Low Phosphate Diet Upregulates the Renal and Intestinal Sodium-Dependent Phosphate Transporter in Vitamin D–Resistant Hypophosphatemic Mice (43692)

NOZOMU NAKAGAWA AND FAYEZ K. GHISHAN[1]

Department of Pediatrics, Division of Gastroenterology/Nutrition and Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, Tennessee 37232–2576

Abstract. **Renal and jejunal absorption of inorganic phosphate (P_i) increases with dietary P_i restriction in the rat. The defect in Na^+-dependent phosphate transporter has been localized to the kidney of the Hyp mice; however, the adaptation to low-P_i diet in both kidney and jejunum of the Hyp mice has not been well characterized. Therefore, the current studies were designed to characterize the adaptation of renal and jejunal Na^+-dependent phosphate transport in the Hyp mice and compare it with normal mice. Low-P_i diet significantly increased the slope of the initial rate of renal brush border membrane (BBM) phosphate uptake compared with corresponding values in mice raised on control-P_i diet (0.035 vs 0.017) ($P < 0.01$). Kinetics of renal Na^+-dependent phosphate uptake in Hyp mice showed a V_{max} of 1.00 ± 0.01 and 0.46 ± 0.02 nmoles/mg protein/15 sec in low- and control-P_i diets, respectively ($P < 0.01$), whereas, K_m values were 0.07 ± 0.04 and 0.02 ± 0.01, respectively. Similar kinetic analysis in renal BBM of normal mice showed a V_{max} of 2.4 ± 0.17 and 1.18 ± 0.09 ($P < 0.01$) and K_m of 0.07 ± 0.03 and 0.08 ± 0.03 on low and control P_i diets, respectively. Similarly, low-P_i diet significantly increased the slope of the initial rate of intestinal phosphate uptake (0.013 and 0.007) ($P < 0.01$). Kinetics of jejunal Na^+-dependent phosphate uptake in Hyp mice showed a V_{max} of 0.36 ± 0.01 and 0.2 ± 0.02 nmoles/mg protein/15 sec, ($P < 0.01$) and K_m of 0.13 ± 0.06 and 0.06 ± 0.01 mM in low- and in control-P_i diet, respectively. Kinetic analysis in jejunal BBM of normal mice showed a V_{max} of 0.47 ± 0.04 and 0.18 ± 0.01 nmoles/mg protein/15 sec ($P < 0.01$) and K_m of 0.16 ± 0.04 and 0.11 ± 0.01 in low- and control-P_i diets, respectively. The data indicates that low-phosphate diet upregulates the V_{max} of the renal and jejunal Na^+-dependent phosphate cotransporter in the hypophosphatemic mice.** [P.S.E.B.M. 1994, Vol 205]

It is well established that the intestinal absorption of inorganic phosphate increases in response to low-phosphate diet in several mammalian species (1–4). Adaptation in phosphate absorption is expressed at the level of the luminal membrane of the intestinal and renal epithelium, where Na^+-dependent phosphate transport system has been identified (5). This up-regulation of Na^+-dependent phosphate transport in brush border membrane vesicles (BBMV) by low-phosphate diet is documented in various species including the chick (1) and rat (4).

X-linked vitamin D–resistant hypophosphatemic rickets occurs in both humans and the Hyp mice. In both species, the disease is inherited as an X-linked dominant trait, and it is characterized by reduced renal tubular reabsorption of phosphate, hypophosphatemia, and a rachitic or osteomalacic bone disease. Thus, the Hyp mouse model appears to be an animal model of X-linked hypophosphatemic rickets in humans (6).

The defect in the Na^+-dependent phosphate trans-

[1] To whom requests for reprints should be addressed at Pediatric Gastroenterology/Nutrition, Vanderbilt University School of Medicine, 21st and Garland, S-4322 Medical Center North, Nashville, TN 37232–2576.

Received May 24, 1993. [P.S.E.B.M. 1994, Vol 205]
Accepted October 12, 1993.

0037-9727/94/2052-0162$10.50/0

porter has been localized to the BBMV level of kidney in the Hyp mice (7, 8). Tenenhouse *et al.*, have reported that the Na^+-dependent phosphate transporter at the renal BBMV level in the Hyp mice adapt to low-phosphate diet in a similar manner to normal mice (9). Kinetic analysis of the adaptation to low-phosphate diet in the kidney of the Hyp mice showed an increase in V_{max} without a change in K_m (10, 11). The current studies were designed to extend these findings and explore the adaptation at the BBMV level of intestinal Na^+-phosphate transporter in the Hyp mice.

The aim of our study is 2-fold: (i) to extend the original observation on the renal adaptation, and (ii) to characterize the adaptation of intestinal Na^+-dependent phosphate transporter at the BBMV level in the Hyp and in normal mice.

Materials and Methods

C57 BL/6J +/Y males (normal) and C57 BL/6J Hyp/Y males (genetically hypophosphatemic) were obtained from the Jackson Laboratory (Bar Harbor, ME). After delivery, both mice were raised on a commercial chow diet for three months. The animals were then either maintained on control-P_i diet (0.4% phosphate, 0.45% calcium) or fed a low-P_i diet (0.02% phosphate, 0.45% calcium) for seven days before sacrifice by cervical dislocation. The diets were otherwise similar.

$KH_2[^{32}P]O_4$ (1 Ci/nmole) was purchased from DuPont—New England Nuclear. Enzymes and substrate for leucine aminopeptidase were obtained from Sigma. Cellulose nitrate filters, 0.45-μm pore size were obtained from Sartorius Filter, Inc. (Hayward, CA). All other chemicals were of the highest purity available.

Preparation of BBMV in the Hyp and Normal Mice. Six mice were used for each preparation. After sacrifice, the jejunal segments and kidneys were removed from each mouse. The kidneys were decapsulated and kept in 0.9% NaCl at 4°C. Cortical tissue was removed by slicing the cortex, using a sharp blade. The jejunum extended from the ligament of Treitz to approximately 15 cm aborally. The jejunal segments were washed with ice-cold 0.9% NaCl and everted on a glass rod. The mucosa was scraped from each segment, and BBMV were prepared by using a modified divalent cation precipitation method originally described for renal BBMV and used extensively in our laboratory (12, 13). A sample of each final BBMV preparation was removed for protein determination by the method of Lowry *et al.* (14), using bovine serum albumin as a standard.

Purity of the Membrane Vesicle Preparation. The purity of the membranes was assessed by the measurement of leucine aminopeptidase, an enzyme marker for intestinal brush border membranes, Sigma Kit 251. The procedure is based on the principal that leucine aminopeptidase cleaves a substrate L-leucyl-β-naphthylamide to leucin-β-naphthylamin, which can be measured spectrophotometrically. $(Na^+$-$K^+)$-ATPase was measured according to the method of Scharschmidt *et al.* (15). Cytochrome-C-oxidase and NADPH-cytochrome-C-reductase were assayed as described by Beaufay *et al.* (16).

Marker Enzyme Studies. Leucine aminopeptidase, a marker for brush border enzymes, was enriched 10–12-fold compared with crude homogenate in both jejunal and renal membranes of Hyp and normal mice. The activities of $(Na^+$-$K^+)$-ATPase, cytochrome-C-oxidase, and NADPH cytochrome-C-reductase were depleted to 0.6 ± 0.1-, and 0.8 ± 0.1-fold, respectively.

Transport Measurements. Uptake of phosphate was measured by rapid-filtration technique (17). All experiments were performed at 25°C. Transport was initiated by adding 20 μl of the final vesicle suspension to the desired incubation medium containing labeled substrate. The composition of the incubation medium for each individual experiment is described in the figure legends in Results. At the destined time intervals, the reaction was stopped by the addition of ice-cold stop solution, consisting of 100 m*M* mannitol, 100 m*M* NaCl, 20 m*M* Hepes/Tris (pH 7.4), and 10 m*M* K_2Po_4.

Calculations and Statistical Analysis. Kinetics curves and parameters were fitted by computer program (Enzfitter, Robin J. Leatherbarrow, Elsevier-Biosoft, UK). Statistical significance was determined using Student's paired *t*-test.

Results

Initial Rate of Na^+-Dependent Phosphate Uptake by Renal BBMV in the Hyp and Normal Mice. Figure 1 depicts the initial rate of phosphate uptake by renal BBMV on control-phosphate diet versus low-P_i diet. The initial rate of renal phosphate uptake was linear in both membranes up to 15 sec. The slope of initial rate of Na^+-dependent phosphate uptake into BBMV of Hyp and normal mice maintained in low-P_i diet was significantly greater compared with corresponding mean values in mice maintained in control-P_i diet ($P < 0.01$). To provide evidence that the changes in transport are specific for phosphate uptake, we have carried out experiments to examine D-glucose uptake in Hyp and normal mice under low- and control-P_i diet. D-glucose uptakes at 10 sec were 0.2 ± 0.01, 0.18 ± 0.005, 0.21 ± 0.01, and 0.22 ± 0.008 nmoles/mg protein, respectively.

Kinetics of Na^+-Dependent Phosphate Uptake by Renal BBMV in the Hyp and Normal Mice. In an effort to explore the finding of the initial rate uptake studies, the kinetics of Na^+-dependent phosphate transport was determined. BBMVs from Hyp and nor-

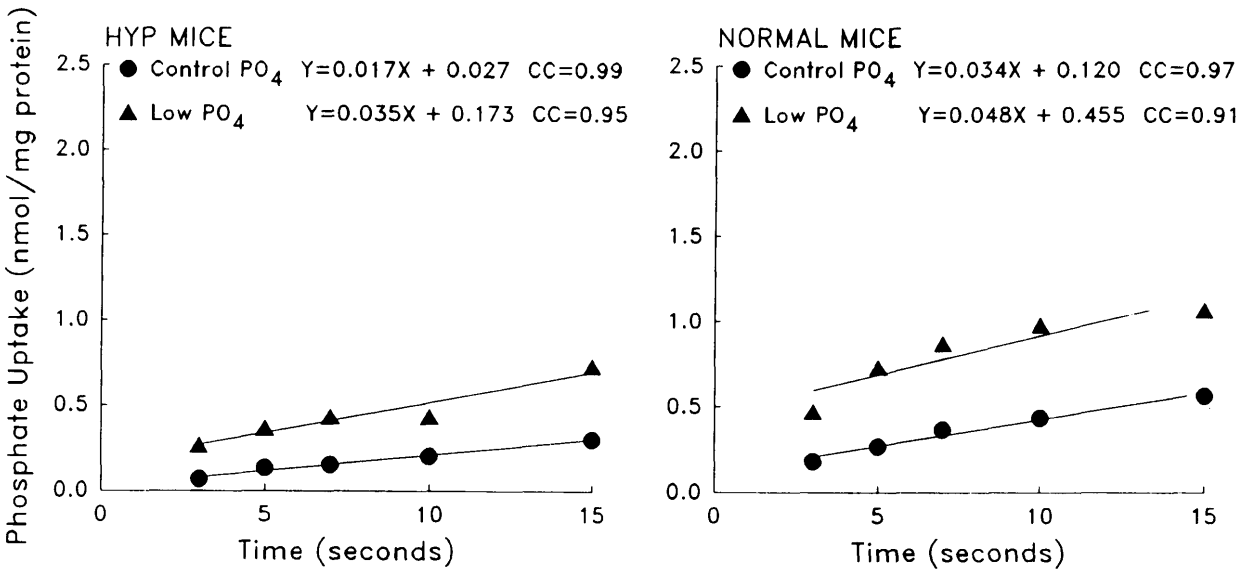

Figure 1. Initial rate on phosphate uptake by renal BBMV on low- and control-phosphate diet in the Hyp and normal mice. BBMV were prepared in 280 m*M* mannitol, 20 m*M* Hepes/Tris buffer pH 7.4. Reaction was started by incubating the vesicles with 100 m*M* NaCl, 100 m*M* mannitol, 20 m*M* Hepes/Tris, 0.1 m*M* KH_2Po_4. Reaction was stopped at desired time intervals. Each experiment was run three times in triplicate.

mal mice were incubated in Na^+-containing and Na^+-free buffers with various phosphate concentrations. Transport was measured at 15 sec during the linear phase of uptake. Figure 2A depicts kinetic parameters of renal Na^+-dependent phosphate uptake in Hyp mice (Na^+-dependent minus Na^+-independent uptake). V_{max} was significantly greater in Hyp mice on low-P_i diet as compared with control-P_i diet ($P <$ 0.01). K_m values were not significantly different. As seen in Figure 2B, similar kinetic analysis in renal BBMV of normal mice showed a significantly greater V_{max} on low-P_i diet compared with control diet ($P <$ 0.001). K_m values were similar on low- and control-P_i diets.

Initial Rate of Na^+-Dependent Phosphate Uptake by Jejunal BBMV in the Hyp and Normal Mice.

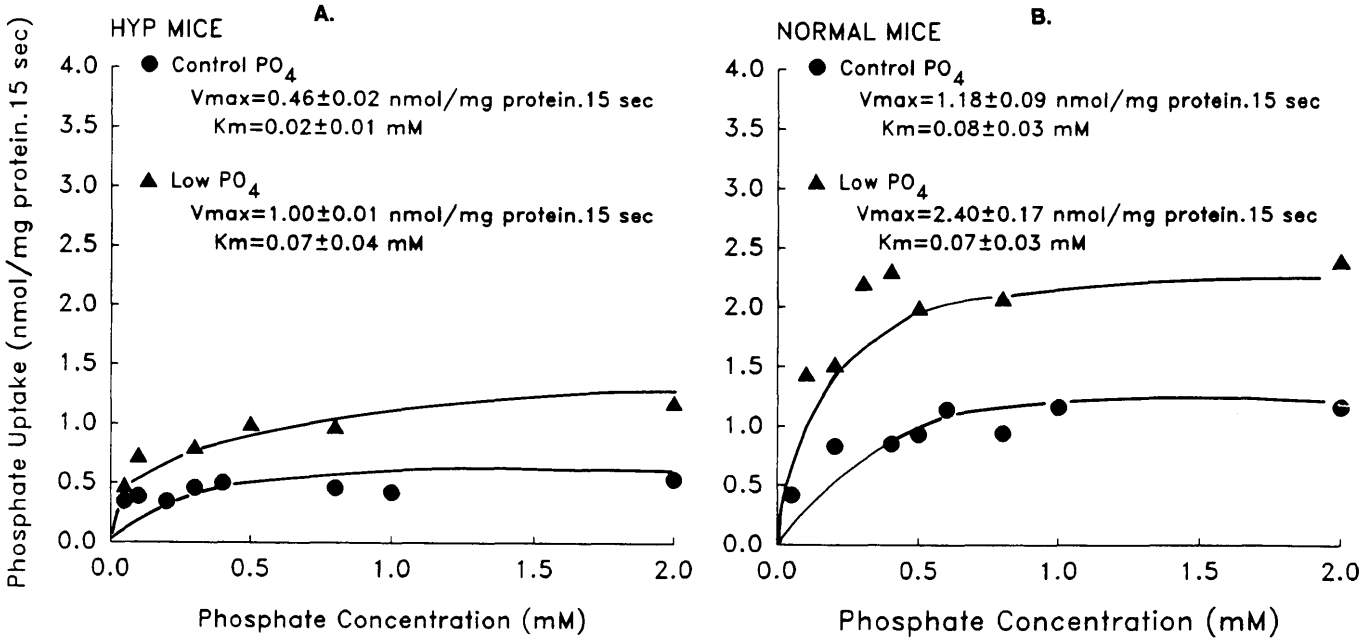

Figure 2. Kinetics of Na^+-dependent phosphate uptake by renal BBMV on low- and control-phosphate diet in the Hyp (A) and normal (B) mice. BBMV were prepared in 280 m*M* mannitol, 20 m*M* Hepes/Tris buffer pH 7.4. Reaction was started by incubating the vesicles with 100 m*M* NaCl or 100 m*M* KCL, 100 m*M* mannitol, 20 m*M* Hepes/Tris, and varying concentrations of KH_2Po_4 and tracer ^{32}P. Reaction was stopped at 15 sec. Mean uptake values in the presence of 100 m*M* KCl were subtracted from mean values in the presence of 100 m*M* NaCl. Kinetic analyses were done utilizing a computerized model of Michaelis-Menten kinetics (Enzfitter-Biosoft, Cambridge, UK). Each experiment was run three times in triplicate.

Figure 3 depicts the initial rate of Na^+-dependent phosphate uptake by jejunal BBMV on control-phosphate diet versus low-P_i diet. In the presence of sodium, the slope of initial rate of P_i uptake into BBMV of Hyp and normal mice was greater in vesicles isolated from low-P_i diet compared with corresponding mean values in mice on control-P_i diet ($P < 0.01$).

Kinetics of Na^+-Dependent Phosphate Uptake by Jejunal BBMV in the Hyp and Normal Mice. Figure 4A depicts kinetic parameters for Na^+-dependent phosphate uptake (Na^+-dependent minus Na^+-independent uptake) by jejunal BBMV of Hyp mice. Kinetics of jejunal Na^+-dependent phosphate uptake in the Hyp mice showed a significantly greater V_{max} on low P_i diet compared to control-P_i diet ($P < 0.01$). K_m values were similar on low- and control-P_i diet. Figure 4B depicts kinetic analysis in jejunal BBMV of normal mice. V_{max} of phosphate uptake was significantly greater in normal mice on low-P_i diet compared with control-P_i diet ($P < 0.01$). K_m values were similar on low and control diets.

Discussion

The present study was undertaken to determine the kinetic parameters of renal and jejunal BBMV of Hyp and normal mice in response to low-P_i diet. Previous studies have reported an adaptive increase in renal brush border membrane phosphate transport in phosphate restricted Hyp mice (8). This increase was thought to be due to an increase in the high-affinity phosphate transporter (10). Another study also suggested an increase in V_{max} of Na^+-dependent phosphate uptake (11). This increase occurs as early as one day after P_i restriction and continues for at least 14 days. The mechanism for this adaptive response is not known. Boneh *et al.* suggested that protein kinase C may play a role in the renal adaptive responses to phosphate restriction in normal mice, however, it is not clear whether protein kinase C influences the expression of the renal abnormalities in phosphate transport in Hyp mice (11). It has been suggested that protein kinase C is modulated in response to parathormone (PTH). However, Quamme *et al.* demonstrated that the regulation of Na^+-dependent cotransport by PTH and the adaptive response to low phosphate have distinct regulatory control mechanisms (18).

Alternative mechanisms involve $1,25(OH)_2$ vitamin D_3 metabolism as it is well known that low-P_i diet elevates plasma $1,25(OH)_2D_3$. $1,25(OH)_2D_3$ has also nongenomic effects, as reflected by altered membrane composition and an increase in membrane fluidity (19), as well as genomic effects resulting in stimulation of calcium and phosphate uptake (19–21). Danisi *et al.* demonstrated that the adaptive response in phosphate transport is temporally related to the increase in the plasma level of $1,25(OH)_2D_3$ (22). The elevation in plasma $1,25(OH)_2D_3$ might be involved in the adaptation of the intestinal phosphate transport system in response to phosphate restriction. However, the role of $1,25(OH)_2D_3$ in modulating the transport of phosphate in the Hyp mice remains controversial. Some studies suggest that serum level of $1,25(OH)_2D_3$ does not rise in response to low-phosphate diet in the Hyp mice (23).

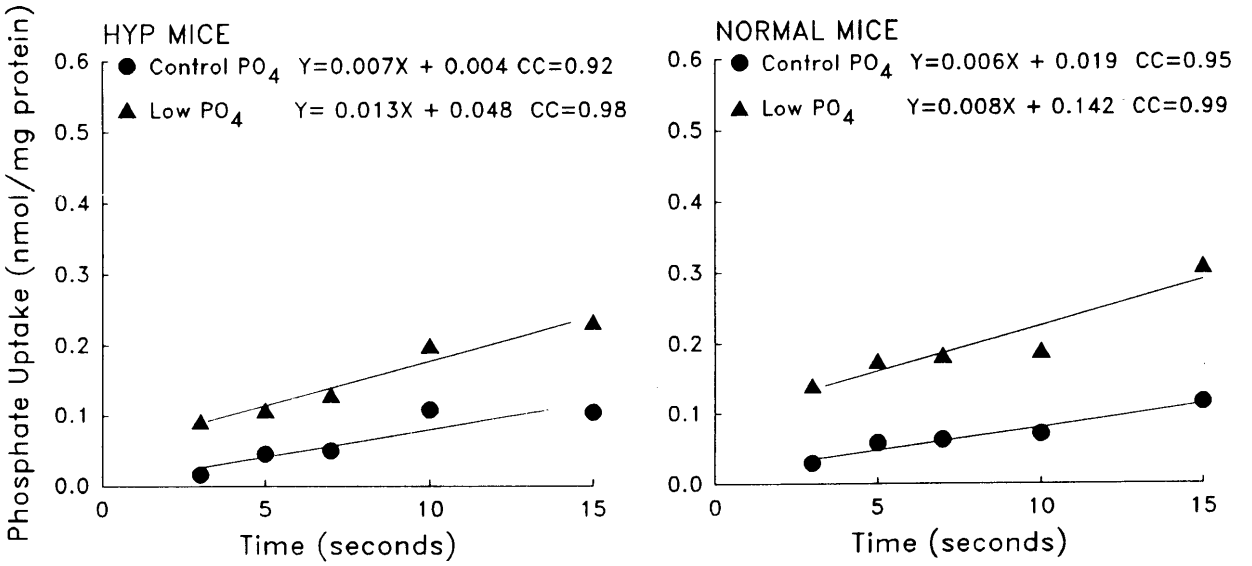

Figure 3. Initial rate on phosphate uptake by jejunal BBMV on low- and control-phosphate diet in the Hyp and normal mice. BBMV were prepared in 280 m*M* mannitol, 20 m*M* Hepes/Tris buffer pH 7.4. Reaction was started by incubating the vesicles with 100 m*M* NaCl, 100 m*M* mannitol, 20 m*M* Hepes/Tris, 0.1 m*M* KH_2Po_4. Reaction was stopped at desired time intervals. Each experiment was run three times in triplicate.

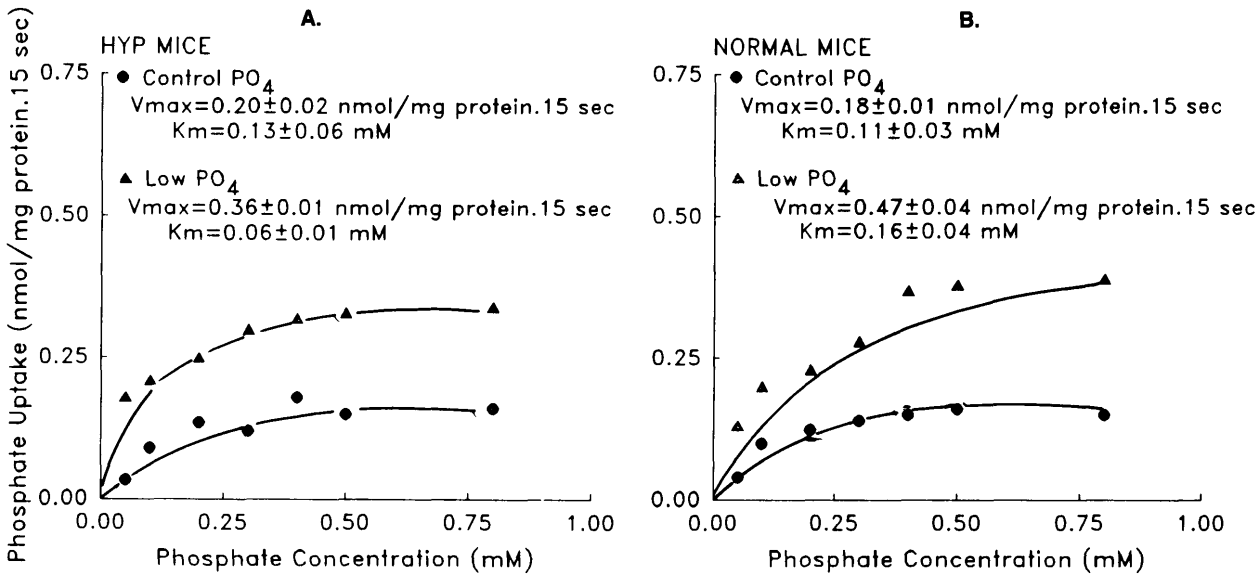

Figure 4. Kinetics of Na^+-dependent phosphate uptake by jejunal BBMV on low- and control-phosphate diet in the Hyp (A) and normal (B) mice. BBMV were prepared in 280 m*M* mannitol, 20 m*M* Hepes/Tris buffer pH 7.4. Reaction was started by incubating the vesicles with 100 m*M* NaCl or 100 m*M* KCL, 100 m*M* mannitol, 20 m*M* Hepes/Tris and varying concentrations of KH_2Po_4 and tracer ^{32}P. Reaction was stopped at 15 sec. Mean uptake values in the presence of 100 m*M* KCL were subtracted from mean values in the presence of 100 m*M* NaCl. Kinetic analyses were done utilizing a computerized model of Michaelis-Menten kinetics. Each experiment was run three times in triplicate.

Regardless of the mechanism involved in the up-regulation of phosphate transport by low-P_i diet, our studies show clearly that low-phosphate diet up-regulates the V_{max} of the renal and jejunal Na^+-dependent phosphate cotransporter in the hypophosphatemic mice. These findings suggest that the Na^+-phosphate transporter in the kidney of the Hyp mice is responsive to low-phosphate diet and argues strongly against a defect in the regulation of the transporter function. It should be emphasized that our studies are *in vitro* experiments and may not reflect the *in vivo* situation.

This study is supported by NIH Grant DK 33209-08

1. Fox J, Care AD. Stimulation of duodenal and ileal absorption of phosphate in the chick by low-calcium and low-phosphorus diet. Calcif Tissue Res **26**:243–245, 1978.
2. Quamme GA. Phosphate transport in intestinal brush border membrane vesicles: Effect of pH and dietary phosphate. Am J Physiol **249**:G168–G176, 1985.
3. Fox J, Care AD. Effect on low-calcium and low-phosphorus diets on the intestinal absorption of phosphate in intact and parathyroidectomized pigs. J Endocrinol **77**:225–231, 1978.
4. Caverzasio J, Danisi G, Straub RW, Murer H, Bonjour JP. Adaptation of phosphate transport to low-phosphate diet in renal and intestinal brush border membrane vesicles: Influence of sodium and pH. Pflugers Arch **409**:333–336, 1987.
5. Berner W, Kinne R, Murer H. Phosphate transport into brush border membrane vesicles isolated from rat small intestine. J Biochem **160**:467–474, 1976.
6. Eicher EM, Southard JL, Scriver CR, Glorieux FH. Hypophosphatemia: Mouse model for human familial hypophosphatemic rickets. Proc Natl Acad Sci USA **73**:4667–4671, 1976.
7. Nakagawa N, Arab N, Ghishan FK. Characterization of the defect in the Na^+-phosphate transport in vitamin D–resistant hypophosphatemic mice. J Biol Chem **266**:13616–13620, 1991.
8. Tenenhouse HS, Scriver CR. The defect in transcellular transport of phosphate in the nephron is located in brush border membranes in X-linked hypophosphatemia. Can J Biochem **56**:640–646, 1978.
9. Tenenhouse HS, Scriver CR. Renal brush border membrane adaptation to phosphorus deprivation in the Hyp/Y mouse. Nature **281**:225–227, 1979.
10. Tenenhouse HS, Klugerman AH, Neal JL. Effect of phosphonoformic acid, dietary phosphate and the Hyp mutation on kinetically distinct phosphate transport processes in mouse kidney. Biochim Biophys Acta **984**:207–213, 1989.
11. Boneh A, Tenenhouse HS. Protein kinase C in mouse kidney: Effect of the *Hyp* mutation and phosphate deprivation. Kidney Int **37**:682–688, 1990.
12. Ghishan FK, Wilson FA. Developmental maturation of D-glucose transport by rat jejunal brush border membrane vesicles. Am J Physiol **248**:G87–G92, 1985.
13. Barnard JA, Ghishan FK, Wilson FA. Ontogenesis of taurocholate transport by rat ileal brush border membrane vesicles. J Clin Invest **75**:869–873, 1985.
14. Lowry DH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**:265–275, 1951.
15. Scharschmidt BF, Keeffe EB, Blankenship NM, Ockner RK. Validation of a recording spectrophotometric method for measurement of membrane-associated Mg and Na^+-K^+-ATPase activity. J Lab Clin Med **93**:790–799, 1979.
16. Beaufay HA, Amas-Costesec AA, Feytmans E, Thinessempoux D, Wibo M, Robbi M, Berthet J. Analytical study of microsomes and isolated subcellular membranes from rat liver. J Cell Biol **61**:188–200, 1974.
17. Hopfer U, Nelson K, Penott J, Isselbacker KJ. Glucose transport in isolated brush border membrane from rat small intestine. J Biol Chem **247**:25–32, 1973.

18. Quamme G, Biber J, Murer H. Sodium-phosphate cotransport in OK cells: Inhibition by PTH and "adaptation" to low phosphate. Am J Physiol **257**:F967–F973, 1989.
19. Brasitus TA, Dudeja PK, Eby B, Lau K. Correction by 1,25-dihydroxycholecalciferol of the abnormal fluidity and lipid composition of enterocyte brush border membranes in vitamin D–deprived rats. J Biol Chem **261**:16404–16409, 1986.
20. Lee DBN, Walling MW, Corry DB. Phosphate transport across rat jejunum: Influence of sodium, pH and 1,25-dihydroxyvitamin D_3. Am J Physiol **251**:G90–G95, 1986.
21. Lee DBN, Walling MW, Brautbar N. Intestinal phosphate absorption: Influence of vitamin D and non–vitamin D factors. Am J Physiol **250**:G369–G373, 1986.
22. Danisi G, Caverzasio J, Trechsel U, Bonjour JP, Straub RW. Phosphate transport adaptation in rat jejunum and plasma level of 1,25-dihydroxyvitamin D_3. Scand J Gastroenterol **25**:210–215, 1990.
23. Meyer RA, Gray RW, Meyer MH. Abnormal vitamin D metabolism in the X-linked hypophosphatemic mouse. Endocrinology **107**:1577–1581, 1980.