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Transport of Phosphate by Renal Brush Border Membrane Vesicle (BBMV) during Development — Role of the Growth Hormone —

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=Abstract=**It is well documented that plasma concentrations of Pi (inorganic phosphorus) are higher in developing subjects than in adults. In a previous study, we demonstrated that the Vmax (capacity) of the Na-Pi cotransport mechanism of the renal brush border membrane vesicles was higher in immature than mature rats.**

In this study, we evaluated the role of a growth hormone in the maintenance of a higher Vmax observed in immature rats.

In mature rats, serum Pi, and the tubular reabsorption of Pi (TRPi) increased in the growth hormone treated animals. On the other hand, those values were not changed by growth hormone treatment in immature rats.

In kinetic analysis, the Km (affinity) values were not different between the control (growth hormone-untreated) and growth hormone-treated renal brush border membrane vesicles in both immature and mature rats. The Vmax of the immature rats also was not changed by growth hormone treatment. On the contrary, Vmax increased significantly in the growth-hormone treated than the control mature rats.

With the above findings, it seems that immature rats reabsorb Pi maximally even in the control state, and it is likely that a growth hormone is responsible for the phenomenon.

Key Words: *Renal brush border membrane vesicles, Development, Growth hormone, Na-Pi transport mechanism*

INTRODUCTION

It is well-documented that plasma concentrations of Pi (inorganic phosphate) bear a direct relationship to the rate of growth both in humans (Richmond *et al.*, 1951; McCrory *et al.*, 1952; Connelly *et al.*, 1962; Brodehl *et al.*, 1982) and in various animals (Altman and Ditter, 1974). It might be considered an advantage for growing cells to be exposed to a rich Pi environment, which has been suggested as essential to the accretion of new tissue (Spitzer *et al.*, 1983).

It is likely that the kidney contributes to the phenomenon, and there is evidence that fractional reabsorption of Pi is significantly higher in the newborn than in the adult (Caverzasio *et al.*, 1982; Johnson and Spitzer, 1986; Haramati *et al.*, 1988). There is ample evidence that Pi is absorbed almost exclusively in the proximal tubule, especially in the earliest portion (S1 segment), at least in intact animals (Knox *et al.*, 1977). Using a micropuncture technique, Kaskel *et al.* (1988) identified the proximal

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tubule as the site where most of the enhanced Pi reabsorption takes place in immature guinea pigs.

In the proximal tubule of the kidney, it has become apparent that Pi is cotransported with sodium, most evidently by the brush border membrane vesicle experiment (Hoffman *et al.*, 1976), and this mechanism is thought to be the rate limiting step in the regulation of tubular Pi reabsorption (Cheng and Sactor, 1981; Gmaj and Murer, 1986). We (1987) and Neiberger *et al.* (1989) demonstrated that the Vmax (capacity) of the Na-Pi transport mechanism was significantly higher in the immature than the mature animals. However, how the higher Vmax is maintained in the developing subject is not understood.

Several investigators have advanced the notion that the high rate of renal phosphate reabsorption in the immature period is a consequence of the increased demand for phosphate associated with rapid growth (Caverzasio *et al.*, 1982; Johnson and Spitzer, 1986; Haramati *et al.*, 1988).

Although the precise hormonal and metabolic signals that interface between the growth process and phosphate handling have not been elucidated, it is likely that a growth hormone is an important factor (Haramati *et al.*, 1990).

In order to find out whether a growth hormone (GH), which is known to reduce urinary phosphate excretion (Henneman *et al.*, 1960; Corvilain and Abramov, 1964; Camanni, 1968), affects the observed developmental change in the Na-Pi cotransport, an experiment using brush border membrane vesicles from rat kidneys was carried out. The advantages of a microvesicle experiment are its absence of metabolism and the ease with which the composition of the solutions on both sides of the membrane can be controlled (Kinne and Schwarz, 1978).

MATERIALS AND METHODS

Immature (4-5 weeks old) or mature (10-12 weeks old) male Sprague-Dawley rats were used for the experiment. The rats were allowed free access to food (〉 0.4% Pi diet) and water until sacrifice. The experimental rats were given GH (Lucky Pharmaceutical Co., human methylated GH, 2 units/kg/day for 5 days), and the control rats received vehicles. The dose will result in blood concentrations well above physiologic levels (Frohman and Bernardis, 1970) thus minimizing the effect of spontaneous release of the hormone by the pituitary gland, and the duration has been shown sufficient to induce a noticeable increase in TRPi (Henneman *et al.*, 1960). To get the chemical measurements for the whole animals, urine was obtained by putting the rats in metabolic cages individually, and blood was drawn by intracardiac puncture just before sacrifice by cervical dislocation.

Preparation of Brush Border Membrane Vesicles (BBMV)

BBMV were prepared by a modification of the Mg precipitation method (Booth and Kenny, 1974). The animals were sacrificed by a sudden cervical dislocation, followed by rapid removal of the kidneys, and placed in an ice-cold mannitol buffer (10 mM mannitol, 2 mM Tris at pH 7.1). All succeeding steps were carried out at 0-4℃.

For each experiment, 2-4 rats of each age were required in order to obtain a sufficient amount (= 2 gm) of superficial cortex. After the kidneys were removed, the superficial cortex (approximately 1/2 of the cortex width) was carefully isolated by hand cutting with a # 10 blade and put in 10 cc of ice-cold mannitol buffer for each gram of tissue. The tissue was homogenized for 2 minutes in a Sorvall Omni mixer (initial homogenate).

Magnesium chloride was added to the homogenate to give a concentration of 12 mM, and the suspension was allowed to stand with occasional mixing for 15 minutes. Then a series of alternating low and high speed centrifugations was carried out to obtain the final brush border vesicle pellet. A sufficent volume of HEPES-mannitol buffer (300 mM mannitol, 20 mM HEPES at pH 7.45) was added to give a protein concentration of 10-20 mg/ml in the final suspension. The mixture was aspirated 10 times through a 27-gauge needle and used for measurements of protein concentrations, enzyme activities, and transport studies. Solutions were filtered through 0.45 μm filters, and the containers were sterilized before use.

Analytical Method

The purity of the BBMV prepartions was determined by measuring the alkaline phosphatase (Andersch and Szczypinski ,1947) in both the initial homogenate and final BBMV preparations. For transport studies, only the vesicle preparations with more than 8 times the alkaline phosphatase enrichment were used.

Protein concentrations were measured by Lowry method (Lowry *et al.*, 1951) using bovine serum albumin as the standard. Inorganic phosphorus was measured by a modification of the Fiske and Subbarow (1925) method. Calcium and creatinine were measured by a Hitachi 736-40 and by a Beckman creatinine autoanalyzer, respectively.

Transport Studies

An incubation medium (mannitol 300 mM, NaCl or KCl 100 mM, at pH 7.4) with various concentrations of Pi was used for the study. A sufficient quantity of ^{32}P-K2 HPO4 (Dupont) was added to achieve the concentrations of 0.05 to 3 mM Pi (final concentrations of 0.04 to 2.4 mM). Transport experiments were carried out essentially according to the method of Hopfer *et al.* (1973) at room temperature.

For the kinetics of the transport study, 10-second uptakes were carried out. The 10-second period was chosen because it was shown that the uptake of Pi was linear up to 15 seconds (Lelivre-Pegorier *et al.*, 1983) and also because it was more reproducible at 10-second than at shorter times. The Na dependent Pi uptake was calculated by subtracting the average influx in the presence of KCl from the average influx in the presence of NaCl

To see the overshoot uptake of Pi with inward Na gradient, the uptakes were terminated at 10-second, 1 minute, 2 minute, and 120 minute by withdrawing 25 ml of the incubation mixture and adding it to 1 cc of the ice-cold stop solution (300 mM mannitol, 100 mM NaCl, 20 mM HEPES, 10 mM Arsenate at pH 7.4). The resultant suspension was rapidly passed through a 0.45 mm filter (Millipore) under continuous suction and washed twice with 5 ml of ice-cold stop solution.

The filters were dried out, covered with 10 ml of Aquasol (DuPont) scintillation fluid, and counted in a liquid scintillation counter (Beckman Co.). All incubations were performed in triplicate. A sample of the incubation medium was also counted with each experiment to determine the specific activity. Chemical reagents of high purity were obtained from Sigma.

Stastical Analysis

Values were expressed as the means ± SE. The significance of the differences between the group means was assessed by a Mann-Whitney U test. Kinetic data were analyzed by ANOVA (Scheffe test) using a Stat view 512 (Microsoft) program. A value of less than 0.05 was considered stastically significant.

RESULTS

Whole Animal Data

Body weight at Days 1 and 5, kidney weights, % kidney weight per body weight, and dietary intake of the rats are shown in Table 1.

Table 1. Body weight, kidney weight, and dietary intake in the rats

Age	4-5 Weeks			10-12 Weeks		
GH	Control (n = 10)	Treated (n = 10)	p	Control (n = 6)	Treated (n = 6)	p
Body weight (gm, Day 1)	111 ± 1.6	109 ± 1.7	ns	254 ± 2.4	259 ± 1.0	ns
Body weight (gm, Day 5)	124 ± 3.9	120 ± 3.7	ns	266 ± 10.8	282 ± 4.5	0.024
Kidney weight (gm)	1.09 ± 0.04	1.06 ± 0.03	ns	1.99 ± 0.05	2.10 ± 0.10	ns
KWt/BWt (%)	0.88 ± 0.03	0.88 ± 0.04	ns	0.75 ± 0.04	0.76 ± 0.01	ns
Dietary intake (gm/kg/d)	109 ± 3.3	109 ± 3.4	ns	103 ± 3.7	98 ± 2.8	ns

Table 2. Serum Ca, Pi, Cr, and tubular reabsorption of Pi (TRPi) in the rats

Age	4-5 Weeks			10-12 Weeks		
GH	Control (n = 10)	Treated (n = 10)	p	Control (n = 6)	Treated (n = 6)	p
Serum Ca (mg/dl)	9.4 ± 0.76	10.1 ± 0.07	ns	10.0 ± 0.96	10.6 ± 0.41	ns
Serum Pi (mg/dl)	9.5 ± 0.38	9.3 ± 0.15	ns	6.3 ± 0.2	7.7 ± 0.42	0.023
Serum Cr (mg/dl)	0.38 ± 0.03	0.36 ± 0.02	ns	0.58 ± 0.06	0.52 ± 0.06	ns
TRPi	8.36 ± 0.92	85.5 ± 2.89	ns	70.2 ± 2.72	81.1 ± 3.42	< 0.01

Body weights at Day 1 and Day 5 were not different between the GH-treated and the control (GH untreated) in the immature rats. In the mature rats, the body weights at Day 1 were not different, but at Day 5 the body weights of the GH-treated rats were significantly heavier than the control animals.

Kidney weights at Day 5 were not different between the GH-treated and the control rats both in the immature and in mature rats. The percent of the kidney weight per body weight also was not different between the GH-treated and the control animals both in the immature and mature animals.

The dietary intake (gm/kg/d) was not significantly different among all the animal groups.

Biochemical Data

The results of serum Pi, calcium, creatine, and TRPi are shown in Table 2. Serum Pi levels were 9.5 ± 0.38 mg/dl and 9.3 ± 0.15 mg/dl in the control and GH-treated immature rats, respectively and were not different significantly. In the mature rats, the serum Pi of control was 6.3 ± 0.2 mg/dl, which was significantly lower than that of the GH-treated rats (7.7 ± 0.42 mg/dl). The serum Pi levels were significantly higher in the immature rats than in the mature rats in both the control and GH-treated animals.

The range of serum calcium levels was 9.4 to 10.6 mg/dl and was not different among the all 4 groups.

Serum creatinine levels were higher in the mature than immature rats. However, serum creatinine levels were not changed by the GH treatment.

TRPi were 83.6 ± 0.92% and 85.5 ± 2.89% in the control and GH-treated immature rats, respectively, and were not different significantly. In the mature rats, TRPi of the control rats was 70.2 ± 2.72%, which was significantly lower than that of the GH-treated rats (81.1 ± 3.42%).

Uptake Study according to Incubation Time (Fig. 1 and Fig. 2)

The uptake studies at a Pi concentration of 0.1 mM in the incubation medium according to the incubation time showed an overshoot (peak uptake at 1 minute) and declined to the equilibrium value at 120 minutes in all 4 BBMV preparations. At

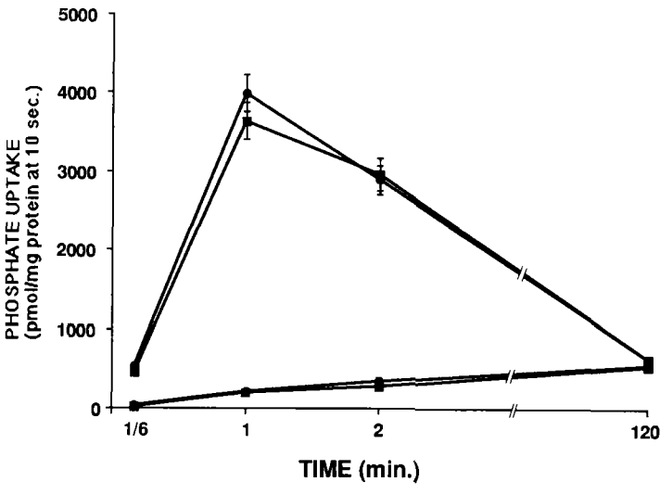

Fig. 1. The uptake of ^{32}Pi by microvesicles of brush border membrane obtained from immature rats either in the control (•) or growth hormone-treated rats (■) according to incubation time. The two upper curves represent 0.1 mM ^{32}Pi uptake in the presence of 100 mM invardly-oriented NaCl gradient, and the lower curves denotd ^{32}Pi uptake in the presence of 100 mM KCl in the incubation media.

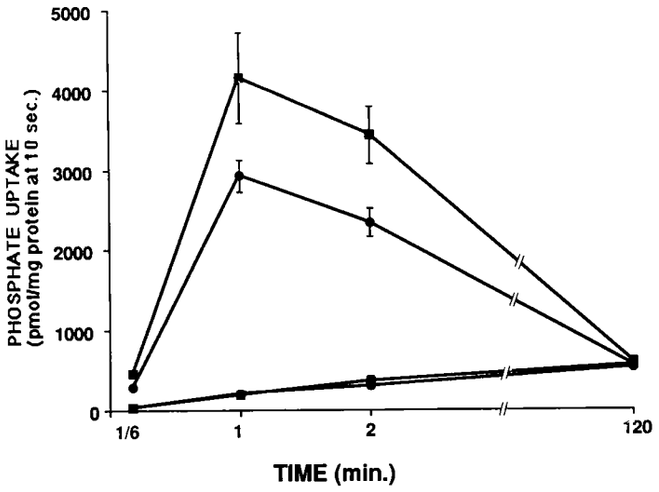

Fig. 2. The uptake of ^{32}Pi by microvesicles of brush border membrane obtained from mature rats either in the control (•) or growth hormone-treated rats (■) according to incubation time. The two upper curves represent 0.1 mM ^{32}Pi uptake in the presence of 100 mM invardly-oriented NaCl gradient, and the lower curves denotd ^{32}Pi uptake in the presence of 100 mM KCl in the incubation media.

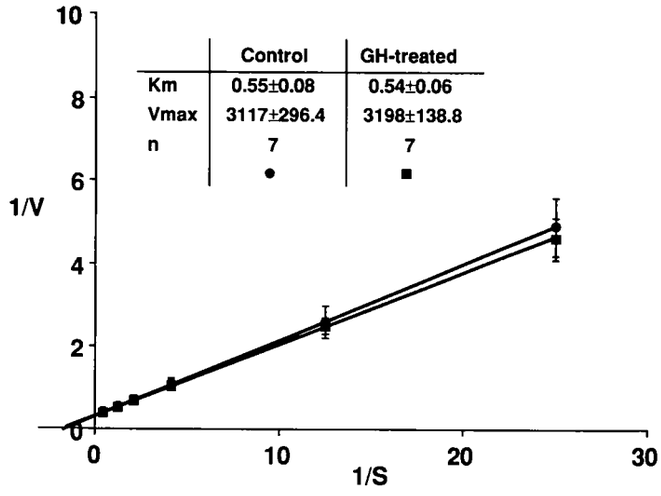

Fig. 3. Double reciprocal plots (Lineweaver-Burke) of the 100 mM Na gradient ^{32}Pi uptake studies in immature rat renal BBMV are shown at concentrations in the media varying from 0.05 to 3 mM (0.04-2.4 mM of final concentrations).

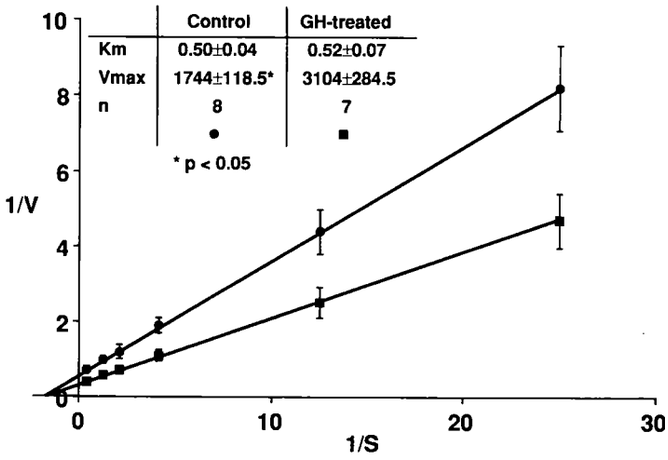

Fig. 4. Double reciprocal plots (Lineweaver-Burke) of the 100 mM Na gradient ^{32}Pi uptake studies in mature rat renal BBMV are shown at concentrations in the media varying from 0.05 to 3 mM (0.04-2.4 mM of final concentrations).

10 seconds, the uptake with an inward 100 mM Na gradient of BBMV from the immature control rats was 451 ± 46.3 pmol/mg protein, which was not different significantly from that of the GH-treated immature rats (523 ± 15.4 pmol/mg protein). One minute BBMV Pi uptakes were also not different between the control and GH-treated immature rats.

In the mature rats, the 10-second uptake with an inward Na gradient of BBMV from the control was 287 ± 14.5 pmol/mg protein, which was significantly lower than that from the GH-treated rats (467 ± 32.3 pmol/mg protein). One minute BBMV uptake was also significantly lower in the control than GH-treated mature rats.

Between the immature and mature control rats BBMV, 10-second and 1-minute uptakes were significantly higher in the immature rat BBMV.

At 120 minutes, the Pi uptakes were not different among the 4 groups, which means the vesicle volumes were not different from each other.

Kinetic Studies (Fig. 3 and Fig. 4)

Kinetic data were obtained by double reciprocal plots (Lineweaver-Burke plot) of the uptake studies at concentrations of ^{32}Pi in the media varying between 0.05 and 3 mM (final concentrations varying between 0.04 and 2.4 mM).

Km (affinity) values were 0.55 ± 0.08 and 0.54 ± 0.06 mM of Pi in the control and GH-treated immature rat BBMV preparations respectively. In the mature rats, the Km of the control rat BBMV was 0.50 ± 0.04, and that of the GH-treated rat BBMV was 0.52 ± 0.04 mM of Pi. The Km values were not stastically different among all 4 groups ($p=0.31$).

However, the Vmax values were significantly

different among the groups ($p < 0.01$). Vmax (capacity) values were 3117 ± 296.4 and 3189 ± 138.8 pmol/mg protein at 10 seconds in the control and GH-treated rat BBMV preparations respectively, and they were not different significantly. In the mature rat BBMV, the Vmax of the control was 1744 ± 118.5 pmol/mg protein at 10 seconds, which was significantly lower than that of the GH-treated rat BBMV (3104 ± 284.5 pmol/mg protein at 10 seconds).

The Pi influx in the presence of a 100-mM inwardly-directed KCl gradient was linearly related to the extravesicular Pi concentrations (0.05-0.3 mM) and did not vary as a function of age or by growth hormone. The Pi uptake values according to incubation times in the presence of a 100-mM inwardly directed KCl gradient were significantly lower than those of the values in the presence of a 100-mM inwardly directed NaCl gradient ($p < 0.01$).

DISCUSSION

Intrinsic to the process of growth is the maintenance of a positive balance of a variety of substances including minerals like sodium and phosphorus. Pi is an important constituent of all body tissues, and is located mostly (85%) in the skeleton (Stoff, 1982).

Serum Pi concentrations were shown to be higher repeatedly in growing subjects (Connelly *et al.*, 1962; Brodehl *et al.*, 1982) , and it has been suggested that an environment high in phosphate is essential to the accretion of new tissue (Spitzer *et al.*, 1983). We also observed a higher serum Pi in immature than mature control rats.

As for the underlying mechanism by which the kidneys of growing subjects preserve the positive external balance of Pi, McCrory *et al.* (1951) suggested that the retention of phosphate is due to the lower rate of GFR in growing subjects by clearance study. However, the role of the tubular function in the maintenance of a phosphate balance in the growing period has been more emphasized recently (Caverzasio *et al.*, 1982; Johnson and Spitzer, 1986). The higher TRPi in the immature than in the mature control rats observed in this experiment also suggests the significance of the role of the renal tubule in the maintenance of positive Pi balance in the immature animals.

In the kidney, filtered Pi is almost exclusively reabsorbed in the proximal tubule, mostly by the Na-Pi cotransport system, which is thought to be the main limiting step in the regulation of the tubular transport of Pi (Baumann *et al.*, 1975; Stoll *et al.*, 1979; Stoll *et al.*, 1980).

Choi and Ko (1987) report that the Vmax of the Na-Pi cotransport mechanism was higher in the immature than mature rats BBMV, while the Km of the mechanism was not different. They also suggest that the maintenance of a positive balance of Pi is attributed to a higher Vmax in the developing subject. This finding was recently confirmed by Neiberger *et al.* (1989) in guinea pigs and by our current experiment in rats. The higher capacity for a transfer of Pi in the proximal tubule microvilli of immature animals may be due to a larger number of transporters or a higher rate of substrate turnover on each transporter, i.e., a higher mobility of the cotransporters in the membrane. However, the mechanisms by which the higher Vmax of the Na-Pi cotransport mechanism in the immature animal kidney BBMV are maintained are not understood.

Various factors such as the growth hormone (GH), parathyroid hormone (PTH), 1,25-dihydroxy Vitamin D, insulin, glucocorticoid, and dietary intake are known to influence the tubular handling of Pi (Spitzer *et al.*, 1983; Gmaj and Murer, 1986). Of the many factors listed above, GH is acknowledged to be a primary driving force for the growth of animals.

A role of the growth hormone in the regulation of phosphate reabsorption was predicted on the basis of clinical observations made in patients with pituitary dwarfism and acromegaly (Corvilain and Abramov, 1972). Moreover, Na-stimulated Pi transport is enhanced in BBMV preparations from adult dogs subjected to chronic administration of GH (Hammerman *et al.*, 1980). Recently, Mulroney *et al.* (1989) observed a decreased tubular reabsorption of phosphate when immature rats were given a new GH-releasing factor antagonist. They concluded that the growth hormone, directly or indirectly, plays a central role in the regulation of renal phosphate handling during development.

In this experiment, we observed the effect of the growth hormone on the Pi handling in vivo and at the level of BBMV during development. In mature rats, serum Pi and TRPi were significantly higher in the GH-treated rats than in the control rats. The Vmax was also significantly higher in the BBMV of the growth hormone-treated rats than that of the control rats. The Vmax of growth hormone-treated rats was comparable to those of the immature rat groups. On the other hand, the serum Pi, TRPi, and Vmax of the renal BBMV Na-Pi cotransport mechanism were not different between the control and GH-treated immature animals. The Km values were not different between the GH-treated and control rat renal BBMV preparations in both the immature and mature rats.

It is suggested that the growth hormone exerts a maximal effect on the Pi balance already in the control immature rats. Based on the above findings, we concluded that the observed higher Vmax of the Na-Pi cotransport mechanism of BBMV in the immature animals was due to the effect of the GH. But curiously enough the pulsatile release of the growth hormone was lower in the immature than mature animals (Lumpkin *et al.*, 1989).

The findings suggest that immature animals are more sensitive to the growth hormone. Therefore, the higher Vmax in the GH-treated mature rats was probably due to the fact that the relative insensitivity to GH was overcome by the administration of a pharmacological dose of GH. For a better understanding of the role of the growth hormone in Pi in the maintenance of a positive Pi balance in immature animals, a growth hormone inhibition study would be more ideal but seems unfeasible for microvesicle study.

The parathyroid hormone is a well known factor in reducing the tubular reabsorption of Pi. Johnson and Spitzer (1986) showed that the tubules of immature guinea pigs were unresponsive to PTH by isolated perfused kidney technique and concluded that the failure of the kidney to mount a phosphaturic response to PTH in the immaure guinea pigs may be due to the higher demand for Pi consequent to the increase in the number and size of the cells (This may simulate a state of Pi depletion). This assumption was confirmed by Corn *et al.* (1989) who showed that phosphaturic response was restored by Pi infusion in immature animals. So they concluded that the unresponsiveness to PTH observed in immature animals is not due to the immaturity of the kidney, but rather to other factors serving to promote phosphate retention. However, we cannot disregard completely the role of PTH in the developmental changes in the Pi balance in this experiment because our rats were not parathyroidectomized.

Dietary Pi intake is also an important modulator of the Na-Pi cotransport mechanism (Stoll *et al.*, 1979; Cheng *et al.*, 1983), but dietary intake of Pi was the same in all 4 groups of animals in this experiment. However, it has been suggested that immature animals behave like Pi-depleted mature animals (when Vmax becomes higher) because of rapid growth (Neiberger *et al.*, 1989). In this respect, a growth hormone may be responsible for a relatively Pi-depleted state and for an avid phosphate reabsorption in the immature animals even at the same normal dietary Pi intake.

In summary, the Km (affinity) values of the Na-Pi cotransport mechanism were not different in all 4 groups of rats. But the Vmax (capacity) increased significantly by growth hormone treatment in mature rats. In immature rats, the Vmax did not increase by growth hormone treatment. With the above findings, we concluded that the growth hormone seems to play a significant role in the maintenance of positive Pi balance in immature rats.

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