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# ANALYSIS OF FACTORS INFLUENCING THE IN VITRO DEVELOPMENTAL PATTERN OF *p*-AMINOHIPPURATE TRANSPORT BY RABBIT KIDNEY

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## SUMMARY

The ability of rabbit renal cortical tissue to accumulate *p*-aminohippurate was low at two weeks, increased to a peak at four weeks and then declined to an intermediate value in adult tissue. These differences are not due to differences in the metabolism of *p*-aminohippurate, tissue composition or tissue viability but reflect real differences in the ability of the tissue to transport or retain *p*-aminohippurate. The estimated maximal rate of *p*-aminohippurate transport into slices increased to four weeks and declined whereas runout from tissue was greatest in adult tissue. Both of these changes could contribute to the decline in the slice to medium (S/M) concentration ratio after four weeks. *p*-Aminohippurate S/M ratio from tissue of all ages was enhanced by acetate. Inasmuch as the *p*-aminohippurate S/M ratio was greatest in adult tissue in the presence of acetate, the data suggest that the decline normally seen after four weeks reflects the presence of an inhibitor or changes in metabolic requirements in the tissue rather than a decline in intrinsic transport capacity.

Preventing contact with solid food during development appeared to retard development of maximal transport capacity. Neomycin was without effect. These data suggest that substances in the diet might serve as substrates to "trigger" the development of anion transport in normal newborn kidneys. Conformation of this suggestion awaits experimental verification.

## INTRODUCTION

The kidneys of most newborn animals are immature at birth. This immaturity has been demonstrated utilizing both in vitro and in vivo techniques [1–3]. Several studies have focused on describing the development of one particular function of the kidney, the active transport of organic acids. The majority of in vitro studies have employed the slice method of Cross and Taggart [4] in which transport of a prototype acid, *p*-aminohippurate is represented as the slice/medium (S/M) concentration ratio developed after incubation for varying periods of time. Studies employing this

technique have produced a characteristic pattern of development in tissue from rabbits [5], rats [6], and dogs [7, 8]. The *p*-aminohippurate S/M ratio is low immediately following birth and progressively increases with age. Peak values are obtained at 1–4 weeks, depending on the species. The S/M ratio then declines with age until adult levels are obtained. The purpose of this study was to elucidate factors responsible for this pattern of development in newborn rabbit kidneys.

The slice technique is not a true measure of organic acid transport capacity. Rather, the S/M ratio is determined by intracellular accumulation and the respective rates of influx and efflux of the anion studied. Therefore, the uptake and runout components of the slice technique were quantified independently in tissue from rabbits of different ages in order to determine the role each played in establishing the developmental pattern. It was also of interest to determine environmental factors that might influence or trigger changes seen in the maturation of this transport system.

## METHODS

Experiments were conducted on New Zealand white rabbits bred and raised in the department animal quarters or purchased from a local breeder. The young animals were kept with their mothers until the time of experimentation. With experiments utilizing young animals, an entire litter was used for a single experiment. In the experiments employing adults, females were used exclusively.

Animals were killed by a blow on the head. The kidneys were quickly removed and placed in ice-cold saline. Thin renal cortical slices were prepared freehand and distributed evenly (approximately 100–200 mg of slices per beaker) into beakers containing 2.7 ml of the phosphate buffer devised by Cross and Taggart [4]. All incubations were carried out in a Dubnoff metabolic shaker at 25 °C under a gas phase of 100% oxygen. The duration of incubation and the concentration of *p*-aminohippurate in the medium varied with the experiment being conducted. After incubation, the slices were removed from the medium, blotted on gauze and weighed. The tissue and, when necessary, a 2-ml aliquot of medium were treated as outlined by Cross and Taggart [4]. *p*-Aminohippurate concentrations were determined by the method of Smith et al. [9] except when *p*-amino-[$^{14}$C]hippurate was employed.

To quantify maximal ability of renal cortical slices to accumulate *p*-aminohippurate, slices were incubated for 90 min in $7.4 \cdot 10^{-5}$ M *p*-aminohippurate. The data were expressed as the slice to medium (S/M) ratio, calculated by dividing the concentration of *p*-aminohippurate per g of tissue by the concentration of *p*-aminohippurate per ml of incubation medium. Recovery of *p*-aminohippurate from the slices was determined by comparing the recovery of *p*-aminohippurate from beakers containing *p*-aminohippurate medium incubated without slices to those incubated with slices.

To estimate maximal rate of uptake into slices, beakers were incubated for 2 and 12 min in medium containing *p*-aminohippurate in concentrations of 1, 2, 4 and $8 \cdot 10^{-4}$ M. Uptake was calculated by subtracting the 2-min from the 12-min *p*-aminohippurate uptake, dividing by 10 and expressing the results as $\mu$g *p*-aminohippurate per g tissue per min. The data were plotted on a Hofstee plot.

Runout of *p*-aminohippurate was determined using the method of Farah et al. [10] with some of the modifications devised by Berndt [11]. Slices were preloaded

by incubating 300–600 mg of tissue slices in 6 ml of medium containing $6.3 \cdot 10^{-4}$ M *p*-amino-[$^{14}$C]hippurate for 90 min. After incubation the slices were blotted on gauze and placed in a runout net similar to the one described by Berndt [11]. Slices in the net were transferred at 1-min intervals through a series of 20 beakers plus an initial rinse beaker, each containing 4.0 ml of *p*-aminohippurate-free medium. At the conclusion of the runout experiment the slices were removed from the net, blotted, weighed and treated as usual. 1 ml of 10% trichloroacetic acid was added to each runout beaker and the samples centrifuged before assaying. Radioactivity in the slices and runout beakers was determined by placing a 1-ml aliquot into a vial containing 10 ml modified Bray's solution (2.5 g of 2,5-diphenyloxazole and 100 g of naphthalene per l of dioxane) and then counting the vials in a Beckman LS-100 liquid scintillation counter. The data were expressed as μg *p*-aminohippurate remaining in the slices per 100 mg of tissue as a function of runout beaker number (time) and plotted semi-logarithmically.

In 9 litters young animals were prevented from obtaining solid food by feeding the mother rabbit separately for approximately 2 h, twice daily. Fresh water was provided for the young animals at all times but solid food contact was not permitted until after the litter reached the 4th week of age. *p*-Aminohippurate S/M ratio experiments were performed on representative animals from each litter at 3, 4 and 5 weeks of age.

The effect of oral neomycin sulfate was determined in 4 litters. The drug was administered orally to half of each litter in a dose of 1 g/kg body weight once daily for 7 days. Control littermates received equal volumes of water. S/M ratio experiments were conducted at 3 and 4 weeks of age, 24 h after the last dose of neomycin.

The effect of acetate on *p*-aminohippurate accumulation by renal cortical slices from 2-week, 4-week and adult rabbits was determined by adding sodium acetate (final concentration, $10^{-2}$ M) to the incubation medium, then conducting S/M ratio experiments.

Statistical analyses were performed using student's "*t*" test, paired and group comparisons [12]. The 0.05 level of probability was used as the criterion of significance. Variability was expressed as the standard error (S.E.).

## RESULTS

The *p*-aminohippurate S/M ratio was low at 2 weeks ($4.05 \pm 0.39$) and increased to 4 weeks ($12.54 \pm 0.69$). The S/M ratio in adult tissue ($6.41 \pm 0.57$) was significantly less than the peak at 4 weeks (Fig. 1). Recovery of *p*-aminohippurate after 90 min of incubation was 102, 101 and 102% for 2-week, 4-week and adult rabbits respectively; all values had a S.E. of less than 1.5%.

Uptake of *p*-aminohippurate by slices was determined at 2 and 12 min of incubation at four different concentrations of the anion (Fig. 2). After 12 min incubation 2-week tissue demonstrated the lowest uptake followed by the adult tissue. Maximum uptake occurred in 4-week tissue. The same pattern of uptake was established after only 2 min of incubation and was the same at all concentrations of *p*-aminohippurate (Fig. 2). The data were analyzed kinetically utilizing a Hofstee plot, where velocity (uptake in μg/100 mg per min) was plotted against velocity over substrate concentration (Fig. 3). The apparent affinity for the carrier ($K_m$) is

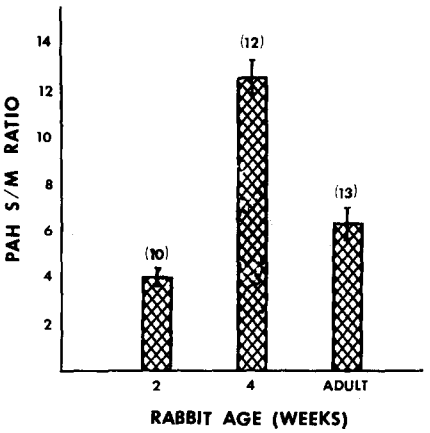


Fig. 1. Accumulation of *p*-aminohippurate (PAH), represented as the slice to medium ratio, by 2-week, 4-week and adult rabbit renal cortical slices. Bars represent means ±S.E. of the number of experiments (litters tested) indicated in parentheses. All groups are significantly different from each other ($p < 0.05$).

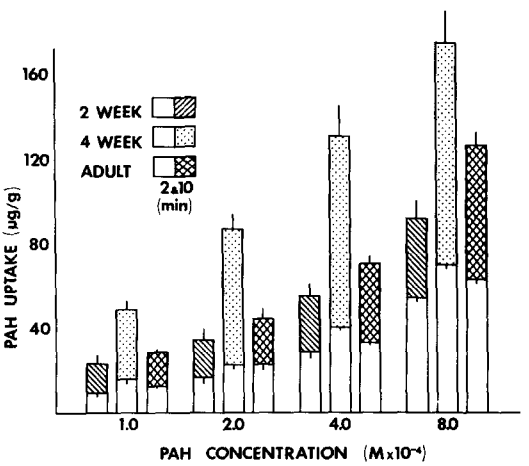


Fig. 2. *p*-Aminohippurate (PAH) uptake in 2-week, 4-week and adult rabbit renal cortical slices. In each experiment slices from several animals were pooled and then distributed into 16 beakers, 4 at each concentration of *p*-aminohippurate and 2 at each time. Duplicate values were averaged. Each point represents mean ±S.E. from four separate experiments at each age.

represented graphically as the negative slope and the apparent maximal rate of uptake ($V$) is represented by the Y intercept. There were no differences in $K_m$ but the maximal rate of transport was significantly different between age groups. The 2-week values were lowest followed by adult, with 4-week tissue demonstrating the highest rate of uptake.

Runout of *p*-aminohippurate from 2-week, 4-week and adult tissue is plotted in Fig. 4. The initial concentration of *p*-aminohippurate was highest in 4-week, followed by adult and 2-week tissue. Runout of *p*-aminohippurate was slowest at 2 weeks followed closely by the 4-week value. The first order runout constant for 4-

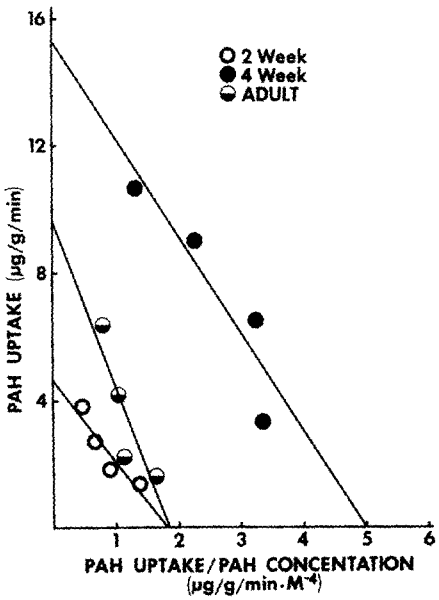


Fig. 3. Hofstee plot of *p*-aminohippurate (PAH) uptake between 2 and 12 min from the data in Fig. 2. There were no significant differences between the slopes of the calibrated regression lines.

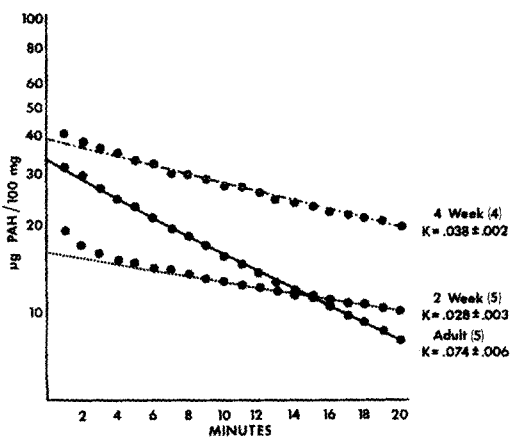


Fig. 4. Runout of *p*-aminohippurate (PAH) from renal cortical slices from 2-week, 4-week and adult rabbits. Slices were pre-loaded with *p*-aminohippurate for 90 min, rinsed and transferred through a series of *p*-aminohippurate-free beakers at 1-min intervals. Concentration of *p*-aminohippurate in the slice as a function of runout time was determined and a first-order rate constant ($k$) calculated. Points and regression lines represent means from 4 or 5 experiments. Constants represent means $\pm$S.E. and are significantly different from each other ($p < 0.05$).

week tissue ($0.038 \pm 0.003\ min^{-1}$) was significantly greater than that from 2-week tissue ($0.028 \pm 0.003\ min^{-1}$). The adult runout constant ($0.074 \pm 0.006\ min^{-1}$) was much higher than either the 2-week or 4-week value.

When contact with solid food was prevented by feeding the mother rabbit separately, the *p*-aminohippurate S/M ratio at 4 weeks of age was $9.3 \pm 0.9$, which was

significantly lower than the normal 12.5±0.7 (Table I). Some animals were not used at 4 weeks of age but were permitted solid food contact from 4–5 weeks. The average S/M ratio at 5 weeks was 11.7±1.7, similar to the normal S/M value reported for 4 weeks of age.

TABLE I

EFFECT OF DIET ON *p*-AMINOHIPPURATE ACCUMULATION (S/M RATIO) IN RENAL CORTICAL SLICES FROM NEWBORN RABBITS

Values represent means ±S.E. of 4-9 duplicated experiments, each representing a different litter.

| Litter age (weeks) | Restricted diet* | Normal diet |
|---|---|---|
| 4 | 9.3±0.9 | 12.5±0.7 |
| 5 | 11.7±1.7 | 11.0±0.6** |

* *p*-Aminohippurate S/M ratio in rabbit renal cortical slices. 4-week-old rabbits were denied solid food entirely. 5-week-old rabbits were exposed to food between weeks 4 and 5 of age.

** *p*-Aminohippurate S/M ratio in rabbit renal cortical slices from previous data [5].

When animals were treated orally for one week with 1.0 g/kg neomycin sulfate, there was no difference in the *p*-aminohippurate S/M ratio between the treated animals and the water-treated controls, at either 3 or 4 weeks of age (Table II). Higher doses of neomycin sulfate were attempted but proved to be excessively toxic.

TABLE II

*p*-AMINOHIPPURATE ACCUMULATION (S/M RATIO) IN RABBIT RENAL CORTICAL SLICES AFTER TREATMENT WITH NEOMYCIN SULFATE

Rabbits were treated with 1.0 g/kg neomycin sulfate orally for 7 days prior to experimentation. *p*-Aminohippurate uptake was determined 24 h after the last injection. Each value represents the average value obtained for a group of 2–4 animals within a litter. Control animals within the litter received equal volumes of distilled water (1.0 ml/100 g body weight).

| Litter age (weeks) | Controls | Neomycin-treated |
|---|---|---|
| 3 | 10.7 | 12.0 |
| 3 | 14.0 | 15.0 |
| 4 | 16.5 | 18.2 |
| 4 | 13.8 | 14.8 |

Acetate added to the incubation medium stimulated the *p*-aminohippurate S/M ratio at all ages (Table III). The acetate-stimulated in vitro development curve increased progressively with age, without the characteristic peak normally observed at 4 weeks of age.

TABLE III

EFFECT OF ACETATE ($10^{-2}$ M) ON *p*-AMINOHIPPURATE ACCUMULATION (S/M RATIO) BY RABBIT RENAL CORTICAL SLICES

Values are means ±S.E.

| Age (weeks) | *N** | Control | Acetate |
|---|---|---|---|
| 2 | 4 | 4.3 (±0.5) | 10.0 (±1.3)** |
| 4 | 4 | 12.3 (±2.8) | 17.0 (±1.0)** |
| Adult | 4 | 7.3 (±1.1) | 19.8 (±2.0)** |

* *N*, number of litters or adults tested.
** Significantly different from the control ($p < 0.05$).

## DISCUSSION

The peak in *p*-aminohippurate S/M ratio which occurs in the first few weeks of life has been described several times in different species, but no explanations have been found for the pattern of development [5–8]. The decline in the *p*-aminohippurate S/M ratio after 4 weeks in rabbits can not be attributed to differences in electrolyte concentrations [13] or tissue water content [5]. Optimal conditions for incubating slices from newborn appeared to be the same as for adult slices [5–7]. Recovery of *p*-aminohippurate was essentially 100% in all three age groups indicating that metabolism of the anion probably did not influence the developmental pattern. Baerlocher et al. [14] observed an age-related quantitative difference in optimal temperature for steady state accumulation of amino acids into renal tissue. Temperature sensitivity of *p*-aminohippurate uptake, however, does not appear to vary with age. In a series of 8 paired experiments increasing incubation temperature to 37 °C reduced *p*-aminohippurate uptake by 55% in 2-week tissue, 49% in 4-week tissue and 50% in adult tissue. Thus, age-related differences in *p*-aminohippurate S/M ratio must reflect real differences in the ability of tissue to transport and/or retain *p*-aminohippurate.

Uptake of *p*-aminohippurate into renal cortical slices is linear with time during short periods of incubation and thus reflects maximal transport capacity [15, 16]. The rate of uptake between 2-week, 4-week and adult tissue in the present study follows the same pattern as the S/M ratio, with maximal uptake rate observed at 4 weeks of age (Fig. 3). If the analogy can be drawn from a pure enzyme system, the apparent affinity for the carrier is similar between age groups while the number of functional transport sites changes with age [17]. On the other hand, runout of *p*-aminohippurate from pre-loaded slices appears to increase steadily with age (Fig. 4). The development of runout with age could contribute to the decline in *p*-aminohippurate S/M ratio seen after 4 weeks.

The S/M ratio was reduced at 4 weeks in animals without solid food contact (Table I). With exposure to food after 4 weeks, the S/M ratio at 5 weeks approached the peak value normally obtained at 4 weeks, suggesting that substances present in solid food stimulate development of the transport system. These observations must be tempered by the fact that the animals with limited food contact weighed slightly less than those fed normally. However, no correlation was found between body weight

and the S/M ratio obtained at 4 weeks in either the limited food or normally fed groups.

Goldberg et al. [18] demonstrated stimulation of *p*-aminohippurate S/M ratio in rats 48 h following uninephrectomy and that this effect was blocked by post-operative feeding of neomycin sulfate. Neomycin had little or no effect on the S/M at the ages tested (Table II). Either the drug was ineffective in sterilizing the gut or gut bacteria are not a significant source of organic acid substrates in young rabbits.

The *p*-aminohippurate S/M ratio in adult renal cortical slices is enhanced in the presence of acetate [4]. Schacter et al. [19] suggested that acetate reverses inhibition produced by endogenous inhibitors. Acetate enhanced *p*-aminohippurate uptake, but not to the same magnitude at each age (Table III). The maximal S/M ratio obtained was in adult tissue in the presence of acetate. These results are consistent with the supposition that endogenous inhibitors are influencing the normal adult S/M ratio resulting in the decline seen after 4 weeks of age. Alternatively, acetate may serve as an energy source for the transport system. *p*-Aminohippurate uptake by adult tissue may be more limited by energy availability (e.g. another transport system in adult tissue competing for the same energy source). If there are fewer demands for energy in newborn tissue acetate would have a lesser effect.

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REFERENCES

1 Horster, M. and Lewy, J. E. (1970) Am. J. Physiol. 219, 1061–1065
2 Rennick, B. R. (1969) Am. J. Physiol. 217, 247–250
3 Williamson, R. C. and Hiatt, E. P. (1947) Proc. Soc. Exp. Biol. Med. 66, 554–557
4 Cross, R. J. and Taggart, J. V. (1950) Am. J. Physiol. 161, 181–190
5 Hirsch, G. H. and Hook, J. B. (1970) J. Pharmacol. Exp. Ther. 171, 103–108
6 Kim, J. K., Hirsch, G. H. and Hook, J. B. (1972) Pediatr. Res. 6, 600–605
7 Hook, J. B., Williamson, H. E. and Hirsch, G. H. (1970) Can. J. Physiol. Pharmacol. 48, 169–175
8 Rennick, B., Hamilton, B. and Evans, R. (1961) Am. J. Physiol. 201, 743–746
9 Smith, H. W., Finkelstein, N., Aliminosa, L., Crawford, B. and Graber, M. (1945) J. Clin. Invest. 24, 388–404
10 Farah, A., Frazer, M. and Stoffel, M. (1963) J. Pharmacol. Exp. Ther. 139, 120–128
11 Berndt, W. O. (1965) J. Pharmacol. Exp. Ther. 150, 414–419
12 Lewis, A. E. (1966) Biostatistics, Reinhold Publ. Co., New York
13 Cole, B. R., Bartlett, K. G. and Robson, A. M. (1972) 5th Int. Congr. Nephrol. (Abstracts) p. 133
14 Baerlocher, K. E., Scriver, C. R. and Mohynddin, F. (1971) Biochim. Biophys. Acta 249, 353–363
15 Ross, C. R. and Farah, A. (1966) J. Pharmacol. Exp. Ther. 151, 159–167
16 Hirsch, G. H. and Hook, J. B. (1970) Can. J. Physiol. Pharmacol. 48, 550–556
17 Huang, K. C. and Lin, D. S. T. (1965) Am. J. Physiol. 208, 391–396
18 Goldberg, V. J., Weiss, F. R., Keller, A. I. and Preuss, H. G. (1970) Am. J. Physiol. 218, 1065–1069
19 Schachter, D., Manis, J. G. and Taggart, J. V. (1955) Am. J. Physiol. 182, 537–544