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Biochemical and Ultrastructural Correlates of Substrate Stimulation of Renal Organic Anion Transport (39293)

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The kidneys of most newborn animals are functionally immature. Horster and Lewy (1) observed that the clearance of *p*-aminohippuric acid (PAH) in newborn rats was less than in adults. Transport capacity measured as the accumulation of PAH in renal cortical slices or separated proximal tubules was also less in the newborn (2-5). The relatively low functional capacity in young laboratory animals has been attributed to structural immaturity and to the presence of nephrogenic and incompletely differentiated tissue in the outer cortex of the kidney. Rennick and her colleagues (2) have shown, for example, greater *in vitro* ability of inner, juxtamedullary tissue to transport PAH than the histologically immature, outer cortical tissue. Renal tubular cells in young animals appear by light microscopy to be small (6), but electron microscopic studies in newborn and fetal animals have shown complete ultrastructural differentiation, with only small quantitative differences from mature kidneys (7). Structural development does not, therefore, appear to be the sole or principal determinant of transport capacity. A primary factor is the availability of substrate. Hirsch and Hook (3, 8-10) observed that treatment of newborn animals with penicillin, an anion rapidly transported in the kidney, increased the ability of cortical slices to accumulate PAH *in vitro*. It was suggested that substrate stimulation of PAH transport in the kidney occurred through increased synthesis of transport carrier (10). Histological appearances, as determined by light microscopy, were unaffected by penicillin (6). Therefore, it was of interest to determine the effects of penicillin pretreatment on renal ultrastructure. Tissue slices consist of several cell layers; thus, interior cells depend upon diffusion for contact with incubation medium. Separated proximal tubular cells, however, are constantly bathed in medium and may more closely approximate conditions *in vivo*. Consequently, PAH uptake and ultrastructural analysis in this investigation were conducted in separated proximal tubules. In addition, because the microsomal enzyme, Na, K-ATPase, develops with age in a manner similar to organic acid transport (11) and because anion transport, like other active transport systems, is sodium dependent (12), the possibility was investigated that penicillin acted indirectly through increased Na, K-ATPase activity.

Methods. New Zealand white rabbits were used in these studies. Young animals remained with their mothers until the time of experimentation. At 11 days of age treatment was begun. Half the litter received penicillin and the other half received saline as control. Procaine Penicillin G was administered subcutaneously as a suspension in a dose of 90,000 IU twice daily for 2 days. Separated tubules were prepared by a technique similar to that of Burg and Orloff (13) with some of the modifications employed by Huang and Lin (14). Animals were sacrificed 24 hr after the final injection and the kidneys exposed. Kidneys were perfused with ice-cold saline followed by 0.300% collagenase (Nutritional Biochemical Corp., Cleveland) dissolved in Ringer's solution.[1] Cortical tissue was dissected free and digested under oxygen at 25° in an additional volume of collagenase-Ringer's solution for 45 min. The solution was then centrifuged for 60 sec at 500 rpm and the supernatant was discarded. The pellet was rinsed three times by resuspension in 5% calf serum-Ringer's solution. During the third wash,

[1] 120 m*M* NaCl, 16.2 m*M* KCl, 1.2 m*M* $MgSO_4$, 10.0 m*M* KH_2PO_4-Na_2HPO_4, 1.0 m*M* $CaCl_2$, 5.5 m*M* dextrose.

the tissue suspension was filtered through two layers of surgical gauze prior to centrifugation. The pellet of tubules was resuspended in sufficient Ringer's medium to produce a 2 to 4% (w/v) suspension. PAH uptake was determined by preincubating an aliquot of suspension under 100% oxygen at 25° for 15 min. PAH was then added to produce concentrations in the medium of 1, 4, and 8 × 10^{-4} *M* and the incubation continued for another 15 min. The tubule suspension was then centrifuged at 2° for 10 min at 10,000*g* in the special centrifuge tubes described by Burg and Orloff (13). The tissue pellet was assayed for PAH by the method of Smith *et al.* (15). To estimate trapped medium in the pellet, inulin was added to the suspension immediately prior to centrifugation and concentrations were determined by the method of Schreiner (16). Results were expressed as micrograms of PAH per gram of tissue (dry weight).

Na, K-ATPase activity was determined in a crude homogenate of separated tubules. Freshly prepared tubules were homogenized in a solution containing 0.25 *M* sucrose, 5 m*M* EDTA, and 30 m*M* histidine (pH 6.8). Enzyme activity was measured in 5 m*M* ATP, 30 m*M* Tris, and 5 m*M* $MgCl_2$ at pH 7.4. Half the beakers contained 115 m*M* NaCl and 10 m*M* KCl (total ATPase) and half contained neither NaCl nor KCl (Mg-ATPase) in a total volume of 3 ml. The incubation mixtures were preincubated for 5 min and the reaction was started by adding ATP. The reaction was allowed to proceed for 15 min and then stopped by adding 1.0 ml 10% TCA. After centrifugation, the supernatant was analyzed for phosphate (P_i) and an aliquot of homogenate was analyzed for protein (17, 18). Na, K-dependent ATPase was represented as the difference between total and Mg-dependent ATPase. Results were expressed as micromoles of P_i released per milligram of protein in 10 min.

Aliquots of each homogenate were prepared and shipped to the microscopy laboratory in a double blind fashion. The code was not broken until all analyses were complete. Tissue was prepared for electron microscopy by fixing the suspension of tubules in ice-cold 1% osmium tetroxide in Millionig's buffer at pH 7.3 before dehydration in alcohol. After fixation, tubules were rinsed in buffer and embedded in epoxy resin by standard techniques. Sections for electron microscopy were stained with uranyl acetate and lead citrate by a modified Reynolds technique. Several sections of each tissue sample were taken and multiple photographs were developed from each section.

Results. Penicillin treatment of newborn rabbits significantly increased the rate of PAH uptake at each PAH concentration in the medium when compared to saline control. A double reciprocal plot of the data (Fig. 1) suggested that there was an increase in the apparent maximal rate of uptake from 3.03 in the control to 6.25 μg/g/min after penicillin treatment. There appeared to be, however, no change in the apparent affinity of the carrier for substrate (2.10 × 10^{-4} *M*).

Treatment of 2-week rabbit kidneys with collagenase produced a homogeneous suspension of tubules, with no significant contamination with connective tissue or undifferentiated cells. It was evident from electron microscopy that proximal tubular cells from 2-week animals were differentiated to a degree comparable to adult tissues (Fig. 2). Brush border, apical vacuoles, and intracellular organelles were well formed in the young animals. The brush border was less dense, however, than in the adult, and the basilar plasma membrane was less infolded.

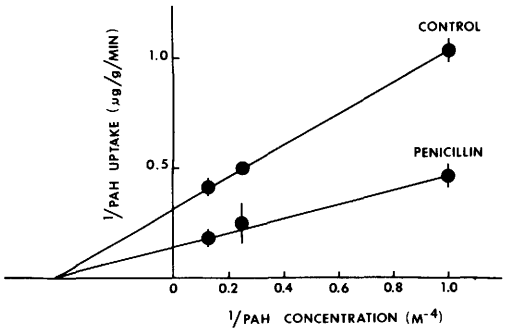


FIG. 1. Double reciprocal plot of *p*-aminohippuric acid (PAH) uptake in separated proximal tubules from 2-week control and penicillin-pretreated rabbits. In each experiment, tubules were preincubated for 15 min without PAH. PAH was then added to produce concentrations in the medium of 1, 4, or 8 × 10^{-4} *M* and the tubules further incubated for 15 min. Each point represents mean ± SE of pups from three litters.

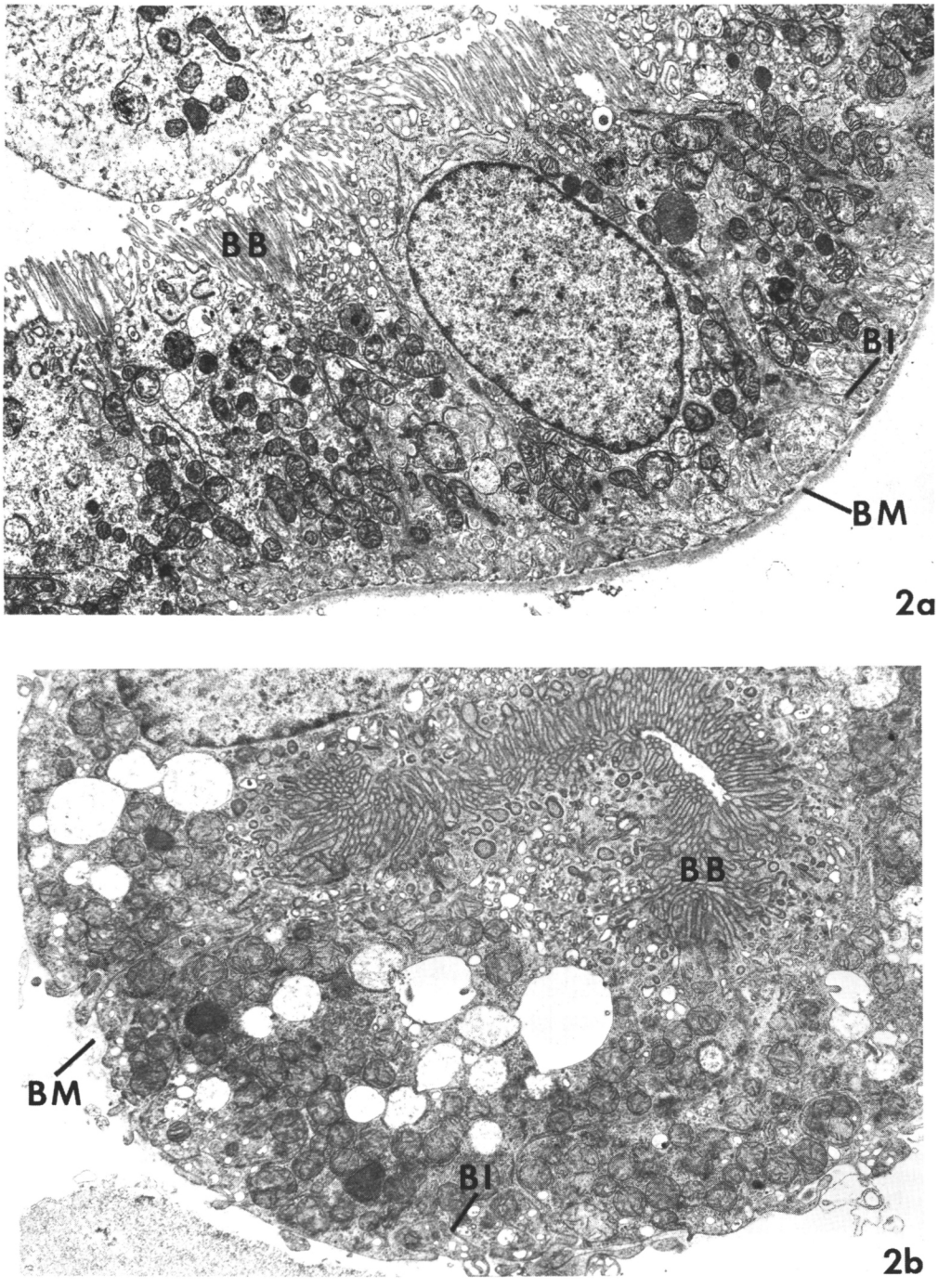


FIG. 2. Electron micrograph of separated proximal tubules from (a) adult and (b) 2-week rabbit (×6020). Both tissues are structurally similar. Differences in structure include a more convoluted brush border (BB), a greater complexity and interdigitation of basilar infoldings (BI) and a thicker basement membrane (BM) in the adult. Due to the lesser complexity of BI less compartmentation and alignment of mitochondria are observed in tissue from young animals. Other structures are comparable.

The basement membranes, which were irregularly preserved in both groups, were not as thick as in adult animals. There was no difference between newborn and adult kidney in the degree of disruption of basement membranes due to the enzymic digestion. Because basilar membrane infoldings were less complex in the newborn, mitochondria were not aligned in basilar compartments as in the adult tubule; they appeared to be unaltered in size. Other structures, including endoplasmic reticulum, free ribosomes, and dense bodies were comparable in both newborns and adults.

Electron micrographs of specimens from control and penicillin-treated animals showed no difference in cellular structure (Fig. 3). The density of brush border and of apical vacuoles, the size and number of lysosomal vacuoles, and the configuration of basilar membranes were comparable in proximal tubules of both groups.

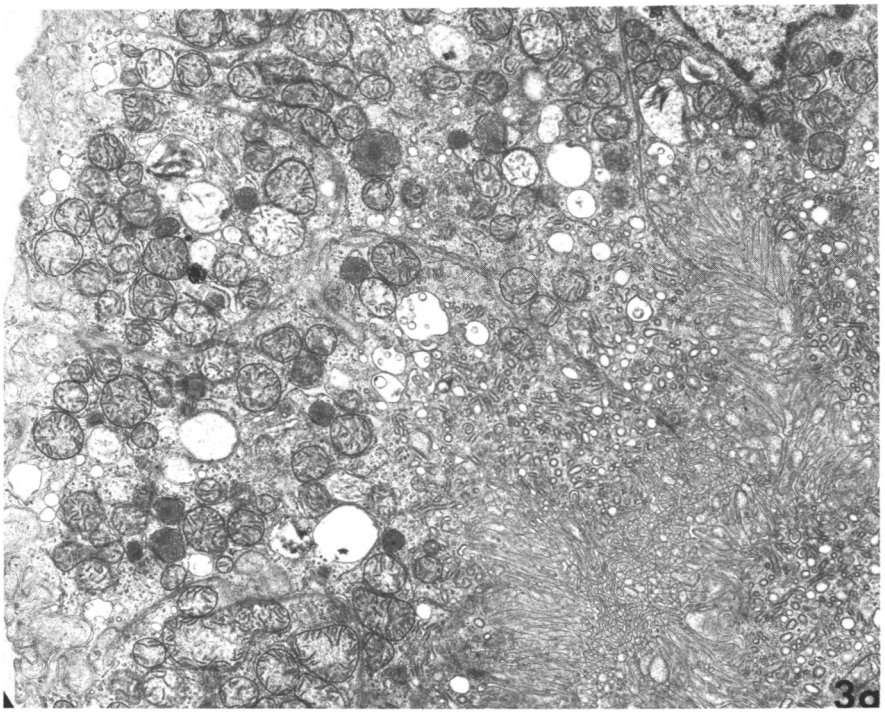
Na, K-ATPase activity in crude homogenates of renal cortex (Fig. 4) was less in the newborn than the adult (0.32 ± 0.08 and 0.69 ± 0.08 μmole PO_4/mg protein/10 min, respectively). Treatment of 2-week animals with penicillin had no effect on enzyme activity (0.30 ± 0.05 μmole PO_4/mg protein/10 min). Similarly, treatment had no effect on magnesium-dependent ATPase.

Discussion. Pretreatment of newborn rats and rabbits with substrates of the organic acid transport system significantly increases the ability of renal cortical slices and separated proximal tubules to accumulate PAH (3, 5, 8–10). Increased steady-state accumulation of PAH in these tissues following penicillin treatment apparently occurs due to stimulation of PAH uptake. Rates of run-out are unaffected (19). It has been proposed that the enhancement of transport capacity following penicillin pretreatment of newborn animals is due to an increased synthesis of transport carrier (10). The present investigation provides further support for this hypothesis in that the apparent theoretical maximal velocity of PAH uptake was increased while there was no change in the apparent affinity of the carrier for substrate (Fig. 1). Conclusions from this analysis, however, must be drawn with caution because unlike cell-free enzyme systems, transport processes in tubular cells are complex and probably do not follow classical Michaelis–Menten kinetics. It may be concluded however, that the change in transport carrier was quantitative (theoretical maximal transport velocity) rather than qualitative (affinity).

The postulated carrier for organic anions has not been isolated or identified. Therefore, the possibility must be considered that penicillin is acting on a process indirectly related to transport, for example, energy transfer. As are many other transport systems, organic anion secretion is dependent upon sodium (12). To determine whether this aspect of function was altered by penicillin, Na, K-ATPase activity was measured. Though enzyme activity was low in the newborn as compared to the adult, penicillin did not increase activity (Fig. 4). Therefore, these data support the hypothesis that the effect of penicillin is specific for the transport system.

Penicillin pretreatment of newborn rabbits has no effect on proximal tubular cell morphology (6). It was of interest to determine whether cellular ultrastructure was changed. Collagenase digestion of renal cortex results in some disruption of proximal tubular cell membranes. Viability of these cells, however, is not severely altered in that they continue to consume oxygen and maintain electrolyte concentration gradients (5). In this study collagenase concentrations in the medium were adjusted to produce adequate yields of tubules with minimal structural damage. By electron microscopy, it was determined that though some damage occurred, cell basement membranes were mostly intact and intracellular structures were normal. In addition, there was not a differential effect of collagenase on adult and 2-week tissues.

Penicillin had no effect on intracellular morphology when compared to controls. Therefore, it was concluded that the effect of penicillin on organic anion transport was either too subtle to be seen or that the induced protein was cytosolic and not membrane bound. Indeed, recent evidence suggests that a soluble enzyme with characteristics similar to the hepatic organic anion binding protein, ligandin, binds PAH and



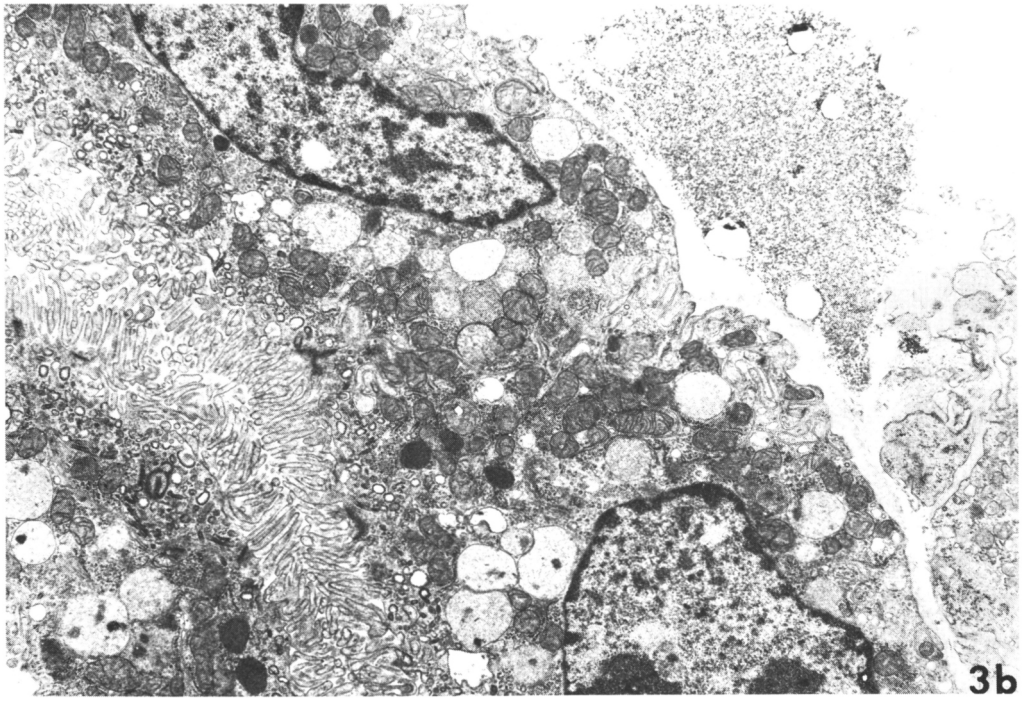


FIG. 3. Electron micrograph of separated proximal tubules from 2-week rabbits (×6400). (a) Saline control. (b) Penicillin treated. Animals received 90,000 IU of procaine penicillin twice daily for 2 days and were sacrificed 24 hr after the final injection. PAH transport capacity was enhanced with no alterations in tubular ultrastructure.

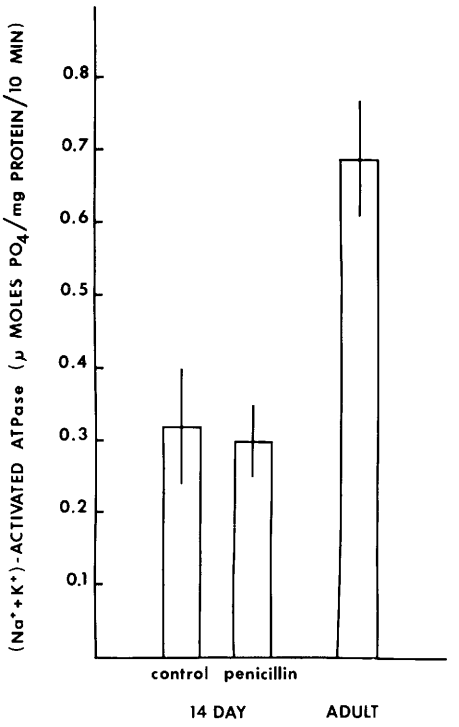


FIG. 4. Effect of penicillin pretreatment and maturation on renal cortical Na^+, K^+-activated ATPase determined from a crude cortical homogenate. Each bar represents mean ± SE of four determinations.

may be implicated in renal organic anion transport (20). Substrate stimulation may then provide a means through which the proposed carrier for organic anions in the kidney may be identified and will facilitate purification of an anion binding fraction lending further understanding to the mechanism of anion transport.

Summary. Penicillin pretreatment enhanced the rate of PAH uptake into separated proximal tubules (collagenase digestion) from 2-week New Zealand white rabbits. A double reciprocal plot of these data suggests that penicillin increases the maximal velocity of PAH uptake. Na, K-ATPase was less in adult tissue but was unaffected by penicillin. No ultrastructural changes could be attributed to the treatment. Thus, substrate stimulation of PAH transport does not involve Na, K-ATPase and probably involves soluble, rather than structural proteins.

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