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Ontogeny of Cell Type-Specific Enzyme Reactivities in Kidney Collecting Ducts

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ABSTRACT. Highly specific antibodies against vital enzymes of the collecting ducts were used to study the appearance of cell type specific enzyme profiles in developing rat kidneys. (Na+K)-ATPase, the abundant enzyme of principal cells, could be detected early *in utero* in most collecting duct cells. However, the characteristic basolateral polarization of this enzyme did not appear until the first hours after birth. After this, the relative amount of (Na+K)-ATPase immunoreactive cells along collecting ducts decreased steadily, to reach the amount found in adult rat kidneys by the 30th postnatal day. Carbonic anhydrase immunoreactivity characteristic for intercalated cells was not detectable in fetal kidneys, but appeared soon after birth, with steadily increasing numbers of cells that were positive. Interestingly, immunoreactive band 3 glycoprotein (anion channel protein of erythrocytes) did not appear until the 5th day of life, with only a slowly increasing number of cells positive for this probe. These results, showing the sequential appearance of cell type-specific enzyme reactivities along collecting ducts, likely reflect a similar pattern of functional development of the respective main cell types. These results may provide an explanation for physiologic neonatal acidosis, as the enzyme profile associated with proton secretion was seen to appear slowly during the first weeks of life in a distinct manner. (*Pediatr Res* 22: 504–508, 1987)

Abbreviations

CA II, carbonic anhydrase
SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis
ABC, avidin-biotin-horseradish peroxidase
DAB, diamino benzidine

The so-called principal cells of the renal collecting ducts have been shown to be vitally involved in the final regulation of urinary electrolyte composition (1–3), whereas a separate population of epithelial cells, the mitochondria-rich (intercalated) cells at this site, preferentially regulate renal acid-secretion (1, 4–6). Recent studies have shown that in rat kidneys, the vital transport enzymes involved in these important functions of collecting ducts, (Na+K)-ATPase (7, 8) and carbonic anhydrase (4, 9, 10) are expressed in the adjacent cells in a specific manner (11). In addition to this remarkable functional specialization, the two main epithelial cell types show distinct morphologic features (1, 2, 12). Further structural-functional specialization of intercalated cells into type A (acidifying) and B (alkalinizing) has been proposed recently on the basis of discoveries in the intracellular polarization of proton secretion in respect to the apex or base of these cells (13, 14). However, little is known of the ontogeny of principal and intercalated cells or of the emergence of cell type-specific functions of collecting ducts, although this knowledge would provide better understanding of the unique functional features of the maturing kidney.

Herein an immunocytochemical approach using highly specific antibodies against (Na+K)-ATPase, CA II (isoenzyme II) and band 3 glycoprotein (the anion channel protein of erythrocytes) was taken to study the evolution of functional specialization of the cell types of rat kidney collecting ducts.

MATERIALS AND METHODS

Tissue samples. Three whole embryos of Sprague-Dawley rats at gestational age of 18 days, and kidney samples collected from three newborn rats at 2 and 12 h of age, and sequentially at 2, 5, 9, 15, 20, and 30 days were used. The animals were sacrificed by decapitation after ether anesthesia and immediately after preparing the kidneys (or whole 18th day embryos cut along sagittal plane) the tissues were placed for 5 h in a fixative consisting of 6% HgCl, 1% sodium acetate, and 0.1% glutaraldehyde which has been shown to provide good retention of the tissue antigenicities studied (11). After fixation, the tissues were routinely embedded in paraffin.

Antibodies. The antibodies against affinity purified CA II isoenzyme were raised in rabbits as described earlier in detail (15). The purity and specificity testing of these antibodies have been documented extensively (4, 16).

Rabbit anti-(Na+K)-ATPase antiserum used was a generous gift from Dr. G. Siegel (Department of Neurology, University of Michigan, Ann Arbor, MI) and was raised in rabbits against a mouse brain (Na+K)-ATPase catalytic unit as described earlier in detail (8). A mouse brain microsomal membrane fraction enriched in (Na+K)-ATPase was prepared according to procedures previously described (8), and from the active preparation thus obtained, the (Na+K)-ATPase catalytic unit was purified using SDS-PAGE with 8.75% separating gel. The eluted catalytic unit fraction was then used to immunize rabbits with complete Freund's adjuvant (8). Immunoblot analysis of specificity showed that the antibody obtained recognized only one 100 kD band in purified preparations of lamb kidney and duck salt gland on 8.75% PAGE. Immunoreactivity of the antibody with various mouse tissues has been characterized earlier (8).

Band 3 glycoprotein antiserum raised in rabbits was a kind gift from Dr. G. Pasternack (Department of Pathology, Johns Hopkins University, Baltimore, MD). The antigen was purified from human erythrocytes after extraction of spectrin, ankyrin, and protein 4.1 using previously described methods (17–19). The cytoplasmic domain of band 3 was further purified from high-

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salt extracted vesicles, and material thus obtained is referred to as 43K peptides. Anti 43 K antibodies were obtained by immunizing rabbits, and were further affinity purified using a 43 K-Sepharose column (20).

Staining procedure. An immunocytochemical staining procedure identical to that previously reported (11) was used. Briefly, 2–3 μm tissue sections were treated with normal rabbit serum to block nonspecific staining, followed by incubation with a primary antiserum, either anti CA II at 1:1000, antiband 3 at 25 μg/ml, or a 1:200 dilution of anti-(Na+K)-ATPase for 1 h at room temperature. After a thorough rinse, the bound antiserum was visualized using a rabbit Vectastain peroxidase ABC kit (Vector Laboratories, Burlingame, CA), including sequential exposure to biotinylated antirabbit immunoglobin and ABC complex. Sites of bound antisera were visualized by incubation in DAB substrate solution.

Immunocytochemical controls included omission of either the primary or secondary antiserum from the staining sequence, as well as substitution of the primary antiserum with nonimmune rabbit serum. These control stainings resulted in no specific tissue reactivity. However, these control sections also served as the basis to evaluate the amount of unspecific background staining. Thus,

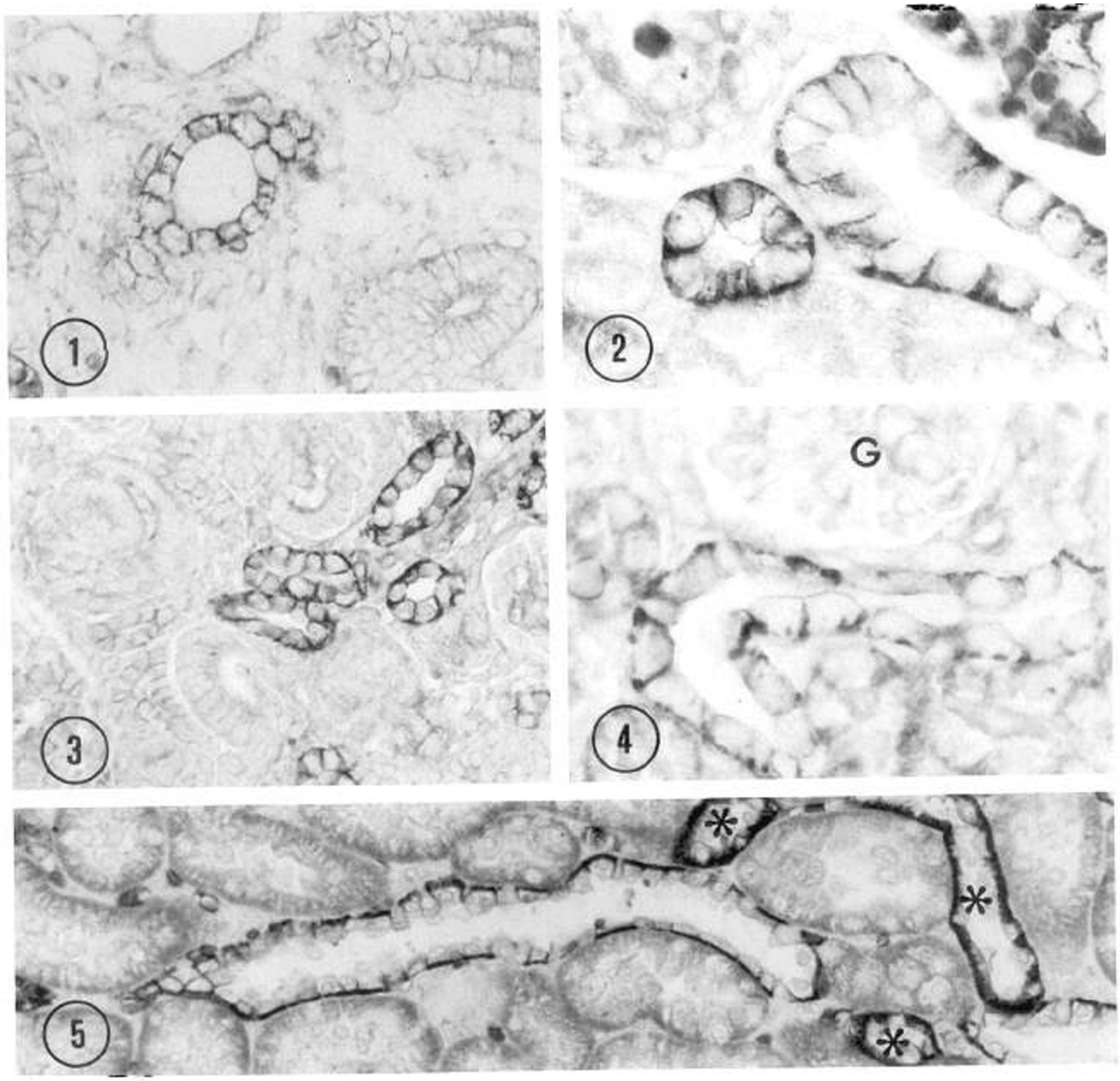

Fig. 1. Paraffin section of fetal rat kidney of 17th gestational day, immunostaining for (Na+K)-ATPase. Most collecting duct cells are positive. Note (Na+K)-ATPase immunoreactivity also in the apical aspect of cells. Original magnification ×150.

Fig. 2. In 12-h postnatal rat kidneys (Na+K)-ATPase immunoreactivity is seen mainly at the basal but also in the lateral membranes of collecting duct cells. Original magnification ×260.

Fig. 3. In 2-day-old rats (Na+K)-ATPase immunoreactivity shows considerable polarization to the basolateral aspect of collecting duct cells. Original magnification ×150.

Fig. 4. (Na+K)-ATPase immunostaining of 7th day postnatal rat kidney shows further polarization of the enzyme reactivity. Note also the presence of cells negative for this enzyme. *G*, glomerulus. Original magnification ×260.

Fig. 5. In 30-day postnatal rat kidney (Na+K)-ATPase immunostaining reveals an adult type of reactivity of cortical collecting duct cells in respect to the intracellular polarization and amount of immunoreactive cells. *Asterisks*, profiles of the loops of Henle. Original magnification ×150.

when present, specific tissue reactivity could be judged on a subjective basis as positive or negative for each cell, by comparing the specific tissue reactivity to the background staining given by the control sections.

RESULTS

Both the embryonic and postnatal rat kidneys showed good morphologic preservation and presence of a cortical zone of continuing nephrogenesis which could be identified up to 15th day of age. All the antibodies used gave a characteristic mosaic-like staining of the collecting duct cell populations (4, 11), and all the samples of similar ages gave closely similar amounts of cells that were positive. In the 30th-day samples, the staining pattern with each antibodies resembled that seen in the adult rat kidney, in respect to the amount of cells positive, subcellular polarization of the antigenicity, and morphologic cell types positive for these antibodies (11).

Appearance of (Na+K)-ATPase immunoreactivity. (Na+K)-ATPase immunoreactivity was seen in the collecting duct cells already in fetal rat kidneys at the 17th gestational day (Fig. 1). At this stage most collecting duct profiles, identified by morphology, showed (Na+K)-ATPase immunoreactivity, although considerable variation in the staining intensity of different collecting duct profiles was observed. Most lining cells of collecting ducts appeared immunoreactive for this antibody, but notably no intracellular polarization could be observed at this stage. Thus, the lateral membranes and base of the cells, as well as the apical cellular aspect were positive for the (Na+K)-ATPase antibodies. In 2-h postnatal kidneys a closely similar pattern of (Na+K)-ATPase expression was seen. In 12-h postnatal samples (Fig. 2), first signs of intracellular polarization appeared as immunoreactive (Na+K)-ATPase was found preferentially at the base of the cells, although considerable immunoreactivity still also persisted at the lateral membranes. On the other hand, only faint to hardly detectable immunoreactivity was seen at the apical cellular aspect. With increasing age (Figs. 3 and 4) the amount of (Na+K)-ATPase positive cells in collecting ducts seemed to decrease in relation to the evolving population of cells positive for CA II. The typical amount of (Na+K)-ATPase immunoreactive cells as found in the adult rat kidney (11) was achieved by the 30th postnatal day (Fig. 5).

Appearance of CA II immunoreactivity. Immunoreactive CA II could not be detected in the fetal rat kidney samples studied (Fig. 6). In the 2-h postnatal kidneys, only occasional CA II immunoreactive cells were seen in collecting ducts (Fig. 7). However, in the 12-h kidney samples the amount of cells rich in carbonic anhydrase increased considerably. After this, a steady increase in the amount of cells immunoreactive for CA II (Fig. 8) could be seen concomitantly with an increase in the intensity of immunoreaction. An adult type of CA II expression in respect to the typical amount of cells positive in each collecting duct segment was reached between 20- and 30-day kidney samples (Fig. 9). No particular pattern of intracellular polarization of CA II immunoreactivity could be observed.

Appearance of band 3 immunoreactivity. No band 3 immunoreactivity could be seen during early postnatal period (Fig. 10), and not until day 5 a few collecting duct cells expressing band 3

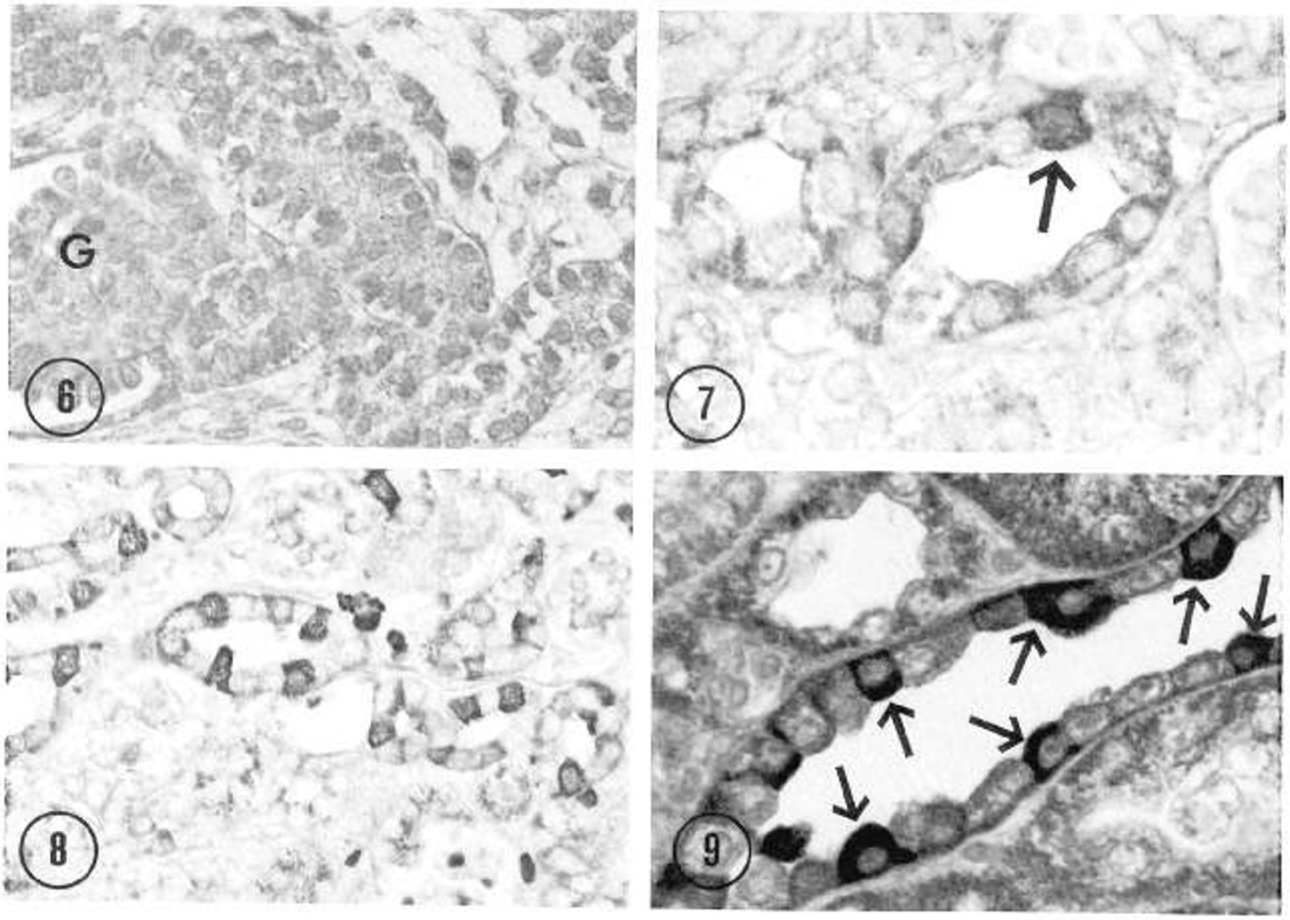

Fig. 6. Seventeenth-day embryonic rat kidney immunostaining for CA II. No positive cells can be seen. *G*, glomerulus. Original magnification ×260.

Fig. 7. In 2-h postnatal kidneys, only occasional collecting duct cells appear immunoreactive for CA II. Original magnification ×260.

Fig. 8. In 7th-postnatal day rat kidneys several CA II-rich cells can be seen along collecting ducts. Original magnification ×150.

Fig. 9. An adult type of CA II immunoreactivity can be seen in cortical collecting duct cells (*arrows*) of 30th-day rat kidneys. Original magnification ×260.

immunoreactivity at the basolateral aspect were seen to appear. After this timepoint, a steady increase in the amount of band 3 positive cells could be observed (Fig. 11). However, as late as day 15 the amount of band 3 positive cells was still relatively low, and did not achieve the amount found in adult rat kidney collecting ducts until at day 30 (Fig. 12). The evolution of cell types with the various immunoreactivities studied is illustrated in Figure 13.

DISCUSSION

Considerable morphologic maturation and increase in the amount of nephrons takes place during the first weeks of life in rats (21). Thus, mature nephrons can be found deep in the cortex, whereas the zone of continuing nephrogenesis is located beneath the renal capsule. Although morphologic maturation of glomerular and tubular elements has been extensively studied (22–24), the evolution of the functional and structural complexity, especially at the collecting duct, which is the site of final control of urinary acidification and electrolyte composition (1, 5), is still poorly understood. Thus, the mechanisms explaining the unique functional features of neonatal kidneys, and for example the transient metabolic acidosis, a phenomenon typical for both full term but especially premature infants (25, 26), remain to be characterized further. The recently introduced immuno- and enzymecytochemical methods detecting key transport enzymes (8, 11, 27) have shown to be useful for providing valuable information on the nephron site and cell type specific location of the transport processes, giving thus new insight into the respective functions. However, a main limitation of those techniques is that they offer only limited possibilities for quantitative analysis of the tissue and cell reactivities.

The present findings show that (Na+K)-ATPase, the enzyme characteristic for principal cells (7), is present early *in utero* in most collecting duct cells. It is also noteworthy that during early postnatal development the amount of (Na+K)-ATPase-positive cells steadily decreased, seemingly in parallel with the increase of cells rich in carbonic anhydrase. This may suggest that the cells responsible for maintaining acid-base balance are recruited from the pool of the morphologically more uniform principal cells, as also suggested earlier (28). Interestingly, the rapid change in the intracellular polarization of (Na+K)-ATPase in principal cells and the appearance of detectable cells with abundant carbonic anhydrase coincided within 12 h after birth, perhaps an indication of functional activation in these cells.

Lonnerholm and Wistrand (29) showed carbonic anhydrase activity in kidneys during early phases of intrauterine life, whereas herein this enzyme was not immunocytochemically detectable in fetal kidneys. However, Lonnerholm and Wistrand (29) used an enzyme histochemical technique which is not specific for the isozyme found preferentially in kidneys (10), and more important, they studied human fetal kidneys. The well-recognized species differences in both the structure and function of collecting ducts (1) may thus explain this controversy. In addition, it has been reported that dark (intercalated) cells as well as their characteristic enzymes are not present in newborn rat or mouse kidneys (21, 30). Whether a fetal type of CA II with different antigenic characteristics and thus undetectable for the antibodies used is present remains to be explored.

Recent results have shown that two types of CA II-rich (intercalated) cells can be identified in rat kidney collecting ducts, based on the simultaneous presence or absence of basolateral band 3 glycoprotein (11). This glycoprotein is involved in the basolateral exchange of intracellular bicarbonate for serosal chloride (17). For counterbalancing, protons are secreted at the apical cellular aspect and these cells would thus be the acidifying type of intercalated cells as proposed by Schuster *et al.* (31). Also, the intercalated cells lacking basolateral band 3 are likely to be of the other identified intercalated cell subpopulation responsible for bicarbonate secretion. This is in line with the acidification model as proposed by Stone *et al.* (6), and is further supported by the findings of Schwartz *et al.* (13) who identified two types of intercalated cells with differing acid secretion characteristics and with reversed intracellular polarization of vacuoles important in proton secretion. Interestingly, in this study band 3 reactivity was not found until day 5 of postnatal life, with only slowly increasing staining intensity and, more important, slowly increasing amount of cells positive for this antibody. This indicates that the intercalated cells with acid secreting capacity evolve slowly during the first weeks of life. This is in agreement with

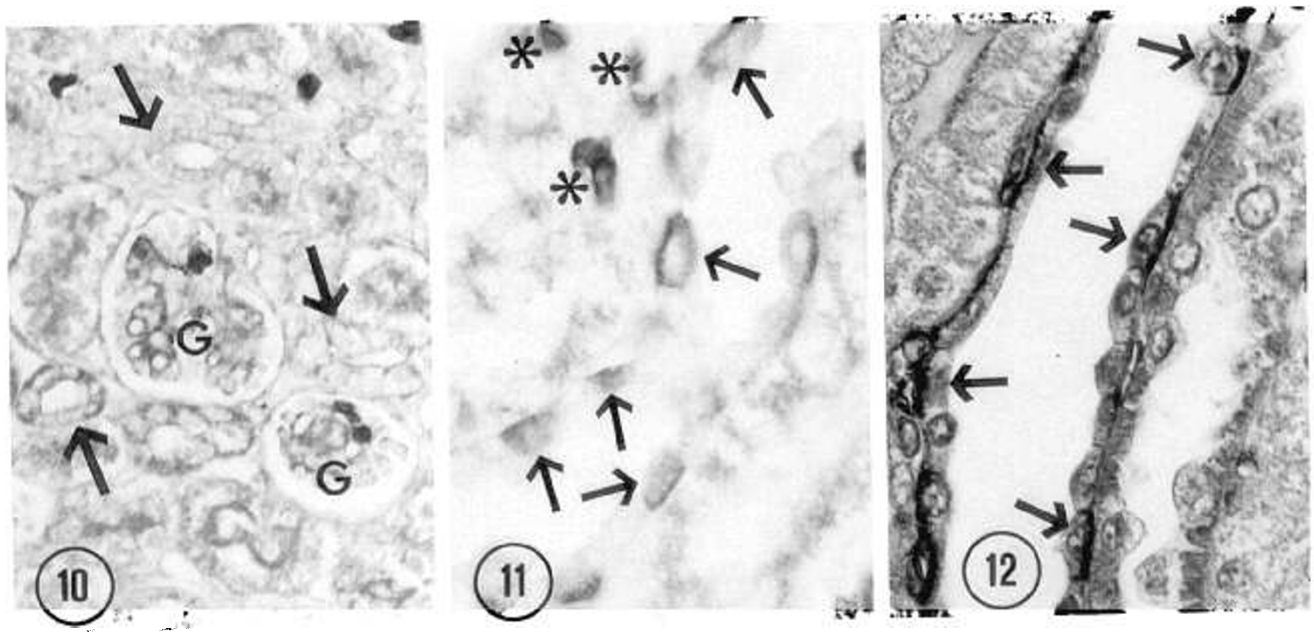

Fig. 10. Paraffin section of kidney of a 2-day-old rat, immunostaining for band 3 glycoprotein. No reactivity at the collecting ducts (*arrows*) can be seen. *G*, glomerulus. Original magnification ×150.

Fig. 11. In the kidneys of 20-day-old rat several cells of cortical collecting ducts show immunoreactivity for band 3 (*arrows*) at the base of the cells. *Asterisks* denote erythrocytes positive for this antibody. Original magnification ×260.

Fig. 12. In 30-day rat kidney, a typical basolateral polarization of band 3 immunoreactivity is seen in a subpopulation of cortical collecting duct cells. Original magnification ×260.

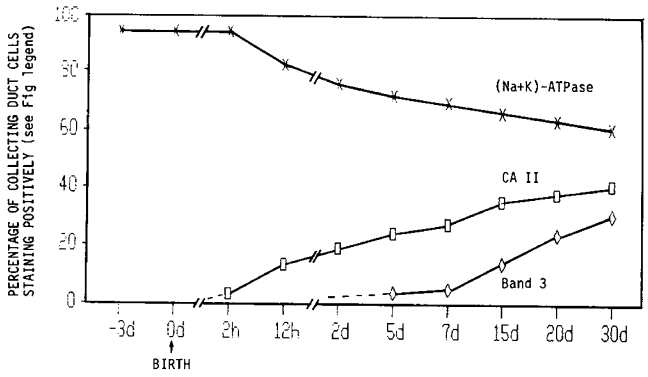

Fig. 13. Amount of collecting duct cells at each timepoint showing positive immunostaining for (Na+K)-ATPase, CA II, and band 3 glycoprotein in fetal (−3 to 0 days) and postnatal (2 h to 30 days) rat kidneys. The amounts given are percentages of total collecting duct cells and are obtained by calculations from five to eight collecting duct profiles from three samples at each timepoint.

previous studies showing that the limitation of collecting ducts to create H^+ gradient disappears within approximately 2 wk of age (32). However, a contributory effect of functionally immature bicarbonate resorption capacity, as shown for the cells of proximal tubules of newborn rabbits (33), cannot be excluded on the basis of the present findings.

The present results show that the immunocytochemically detectable vital enzyme reactivities of collecting duct cell types appear in a sequential manner, reflecting most likely a similar order of functional activation of the respective cell types. Although the current approach cannot provide information of the functional activity or quantitation of the enzymes, these results extend the understanding of the early maturation of the collecting duct cell types and offer also a plausible explanation for the unique functional features of neonatal kidneys. Important areas of further inquiry include the study of signals triggering the rapid expression of the transport enzymes and molecular mechanisms leading to their polarized expression.

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