Methods

For each experiment, 300 mg of papillary, medullary and cortical tissue were collected in 3 ml of 0.25 mmol/l sucrose. Tissue samples were homogenized in glass tubes with a Teflon pestle at 3,000–3,500 rpm. Microsomes were obtained by the method of Low and Zilversmit [10]. The homogenate was centrifuged at 9,000 rpm in Sorvall 10-ml centrifuge tubes for 30 min. The supernatant was separated and the pellet was resuspended in 3 ml of sucrose at 4°C and centrifuged under the same conditions. The pellet was discarded. This supernatant was then pooled with that previously obtained and both centrifuged at 38,500 rpm for 60 min in a Beckman Ti 40 rotor. The supernatant was discarded and the pellet resuspended in 300 ml distilled water.

Protein Determination

Protein determination was carried out by the method of Lowry et al. [11]. Aliquots of the membrane suspension (10 µl) were hydrolyzed with 0.5 M NaOH for 15 min and then 1 ml of fresh copper tartrate was added. After 10 min, 100 µl of Folin reactive was added to each sample and quickly mixed. The color developed in each sample was measured at 750 nm and protein concentration was determined using bovine albumin as standard. The results are expressed as µg/mg dry weight tissue.

Cholesterol Determination

An aliquot of 500 µl of aqueous suspension was extracted by the method of Bligh and Dyer [12]. The organic phase was evaporated at 60°C. The residue was used for the cholesterol quantitation by the method of Searcy and Bergquist [13], which uses as color-reactive a saturate solution of ferric sulfate in acetic acid. Pure cholesterol was dissolved in chloroform and used as standard. Results are expressed as µg/mg dry weight tissue.

Phospholipid Determination

An aliquot of the aqueous suspension of microsomes, equivalent to 20 mg of tissue, was extracted as per Bligh and Dyer [12]. The organic phase was removed and dried under a stream of N_2. The extracts were redissolved in chloroform, applied onto Silica gel G TLC plates (0.25 mm thick) and submitted to a one-dimensional thin-layer chromatography. The solvent system used was the upper phase of ethylacetate: 2,2,4-trimethylpentane:acetic acid:water (90:50:20:100 v/v) [14]. Migration of the lipids was detected by iodine vapors. To quantify the phospholipids, the corresponding zone of the thin-layer chromatographic plates was scraped off and the inorganic phosphorous was determined by a modification of the method of Fiske-Subbarow with the previous mineralization [15]. Areas of silica gel were used as blank values for all samples. The results are expressed as µg/mg dry weight tissue.

DNA Determination

For each experiment, 20 mg of papillary, medullary or cortical slices were collected in 0.5 ml of HCl-Tris buffer, pH 7.4. After homogenization, an aliquot was separated for protein determination. DNA separation began when 0.5 ml of perchloric acid (0.4 N) was added to get the final concentration of 0.2 N of perchloric acid. After centrifugation at 2,400 rpm at 4°C for 10 min, the pellet was washed twice with 1 ml perchloric acid (0.2 N) and then resuspended in 0.5 N perchloric acid and incubated at 70°C during 20 min in order to hydrolyze the DNA. After centrifugation at 2,400 rpm at 4°C for 10 min, the supernatant was utilized for DNA quantitation. An aliquot of 500 µl was assayed by the method of Burton [16]. Results are expressed as µg/mg dry weight tissue or by total kidney zone. All results are expressed per dry weight so as to get values that do not depend on the hydration degree of the tissue.

Statistical Analysis

All values are means ± SEM. Significant differences between means were assessed by two-way analysis of variance (ANOVA) and subsequent Tukey test. Differences were considered statistically significant when p values were < 0.05.

Morphological Studies

For light microscopy, hemilongitudinal blocks of both kidneys were fixed overnight in an absolute alcohol-formol-acetic acid fixative solution. Paraffin sections (5 µm) were stained with the periodic acid-Schiff technique [17].

Mitotic Index

The mitotic index from glomeruli, tubules and interstitial cells was expressed as the ratio between the number of mitoses and the high-power fields evaluated ($\times$ 400).

Density of Glomeruli, Tubules and Interstitial Tissue Compartments

The morphometric analysis was performed as follows: 18 nonconsecutive photographs from cortical, medullary (external zone) and papillary regions, at a final magnification of 312.5, were evaluated using a multipurpose test system grid (M 42), according to the principles and applications of point counting discussed by Weibel et al. [18]. The densities of the glomerular, tubular and interstitial tissue compartments were determined as follows: (a) Density of the glomerular compartment (DGC) = number of the intersecting lines falling on all glomerular profiles/total intersecting lines. (b) Density of the tubular compartment (DTC) = number of the intersecting lines falling on all tubular profiles/total intersecting lines. (c) Percentage of proximal convoluted tubules (% PCT) = number of intersecting lines falling on all proximal convoluted tubule profiles/number of the lines falling on all tubule profiles $\times$ 100. (d) Density of the interstitial tissue compartment (DITC) = number of the intersecting lines falling on interstitial tissue/total intersecting.

Results

Microsomal Protein Content

The microsomal protein concentration differed in the various kidney zones at an early age (fig. 1). The lowest value corresponded to cortex, the amount in papilla was the highest, and the medulla value was in between. During development, no changes were observed in
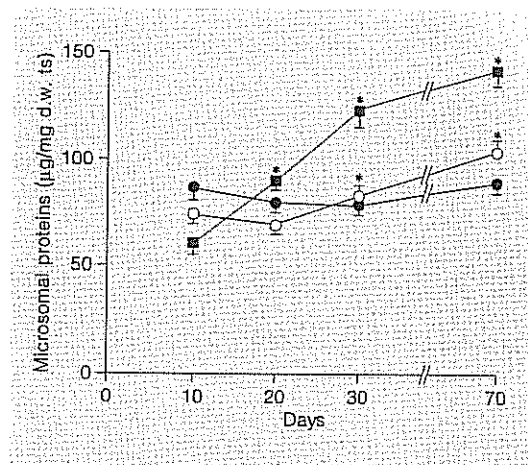
papillary microsomal protein concentration. Medullary concentration did not change from 10 to 20 days. Thereafter, protein concentration increased from 20 days, rising 17% (p < 0.05) and 35% (p < 0.05) after 30 and 70 days respectively. Cortical microsomal protein concentration rose linearly from 10 to 30 days, reaching a 39 and 92% increase at 20 and 30 days respectively. Differences between 10–20 and 20–30 days were statistically significant (p < 0.05). Microsomal protein accumulation reached maximum at 30 days since the value in adulthood did not differ significantly. In adult life, microsomal protein concentration presented a gradient from the cortex to the papilla. Cortical concentration was 35% (p < 0.05) higher and papillary concentration 8% (p < 0.05) lower than medullary protein concentration.

Fig. 1. Microsomal protein content from renal papilla (●), medulla (O) and cortex (■) as a function of age. Each point represents the mean ± SEM of 5 samples. * p < 0.05 vs. the value at 10 days (ANOVA).

Microsomal Cholesterol Content

At the age of 10 days, cortical microsomes were the poorest in cholesterol content. No change was observed until 20 days. Thereafter, a linear increase of 50 and 100% occurred at 30 and 70 days respectively compared to the 10th postnatal day, becoming the renal tissue richest in microsomal cholesterol content. Papillary and medullary microsomal cholesterol concentration was the same at an early age and remained unchanged during development (fig. 2).

Microsomal Phospholipid Content

At 10 days of age, the microsomal phospholipid content (expressed as μg/mg dry weight tissue) did not differ significantly as regards papilla, medulla and cortex (fig. 3). During development, papillary phospholipid concentration remained constant. The medullary concentration rose by 32% at 20 days and then remained constant up to adult life. The evolution of the cortical concentration differed from those of the medulla. From 10 to

20 days, at the time when the medullary changes were evident, cortical concentration did not change. Afterwards, a linear increase of 42% (30 days) and 82% (70 days) was observed. In adulthood, the kidney shows a clear gradient in the microsomal phospholipid concentration with significant phospholipid enrichment of the cortical zone, followed by the medulla and the papilla.

DNA Content

In order to determine if kidney growth was accompanied by changes in the number of cells per unit of mass, DNA content per milligram of dry weight tissue was determined (table 1). No difference was observed in renal papilla between 10 and 70 days. Medullary DNA per unit of tissue mass began to decrease as from 20 days of age. The DNA concentration at 20 days was 20% lower than at 10 days. Such cellular dilution persisted up to adult life. Results obtained from the cortex denote a greater difference than those from the medulla. Cortical
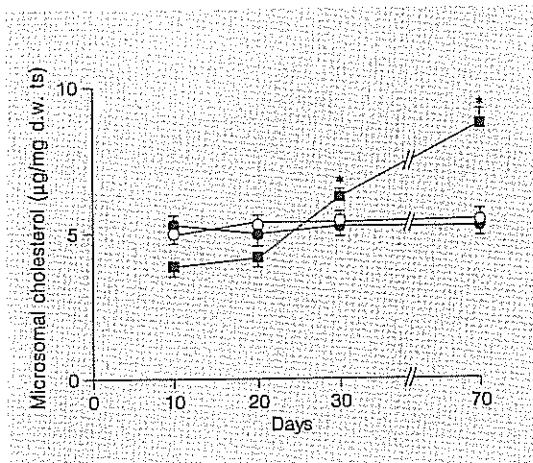
Fig. 2. Microsomal cholesterol content from renal papilla (●), medulla (○) and cortex (■) as a function of age. Each point represents the mean ± SEM of 5 samples. * p < 0.05 vs. the value at 10 days (ANOVA).
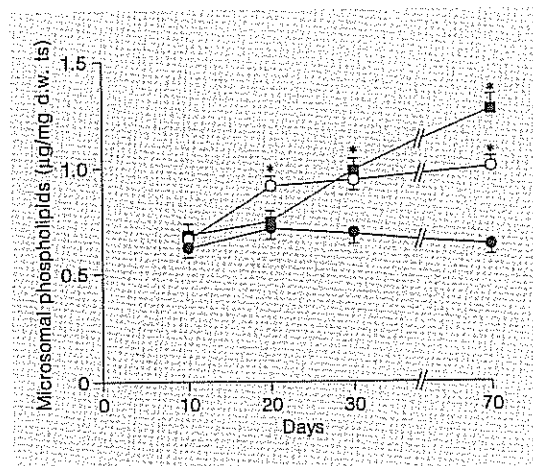
Fig. 3. Microsomal phospholipid content from renal papilla (●), medulla (○) and cortex (■) as a function of age. Each point represents the mean ± SEM of 5 samples. * p < 0.05 vs. the value at 10 days (ANOVA).

Table 1. DNA content and tissue mass in different kidney zones from 10- and 70-day-old rats: results are expressed as mean SEM of 5 samples (DNA/mass was calculated as follows: DNA = DNA_x/DNA_{10d} and mass = $mass_x/mass_{10d}$; x = 20, 30, 70 days)

Zone	Age, days	DNA content		Tissue mass	DNA/mass
		µg/mg d.w.	µg/zone	mg	
Papilla	10	27.0±0.2	18.9	0.70±0.01	–
	20	26.9±2.3	134.5	5.00±0.08	1.00
	30	26.8±2.1	168.8	6.00±0.07	0.95
	70	26.5±2.0	198.7	7.50±0.10	1.00
Medulla	10	36.0±2.3	115.2	3.20±0.09	–
	20	29.2±2.1*	356.2	12.2±0.10	0.81
	30	29.3±2.0*	588.9	20.1±0.20	0.74
	70	29.1±2.7*	646.0	22.2±0.20	0.81
Cortex	10	26.3±3.1	999.4	38.0±1.0	–
	20	22.2±2.5	3,199.8	144.4±5.5	0.84
	30	15.3±2.0*	4,284.0	280.0±8.3	0.57
	70	13.5±2.1	4,212.0	312.0±10.1	0.51

* Significantly different (p < 0.05) than the value at 10 days.

DNA concentration began to drop at 20 days, but became significantly lower at 30 days (42%), in adult life being half of the 10-day value. In order to get an explanation for these results, we determined the mass evolution of the different zones of the kidney, and the relationship between mass and total DNA increment at each development stage was then established (DNA/mass).

As shown in table 1, in the papilla, the ratio was approximately 1.0 thus reflecting that mass growth was accompanied by an increase in cell number. In the medulla, the ratio from 20 to 70 days was 0.8, which means that a moderate dilution of cells occurred during maturation – mainly in the 10- to 20-day-old period. Cortical maturation denoted the greatest cell dilution since the discrepancy between DNA content and mass growth was more marked as development occurred reaching a ratio of 0.5 in adulthood.

Relationship between the Membrane Biochemical Entities

In table 2, protein/phospholipid and cholesterol/phospholipid ratios from 10- to 70-day-old rats are shown. In renal papilla, no differences were observed in values obtained. The lower values obtained at 20 and 30 days in the protein/DNA and protein/phospholipid ratios were mainly a consequence of the nonsignificant decrease in the protein content. In renal medulla, the protein/DNA ratio – which reflects cellular size [7] – fluctuated from 10 to 30 days of age but then a 69% increase was observed in comparison with that of 10 days of age ($p < 0.05$). The protein/phospholipid ratio remained unchanged from 10 to 70 days despite the transient decrease in the ratio during the intermediate period. On the other hand, a 25% decrease ($p < 0.05$) in the cholesterol/phospholipid ratio was observed from childhood to adult life due to the phospholipid increase not accompanied by a

Table 2. Protein/DNA (Pr/DNA), protein/phospholipid (Pr/Phl) and cholesterol/phospholipid (Cho/Phl) ratios in renal papilla, medulla and cortex from 10- and 70-day-old rats

Zone	Age, days	Pr/DNA	Pr/Phl	Cho/Phl
Papilla	10	3.50	148.0	8.33
	20	2.95	110.0	7.81
	30	2.96	110.0	8.13
	70	3.58	146.0	8.13
Medulla	10	2.59	109.8	7.35
	20	2.40	76.5*	5.78*
	30	2.77	86.6*	5.58*
	70	3.51*	103.0	5.49*
Cortex	10	2.42	92.7	5.91
	20	3.98*	126.2*	6.28
	30	6.69*	125.0*	6.20
	70	10.23*	110.5*	7.09*

* Significantly different ($p < 0.05$) than value at 10 days.

rise in the cholesterol content. The renal cortical zone showed a gradual increase in its protein/DNA ratio. In adulthood, the value was 4.1-fold higher than that at 10 days ($p < 0.05$).

Morphometric Analysis

Results expressed as density in this morphometric analysis (table 3) reflected the volume occupied by the various compartments with no discrimination of cell number or size. At 10 days of age, most of the papillary density was occupied by the tubular compartment since the density of the interstitial compartment was low. From the tubular compartment, most were collecting tubules and few were thin limb tubules. From the very immature stage to 70 days of age, collecting tubules were the major structures within the tubular compartment. The interstitial compartment

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Table 3. Density of glomeruli, tubules and interstitial compartments in renal papilla, medulla and cortex from 10- and 70-day-old rats: results are expressed as mean ± SEM of 5 experiments

Zone	Age, days	DGC	DTC	PCT, %	DITC
Papilla	10	–	0.95±0.09	0	0.050±0.001
	20	–	0.94±0.07	0	0.055±0.007
	30	–	0.86±0.02	0	0.127±0.003*
	70	–	0.83±0.01	0	0.160±0.010*
Medulla	10	–	0.40±0.01	15	0.60±0.03
	20	–	0.57±0.02	60	0.30±0.07*
	30	–	0.59±0.02	68	0.13±0.06*
	70	–	0.61±0.03	70	0.14±0.01*
Cortex	10	0.10±0.01	0.62±0.02	40	0.13±0.01
	20	0.11±0.01	0.60±0.02	42	0.11±0.01
	30	0.12±0.01	0.53±0.01	58	0.07±0.01*
	70	0.14±0.03	0.48±0.03	60	0.05±0.01*

DGC = Density of the glomerular compartment; DTC = density of the tubular compartment; PCT = proximal convoluted tubules; DITC = density of the interstitial tissue compartment.

* Significantly different (p < 0.05) than value at 10 days.

acquired significance at 30 days in comparison to that at 10 days.

In renal medulla, the density of the tubular compartment rose from 0.40 (10 days) to 0.60 (70 days). The greatest change occurred between 10 and 20 days. Thereafter, the rise was gradual, and changes between the different maturation stages were not statistically significant. Within this compartment, a significant increase in proximal convoluted tubules was observed. At 20 days, the volume occupied by convoluted tubules was 4-fold that at 10 days, then the differentiation rate diminished accounting for 70% at 70 days. In contrast, the density of the interstitial tissue compartment was significantly higher at 10 than at 70 days of age (0.60 vs. 0.14). Changes between 10–20 and 20–30 days were also significant. However, it appears that these developmental changes were already complete at 30 days.

In the renal cortical zone, the total density was composed of glomerular, tubular and interstitial compartments. The density of the glomerular compartment was not significantly different between 10 and 70 days while the density of the interstitial compartment gradually decreased from 0.13 to 0.05, from 10 to 70 days respectively. In the intermediate development period, changes were significant as from 30 days (with reference to those at 10 and 20 days) with no differences in the 30- to 70-day period. Density of the tubular compartment suffered a slight decrease during development; nevertheless, the percentage of proximal convoluted tubules increased, which denotes a tubular cell differentiation increase (40–60%). Differentiation appeared not to be significant during the first stage (10–20 days) but, after 20 days, maturation became evident.

Table 4. Mitotic index in different zones of kidney from 10- and 70-day-old rats: results are expressed as mean ± SEM of 5 samples

Age days	Papilla T	Medulla		Cortex		
		T	IC	G	T	IC
10	0.7±0.1	4.4±0.1	0.7±0.4	0.2±0.1	2.20±0.40	0.4±0.1
20	0	1.1±0.1*	0	0	0.83±0.11*	0
30	0	0.3±0.1*	0	0	0.68±0.15*	0
70	0	0	0	0	0	0

G = Glomeruli; T = tubules; IC = interstitial cells.
* Significantly different (p < 0.05) than value at 10 days.

Mitotic Index

The different cell types showed also a different mitotic index in each kidney zone (table 4). Mitosis of the various cell types was active in the tissue from 10-day-old rats but not in that from adults. Tubular papillary cells stopped dividing at a very early stage while medullary and cortical tubular cells still showed mitosis at 30 days; however, mitosis dropped dramatically at 20 days of age. Interstitial cells also stopped dividing early since no mitosis was observed in any zone at 20 days of age. Tubular cells were the most active since, in the cortical zone, the mitotic index for this cell type was 11 and 5.5 higher than that for glomerular and interstitial cells. The same thing happened in the medulla where tubular cell mitosis was 4.3-fold higher than that of interstitial cells.

At the 10-day development stage, the medulla was the kidney zone that showed the most active tubular cell mitotic activity, twice that of the cortex and 6.3-fold that of the papilla. However, the medullar mitotic activity loss was faster than that of the cortex.

The cortical tubular mitotic index conserved 30% of its activity at 30 days of age since the remaining mitosis in the medulla was 7%. Papillary cells stopped dividing very early since no mitosis was observed at 20 days of age.

Microscopic Findings

No differences were observed in the microscopic analysis of papillary tissue between 10 and 70 days of age (not shown). On the other hand, the medullary microscopic analysis (fig. 4a, b) shows differences: tubular cells from 10 days are smaller and less differentiated than those from 70-day-old rats. Few brush border membranes are observed at 10 days while a considerable increase can be seen at 70 days. Differences observed in the renal cortex are similar but more evident (fig. 5a, b).

Tissue from 10-day-old rats shows morphological evidence of immaturity. Scanty glomeruli are seen as solid, round avascular masses of density, packed with epithelial cells, with no clefts as evidence of Bowman's space. Tubular cells are not differentiated and very few brush border membranes are observed on the luminal surface of the tubules. At 10 days of postnatal life, tubular cells seem to be smaller and more numerous than in 70-day-old rats. At 70 days, the cortex reaches a com-
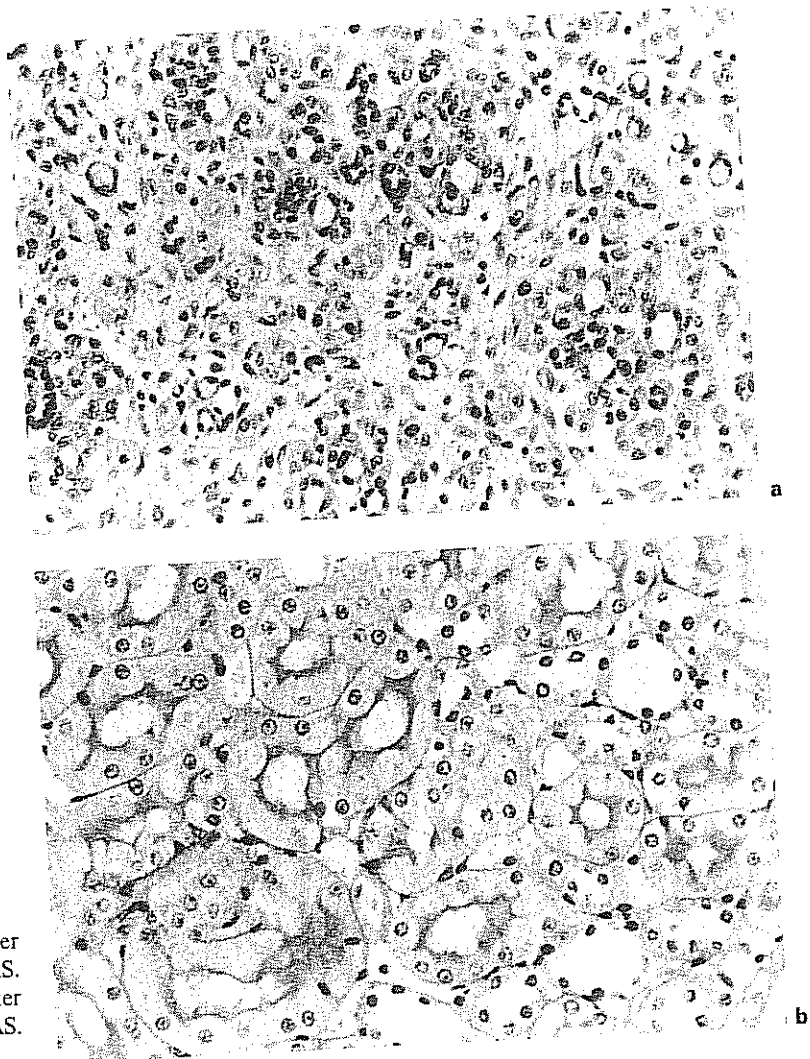
Fig. 4. a Renal medulla (outer zone) from 10-day-old rats. PAS. × 312.5. **b** Renal medulla (outer zone) from 70-day-old rats. PAS. × 312.5.

plete maturity degree. Glomeruli show a complex capillary tuft subdivided into lobules projected into capsular space. The brush border is seen as evidence of differentiated proximal convoluted tubules. The juxtaglomerular apparatus is evident in a great number of glomeruli.

Discussion

This paper clearly indicates that growth of the postnatal kidney is different in the various zones of the organ. Although it has been considered that nephrogenesis is completed in the rat at 10 days of age [5], mitotic activity at this age is present in the three zones of the kidney. Such mitotic activity must account for the
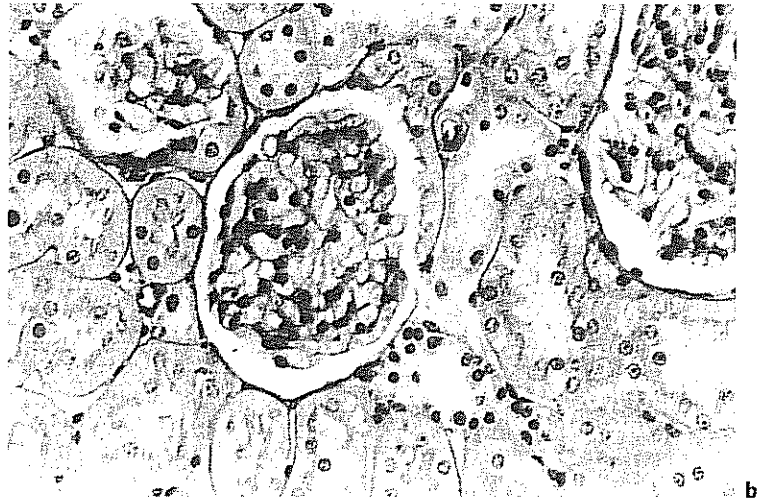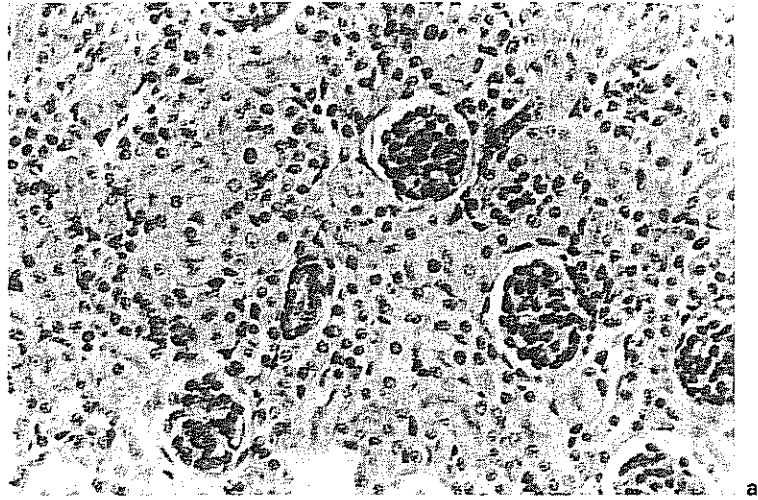
Fig. 5 a Renal cortex from 10-day-old rats. PAS. × 312.5. **b** Renal cortex from 70-day-old rats. PAS. × 312.5.

proliferative process that occurs as growth progresses and is significantly higher in tubular cells from medulla than in those from cortex and papilla (table 3).

The increase in mass, which reflects tissue growth, is different in the various kidney zones and is not always accompanied by a parallel rise in the number of cells as is demonstrated by DNA concentration (table 1). Thus, cortical mass increases 2.0-fold the

DNA concentration and, in the medulla, the mass growth was 1.5 times the DNA increase. On the other hand, the papillary rise in mass is synchronized with the proliferative process since the mass and DNA increase are similar and DNA concentration remains constant during development. The analysis of the protein/DNA ratio is higher in adulthood than at 10 days of age, in cortex as well as in medulla, which suggests changes in cell size in these

two kidney zones but not in papilla where no changes in such ratio were observed.

This biochemical description is confirmed by the microscopic analysis that shows more and smaller cells in the cortex (fig. 4a) and medulla (fig. 5a) of 10-day-old rats than in adulthood (fig. 4b, 5b), while no microscopic changes were observed in the papilla (not shown).

It has been reported that renal growth is due to cell hypertrophy and hyperplasia as measured by the increase in DNA and the protein/DNA ratio, respectively [7]. However, such a conclusion has been reached by experiments performed on renal cortex only. Our results demonstrate that the growth pattern is different for the various kidney zones. Thus, as maturation progresses, the papillary growth is only due to hypertrophy while medullary and cortical growth occurs with hypertrophy and hyperplasia. Although both coexist in cortical and medullary growth, prevalence of the latter over the former is clear since DNA concentration decreases while the protein/DNA ratio increases.

While DNA concentration decreases in cortex and medulla (table 1), phospholipid concentration rises by 81 and 43% respectively (fig. 3). This fact constitutes an indication that new membranes are formed not only due to cell proliferation. This result is also confirmed by microscopic analysis. An increase in brush border membranes in adulthood (fig. 4b, 5b) in cortex as well as in medulla, is observed. Said, rise is reflected by a relative increase in the density occupied by convoluted over other tubular cells (table 2).

Microsomal phospholipid, cholesterol and protein relationships demonstrate that, in the papilla, the biochemical characteristics of the new membranes do not change with maturation. In contrast, medullary cells appear to be poorer in cholesterol in adult than those in newborn rats, while an enrichment in choles-

terol is observed in mature cortical cells. Changes in the cholesterol/phospholipid ratio observed in medullary and cortical microsomes may cause differences in membrane biochemical parameters as in its fluidity known to influence the membrane biological activity [19]. No changes in the relative concentration on microsomal proteins are observed in any of the cases.

The comparative study presented in this paper confirms the concept that the papilla is almost fully developed in newborn rats whereas the renal cortex and also the medulla are very immature and undifferentiated. Our results also proved that the developmental pattern is different and characteristic for each zone of the kidney while it agrees with the high functional compartmentalization of this organ. On the other hand, the membrane biochemical parameters studied in the present work correlate well with the morphologic features of the various kidney tissues from newborn and adult rats as is demonstrated by the microscopic analysis herein presented.

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