# Dietary Regulation of the Renal Sodium-Phosphate ($Na^+/P_i$) Transporter during Early Ontogeny in the Rat (44140)

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*Abstract.* **Phosphates are necessary for proper skeletal growth and function, as well as for growth and development of cells. Phosphate repletion depends partly on the function of the renal sodium-phosphate ($Na^+/P_i$) transport system that functions to recover filtered urinary phosphate. It has been suggested that in order to meet the higher phosphate requirement of the developing animal, the weanling rat would have a greater adaptive response to chronic phosphate deprivation than the adolescent rat. The current study sought to characterize the adaptive response to dietary phosphate deprivation in terms of $Na^+/P_i$ transporter activity, and mRNA and immunoreactive protein levels. Weanling and adolescent rats were pair fed either a low-phosphate diet (LPD) or a control-phosphate diet (CPD) for 1 week. Maximal rates of transport ($V_{max}$) were not different in weanling or adolescent rats on CPD (weanling 2.13 ± 0.29 nmol/mg protein/10 sec, and adolescent 1.41 ± 0.036 nmol/mg protein/10 sec, $n$ = 3). $K_m$ values were not different in either group on CPD (weanling 0.15 ± 0.08 m$M$ $P_i$, and adolescent 0.22 ± 0.13 m$M$ $P_i$). There were no differences in mRNA abundance ($Na^+/P_i$ transporter/1B15 = 0.194 ± 0.12 for weanlings and 0.230 ± 0.03 for adolescents, $n$ = 3) or immunoreactive protein levels ($Na^+/P_i$ transporter/β-actin = 0.232 ± 0.01 for weanlings and 0.300 ± 0.05 for adolescents, $n$ = 3) in the two groups when fed CPD. After chronic $P_i$ deprivation, the weanling rat showed a greater adaptive response than the adolescent as measured by $V_{max}$ values (weanling LPD/CPD = 2.01, $P < 0.01$; adolescent LPD/CPD not different; $n$ = 3), mRNA signal intensity (weanling LPD/CPD = 1.86, $P <$ 0.05; adolescent LPD/CPD not different; $n$ = 3), and protein signal intensity (weanling LPD/CPD = 3.63, $P < 0.01$, and adolescent LPD/CPD 1.91, $P < 0.05$; $n$ = 3). $K_m$ values were not affected by LPD. Immunohistochemical analysis of kidney cortex showed greater apical staining in both groups on LPD, with the increase being noticeably greater in the weanlings.**

**Furthermore, two-way analysis of variance demonstrates a significant adaptive response in the weanling period in regard to maximum transport capacity ($V_{max}$) and immunoreactive protein (Western), suggesting a synergistic effect between the developmental stage and low-phosphate diet. Therefore, it appears that the adaptive response is greater in the more rapidly developing animal (the weanling), and these results suggest a compensatory mechanism to conserve phosphate during periods of rapid growth.** [P.S.E.B.M. 1997, Vol 215]

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Phosphorus is essential for normal mineralization and growth of the skeleton. The repletion of phosphate stores depends on intestinal absorption and the recovery of filtered urinary phosphate through a sodium-dependent phosphate transport system present in the proximal tubular brush border membrane (BBM) of the renal epithelium. The activity of the renal $Na^+/P_i$ transporter varies with dietary levels of phosphate intake, and it has been shown that the maximal rate of transport ($V_{max}$) increases significantly with phosphate deprivation (1–4). The transporter $V_{max}$ also varies with age, but it is not

clear what molecular mechanisms are responsible for these phenomena.

The cDNA encoding the $Na^+/P_i$ transporter has been recently cloned from the rat and mouse kidney (5, 6). These two cDNAs show very high sequence homology with one another (greater than 98%). Polyclonal antiserum has previously been developed against a mouse $Na^+/P_i$ transporter–specific peptide (the peptide shows sequence identity with the rat transporter) (7). Consequently, both physiological and molecular biological techniques can now be used to further our understanding of the renal mechanisms regulating adaptation to chronic phosphate ($P_i$) deprivation in the rat. The current study was undertaken to utilize these molecular tools to characterize the expression and activity of the renal $Na^+/P_i$ transporter in response to chronic phosphate deprivation during the developmental stages of weanling and adolescence.

## Materials and Methods

Sprague-Dawley rats were used for all studies (Harlan, Madison, WI). Eight weanling male rats (21 days old) were taken from two litters and combined into one group (four from each litter). Four rats were chosen at random to receive low-phosphate diet (LPD, 0.02% $P_i$ and 0.45% Ca), and the other four rats received control-phosphate diet (CPD, 0.4% $P_i$ and 0.45% Ca). Similarly, eight adolescent males from two litters (42 days old) were divided into LPD or CPD groups. All experiments were performed on these groups of four animals, with different groups of animals used for repetitive measurements. It should be noted that *n* values given in Results refer to the number of repetitions of the experiment. The diets were identical in content of other ingredients, and these reported $P_i$ levels are final concentrations and are derived from egg white $P_i$ and added calcium phosphate. The rats were pair fed, and the weight of each rat was measured before and after 1 week on the respective diet. Also, the amount of food consumed by each group was recorded. After 7 days on the diet, animals were sacrificed by cervical dislocation, and kidneys were harvested immediately.

**Chemicals and Reagents.** Protein concentration was assayed utilizing the DC method from Bio-Rad (Hercules, CA). $KH_2[^{32}P]O_4$ (1 Ci/nmol) and $[^{32}P]$-dCTP (3000 Ci/nmol) were purchased from NEN/Dupont (Boston, MA). Cellulose nitrate filters used in uptake assays were from Sartorius Filters (Hayward, CA). Ready Protein Plus liquid scintillant for uptake studies was purchased from Beckman (Fullerton, CA). Poly $(A)^+$ RNA was isolated by utilizing the QD kit from 5 Prime–3 Prime (Boulder, CO). Protein gel electrophoresis reagents were from Bio-Rad (Richmond, CA). Immunoblots were processed by the Renaissance chemiluminescent method (NEN/Dupont). Protein molecular weight standards (Rainbow markers), horseradish peroxidase-linked secondary antibodies, and x-ray film (Hyperfilm) were from Amersham (Piscataway, NJ). β-Actin antiserum and accompanying secondary antibodies were from Sigma Chemical Co. (St. Louis, MO). Nitrocellulose membranes were purchased from Micron Separations (Westboro, MA). Control and low-$P_i$ diets were from Dyets Inc. (Bethlehem, PA). Dideoxy chain-termination sequencing was performed using the Sequenase Version 2.0 system (United States Biochemical, Cleveland, OH).

**Brush Border Membrane Isolation and Transport Measurements.** Cortical slices of decapsulated kidneys were pooled from each group of four rats. Renal brush border membrane vesicles (BBMV) were prepared using a well-established magnesium precipitation method that has been extensively described elsewhere (8, 9). Phenylmethylsulfonylfluoride (PMSF) was added to all solutions to 0.5 m*M* final concentration.

A rapid filtration technique was used to measure sodium-dependent phosphate uptake by the vesicles. Uptake was initiated by incubating 20 μl of the BBMV suspension with 100 m*M* NaCl or KCl, 100 m*M* mannitol, 20 m*M* HEPES/Tris, and concentrations of $[^{32}P]$-$KH_2PO_4$ ranging from 0.05 to 2 m*M*. The reaction was stopped at 10 sec by addition of 2 ml of ice-cold stop solution (100 m*M* NaCl, 100 m*M* mannitol, 20 m*M* HEPES/Tris [pH 7.4], and 10 m*M* $KH_2PO_4$). The vesicle suspensions were placed on 0.45-μm nitrocellulose filters under vacuum and washed three times with 2–3 ml of ice-cold stop solution. The radioactivity on the filters was measured by adding a scintillant and determining counts per minute (cpm) in a liquid scintillation counter. Radioactivity bound to the filters in the absence of vesicles was used as background and was subtracted from the counts. Uptake in the presence of KCl was subtracted from the uptake in the presence of NaCl to yield the $Na^+$-dependent component of uptake. Data are expressed as the mean ± SEM for each group and represent the results of at least three separate uptake reactions. Curve fitting and kinetic parameters were determined using the Graft program (Erithacus Software, United Kingdom).

**Reverse Transcriptase–Polymerase Chain Reaction.** The thermostable Taq DNA polymerase (Promega Corp; Madison, WI) and oligonucleotide primers were used in a programmable thermal controller (MJ Research; Watertown, MA). Reverse transcriptase–polymerase chain reaction (RT-PCR) was performed essentially as previously described (10). Primers for amplification of rat $Na^+/P_i$ transporter ($Na^+/P_i$-2) (5) cDNA fragment were as follows: forward primer 5′-ACCTCTGTCACCAACACCAT-3′ at bp 636, and reverse primer 5′-TGAAGAAGAAGTG-GCAGAGG-3′ at bp 1488 (mouse cDNA sequence numbers) (6). Primers were designed from regions that showed nucleotide sequence identity between the rat and mouse cDNAs. Superscript reverse transcriptase (BRL, Bethesda, MD) was used for first-strand cDNA synthesis from 400 ng of rat renal cortex poly $(A)^+$ RNA. Five percent of the cDNA reaction was used for RT-PCR with the following parameters: 45 cycles with 94° C for 45 sec, 58° C for 45 sec, and 72° C for 2 min. PCR products were subcloned

into the TA cloning vector (Invitrogen Corp., San Diego, CA) for sequence analysis and probe generation. The amplified DNA fragment was confirmed by dideoxy chain-termination sequencing utilizing vector-derived and sequence-specific oligonucleotide primers.

**Isolation of mRNA and Northern Blot Analysis.** Following resection, kidneys were immediately frozen in liquid nitrogen. Poly $(A)^+$ RNA was isolated using a commercially available kit. Four micrograms of poly $(A)^+$ RNA from each group was fractionated by 1.5% denaturing agarose gel electrophoresis and transferred to nitrocellulose membranes by standard techniques (11). Probes were generated from rat $Na^+/P_i$ transporter PCR fragment, mouse $Na^+/P_i$ transporter cDNA (6), and 1B15 (encoding rat cyclophilin) (12) cDNAs by random prime labeling. Blots were prehybridized and hybridized as previously described, utilizing $1 \times 10^6$ cpm/ml of buffer (6, 7). High-stringency washes were performed at 65° C with 0.1 × SSC, 0.1% SDS, and blots were placed to film for 4–16 hr. Blots were stripped at 95°C in 0.1 × SSC, 0.1% SDS three times for 15 min. Stripping of the blots was confirmed by overnight autoradiography. Blots were subsequently reprobed with 1B15-specific probes as described above. Quantitation of hybridization signals was done by scanning laser densitometry of the autoradiographs on an UltroScan XL (Pharmacia Biotech, Piscataway, NJ). Experiment was performed in triplicate with poly $(A)^+$ RNA samples from different groups of animals, with hybridization intensities being averaged from the three experiments. $Na^+P_i$ transporter hybridization intensities were normalized for 1B15 levels on the same blot.

**Protein Gel Electrophoresis and Western Blot Analysis.** Rabbit polyclonal antibodies were raised against a mouse $Na^+/P_i$ transporter–specific COOH-terminal peptide ($NH_2$ terminal . . . CRLALPAHHNATRL . . . COOH terminus) (8). The antigenic peptide was generated from a part of the protein which showed amino acid sequence identity between the cloned rat and mouse $Na^+/P_i$ transporter putative proteins. Specificity of this antiserum for the transporter has been documented extensively elsewhere (7, 13).

$Na^+/P_i$ transporter hybridization band intensities were determined by laser scanning densitometry of the autoradiographs, and were normalized for β-actin on the same blot. All experiments were independently performed in triplicate on samples prepared from different groups of animals, with hybridization intensities being averaged for the three experiments.

**Immunohistochemical Analysis of Rat Kidney Cortex.** Kidneys were removed from rats, decapsulated, and cortexes were sliced with a scalpel. Slices were placed in ice-cold phosphate-buffered saline (PBS), pH 7.4, and fixed in paraformaldehyde as previously described (13). Fixed tissue samples were embedded in paraffin, and sections were cut. Tissue sections from the different age groups were placed on separate slides, and slides were processed in identical fashion.

Sections were first deparaffinized by placing slides in a series of xylene and ethanol solutions, followed by rehydration in sterile water. Sections were processed essentially by the manufacturer's protocol (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) as previously described (13). Experiment was performed on tissue sections from two different animals each, and identical results were obtained. Results were analyzed by two independent investigators as blind observers so as to avoid any bias in interpretation.

**Statistical Analysis.** The mean differences were analyzed by analysis of variance (ANOVA) utilizing SPSS/ $PC^+$ (SPSS, Inc., Chicago, IL). Two-way ANOVA was used to test for interaction effects between developmental stage and diet. Results are given as mean ± SEM.

## Results

**Pair Feeding of Rat Groups.** Rats were weighed and given an equal quantity of CPD or LPD. The weight of each animal was again determined after 7 days on the respective diet, and the amount of food consumed by each group was recorded before the animals were sacrificed. There were no significant differences between the weight of animals before and after 1 week on CPD or LPD. Likewise, the amount of diet consumed by each group, regardless of whether the animals were fed the low- or control-phosphate diets, was similar.

**Kinetics of $Na^+$-Dependent Phosphate Uptake in Renal BBMV.** $Na^+$-dependent phosphate transport was determined at phosphate concentrations ranging from 0.05 to 2 m*M* under initial rate conditions (10 sec). Kinetic parameters were derived by using the Michaelis-Menten equation for each group (Fig. 1). The maximal capacity of the transporter ($V_{max}$) and the $K_m$ values are reported in Table I. The $V_{max}$ value of the $Na^+/P_i$ transporter from animals on CPD was 2.13 ± 0.17 nmol/mg protein/10 sec and 1.41 ± 0.21 nmol/mg protein/10 sec in weanling and adolescent rats respectively ($n = 3$). These values were not different. When animals were placed on LPD, the $V_{max}$ values increased to 4.29 ± 0.45 nmol/mg protein/10 sec for weanling rats and 2.28 ± 0.12 nmol/mg protein/10 sec for adolescent rats ($n = 3$; $P < 0.01$). These values represent increases of 2.01-fold in weanlings ($P < 0.01$). In contrast, no difference was observed between adolescent rats on CPD and LPD. The two-way ANOVA results (Table II) demonstrate a synergistic effect between developmental stage and diet, as displayed in Figure 2. The $K_m$ values were not different in either group irregardless of diet. Weanling rats had $K_m$ values of 0.15 ± 0.08 and 0.18 ± 0.10 m*M* on CPD and LPD, respectively. Adolescent rats had $K_m$ values of 0.22 ± 0.13 and 0.24 ± 0.14 m*M* on CPD and LPD, respectively.

**Reverse Transcriptase–Polymerase Chain Reaction.** Rat kidney poly $(A)^+$ RNA was utilized for RT-

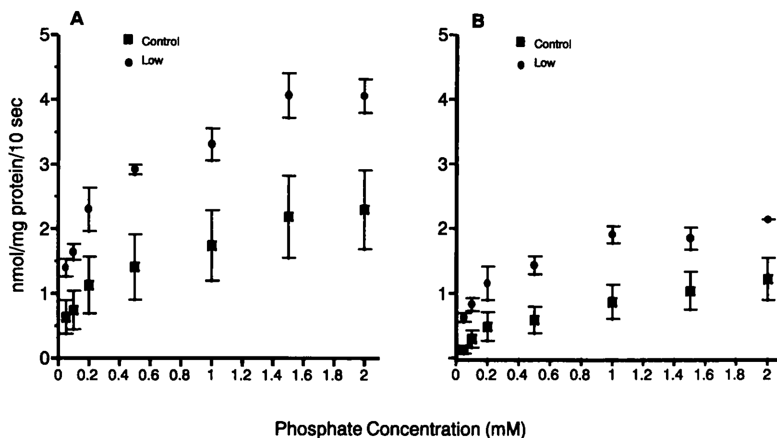



**Figure 1.** Kinetic data of phosphate uptake in 3- and 6-week rats on CPD and LPD. Rats in groups of four were pair fed either CPD or LPD for 1 week, and renal BBMV were prepared. Sodium-dependent uptake of phosphate was assayed at various $P_i$ concentrations under initial rate conditions (10 sec). (A) Uptake data points from 3-week rats. (B) Uptake data points from 6-week rats. ■, data from rats on CPD; ●, data from rats on LPD. Standard error bars are shown for every data point. Kinetic parameters are shown in Table I.

**Table I.** Kinetic Parameters of $Na^+$-Dependent Phosphate Transport in Renal Brush Border Membranes

| Developmental stage–diet | $V_{max}$ | $K_m$ |
|---|---|---|
| Weanling–CPD | 2.13 ± 0.17[a] | 0.15 ± 0.05 |
| Weanling–LPD | 4.29 ± 0.45[b] | 0.18 ± 0.06 |
| Adolescent–CPD | 1.41 ± 0.21 | 0.22 ± 0.08 |
| Adolescent–LPD | 2.28 ± 0.12[c] | 0.24 ± 0.08 |

*Note.* This table depicts the uptake data on renal BBMV isolated from groups of weanling and adolescent rats on the CPD and the LPD. The experiment was repeated in triplicate, and the data was analyzed for statistical significance by one-way ANOVA. No differences were observed between weanling and adolescent rats on CPD, between adolescent rats on CPD and LPD, or in the $K_m$ data.
[a] Statistical significance between weanling rats on CPD and LPD ($P < 0.01$).
[b] Statistical significance between weanling rats on LPD and adolescent rats on CPD ($P < 0.01$).
[c] Statistical significance between weanling and adolescent rats on LPD, between adolescent rats on LPD ($P < 0.01$).

**Table II.** Age and Diet Differences in $V_{max}$ as Analyzed by Two-Way Analysis of Variance

| Two-way ANOVA source of variation | *df* | *MS* | *F* | Significance of *F* |
|---|---|---|---|---|
| Developmental stage | 1 | 5.564 | 25.4 | 0.001 |
| Diet | 1 | 6.924 | 31.6 | 0.000 |
| Developmental stage × diet | 1 | 1.246 | 5.7 | 0.044 |
| Error | 8 | 0.219 | | |

PCR utilizing oligonucleotide primers specific for the mouse $Na^+/P_i$ transporter. These primers were designed from regions of the cDNA that showed sequence identity between the mouse clone and the rat $Na^+/P_i$-2 cDNA (5). PCR reactions showed amplification of a single band of expected molecular mass, while negative control reactions showed no amplification. This DNA fragment was subcloned and sequenced as described. The sequencing results showed that the fragment shared sequence identity with the rat cDNA. This fragment was subsequently used for probe generation for Northern blot analysis.

**Northern Blot Hybridization Analysis.** Northern blot analysis of poly $(A)^+$ RNA isolated from kidneys of rats at different stages of maturation was performed after dietary $P_i$ deprivation. Hybridization of the membranes with the rat $Na^+/P_i$ transporter PCR probe demonstrates a single 2.7-kb transcript in all groups (Fig. 3). Northern blots probed with mouse $Na^+/P_i$ transporter cDNA showed results identical to those obtained with the rat PCR fragment (data not shown). Probes specific for rat cyclophilin (1B15) showed hybridization to a single band at 1.0 kb in all samples. Signal intensity was expressed as a ratio of $Na^+/P_i$ transporter to 1B15 as determined by densitometric analysis (Table III). The signal intensity ratios were not significantly different between the two CPD groups (0.194 ± 0.12 for weanling rats and 0.230 ± 0.03 for adolescent rats; $n = 3$). Chronic $P_i$ deprivation resulted in an increase in the signal intensity ratio in weanling rats, with the signal intensity increasing to 0.360 ± 0.07 ($P < 0.05$). This represents an increase of 1.86-fold. The signal increased to 0.317 ± 0.06 in the adolescent rats with LPD, but it was not statistically higher. No interaction between diet and developmental stage was observed in the two-way ANOVA.

**Western Blot Analysis.** Western blot analysis of rat renal cortex BBM proteins isolated from animals on different diets and at different stages of maturation is shown in Figure 4. An identical pattern of bands is seen in each group. A predominant band was present at 77 kDa in all samples. Other minor protein bands were detected at 126, 97, 81, and 66 kDa (data not shown). All bands are blocked by pretreatment of antiserum with antigenic peptide as extensively documented elsewhere (data not shown) (7, 9, 13). β-Actin antiserum showed hybridization to a single band at 100 kDa, and confirmed near equal loading and transfer of protein (Fig. 4). Signal intensities, expressed as a ratio of the 77-kDa $Na^+/P_i$ transporter bands to β-actin bands, were determined by densitometric analysis (Table III). On the CPD, immunoreactive protein levels were not different between the two groups (0.232 ± 0.01 for weanlings, and 0.300 ± 0.05 for adolescents; $n = 3$). However, when animals were on the LPD, protein levels increased dramatically (0.843 ± 0.03 for weanlings and 0.572 ± 0.07 for adolescents; $n =$

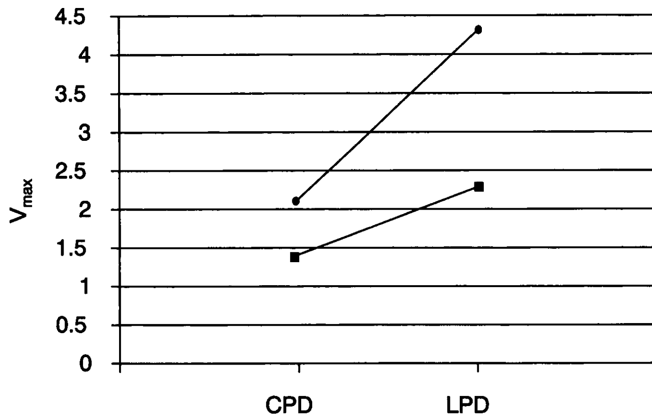



**Figure 2.** Interaction between developmental stage and diet for $V_{max}$ (maximal transport capacity). Developmental stages shown are weanling (W) male rats (21 days old) and adolescent (A) male rats (42 days old). CPD, control-phosphate diet; LPD, low-phosphate diet. The figure indicates significant age and diet interaction. The two-way ANOVA is shown in Table II.

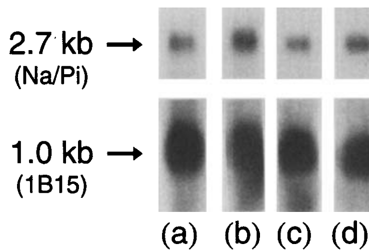



**Figure 3.** Northern blot analysis of rat kidney cortex. Four micrograms of rat renal mRNA was fractionated by denaturing agarose gel electrophoresis and transferred onto nitrocellulose membranes. The blot was probed with an 850-bp DNA fragment specific for the rat renal $Na^+/P_i$ transporter, and was subsequently reprobed with rat cyclophyllin (1B15)-specific probes. The hybridization signal at 2.7 kb represents the $Na^+/P_i$ transporter probes, and the signal at 1.0 kb represents 1B15 probes. Lane a, weanling rats on CPD; Lane b, weanling rats on LPD; Lane c, adolescent rats on CPD; Lane d, adolescent rats on LPD. Signal intensities are compared in Table III.

3). This represents an increase of 3.63-fold ($P < 0.1$) and 1.91-fold ($P < 0.05$) for weanling and adolescent rats on LPD, respectively. The two-way ANOVA results (Table IV) demonstrate further evidence of a synergistic effect between developmental stage and diet. This data is plotted in Figure 5.

**Immunohistochemical Analysis of Kidney Cortex.** Kidney cortexes were obtained from 3- and 6-week rats on CPD or LPD for 1 week. Tissue was fixed, and slices were taken and affixed to slides. Tissue sections were processed with transporter specific antiserum and an immunoperoxidase staining system. Only apical staining was apparent in all samples (Fig. 6A). Three- and six-week samples showed increased apical staining upon dietary phosphate deprivation, with the increase being greater in the weanlings. Additionally, greater numbers of tubules were stained in both groups with LPD. Blocking of the antiserum with antigenic peptide abolished all staining (Fig. 6B). These results were analyzed by the authors and also by two blinded observers, and all were in agreement about interpretation of the data and came to the conclusions stated above.

**Table III.** Quantification of Hybridization Signals from Northern Blots and Western Blots

| Developmental stage–diet | $Na^+$-$P_i$ /1B15 | $Na^+$-$P_i$ /β-actin |
|---|---|---|
| Weanling–CPD | 0.194 ± 0.12[a] | 0.232 ± 0.01[b] |
| Weanling–LPD | 0.360 ± 0.07 | 0.843 ± 0.03[c] |
| Adolescent–CPD | 0.230 ± 0.03 | 0.300 ± 0.05[d] |
| Adolescent–LPD | 0.317 ± 0.06 | 0.572 ± 0.07[e,f] |

*Note.* Mean ± SEM for three independent experiments for Northern blot analyses ($Na^+$-$P_i$/1B15) and Western blot analyses ($Na^+$-$P_i$/β-actin) from the four groups of different age rats were analyzed by one-way ANOVA. No differences were observed were from Northern blot data for adolescent rats on CPD and LPD, between weanling and adolescent rats on LPD, or from Northern and Western blot data between weanling and adolescent rats on CPD.

[a] Statistical significance of Northern blot data from weanling rats on CPD and LPD ($P < 0.05$).

[b] Statistical significance between weanling rats on CPD and LPD ($P < 0.01$).

[c] Statistical significance between weanling rats on LPD and adolescent rats on CPD ($P < 0.01$).

[d] Statistical significance between adolescent rats on CPD and LPD ($P < 0.05$).

[e] Statistical significance between weanling and adolescent rats on LPD ($P < 0.05$).

[f] Statistical significance between weanling and adolescent rats on LPD ($P < 0.01$).

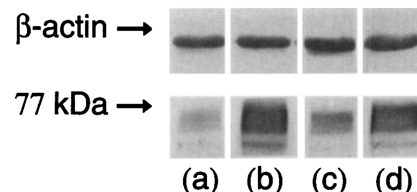



**Figure 4.** Western blot analysis of rat kidney cortex BBM proteins. Thirty-five micrograms of renal BBM protein was fractionated by 10% SDS/PAGE and transblotted onto nitrocellulose membranes. Polyclonal antiserum specific for the mouse and rat $Na^+/P_i$ transporter was used to react with the blots. The blot was stripped and subsequently reacted with β-actin antiserum. The hybridization signal at 77 kDa represents the $Na^+/P_i$ transporter antiserum band, and the signal shown above at 100 kDa represents the β-actin antiserum band. Lane a, weanling rats on CPD; Lane b, weanling rats on LPD; Lane c, is adolescent rats on CPD; Lane d, adolescent rats on LPD. Signal intensities are compared in Table III.

**Table IV.** Age and Diet Differences in Immunoreactive Protein Levels on Western Blots as Analyzed by Two-Way Analysis of Variance

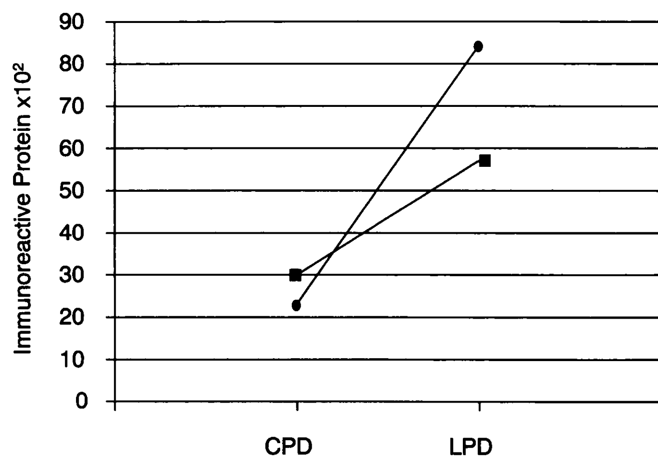
| Two-way ANOVA source of variation | *df* | *MS* | *F* | Significance of *F* |
|---|---|---|---|---|
| Developmental stage | 1 | 0.031 | 4.7 | 0.062 |
| Diet | 1 | 0.584 | 88.6 | 0.000 |
| Developmental stage × diet | 1 | 0.086 | 13.0 | 0.007 |
| Error | 8 | 0.007 | | |

## Discussion

Phosphates are important nutrients for proper mineralization of the skeleton, and deficiency of dietary phosphate results in rachitic bone disease (14). It has been previously suggested that in order to meet the higher phosphate requirements of the developing animal, weanling rats would be able to mount a greater adaptive response to chronic dietary phosphate deprivation than would be seen in phosphate deprived adolescent rats. It was thought that this increased response might lead to higher activity levels, mRNA expression, and immunoreactive protein levels of the renal $Na^+/P_i$ transporter in the weanling animals. The maximal activity of the $Na^+/P_i$ transporter ($V_{max}$) was approximately equal for both groups on CPD. A low-phosphate diet for 1 week resulted in an increase in the $V_{max}$ in both groups. It has been previously determined in a time-course study of 1, 3, and 7 days, that phosphate deprivation for 7 days leads to maximal upregulation of the $Na^+/P_i$ transporter (Collins JF, Ghishan FK, unpublished data). Maximal transporter activity increased 2.01-fold in weanlings and 1.62-fold in adolescents on LPD. $K_m$ values were not different by age and were not affected by dietary $P_i$ levels.

Northern blot analysis of kidney cortical poly $(A)^+$ RNA from groups of weanling and adolescent rats on CPD showed similar levels of the 2.7-kb transporter message. LPD led to an increase of 1.86-fold in mRNA levels in the weanling rats, but the increase in adolescent rats did not reach statistical significance (although an apparent increase was seen in the data). Western blot analysis revealed five bands present at 126, 97, 81, 77, and 66 kDa, with the predominant band being at 77 kDa. The staining appears to be specific, as all protein bands were undetectable upon the addition of antigenic peptide. The rat $Na^+/P_i$ transporter ($Na^+/P_i$-2) has a predicted molecular weight of 64 KDa for the nonglycosylated and 77 kDa for the core-glycosylated form (5). Therefore, it seems likely that the band at 77 kDa represents the active form of the transporter in the rat kidney. The band at 126 kDa may represent dimers, while the other bands are most likely alternatively processed or spliced products, or isoforms of the transporter [a similar situation has been documented in the mouse (7, 9, 13)]. Western blot analysis of renal BBM proteins showed no differences in immunoreactive protein levels of the 77-kDa band in either group on CPD. However, after dietary $P_i$ deprivation, weanlings increased protein levels by 3.63-fold, and adolescents increased protein levels by 1.91-fold. Furthermore, the hybridization intensity differences of the 126-kDa bands seen between samples was approximately proportional to the observed intensity differences in the 77-kDa bands (accurate densitometric analysis of these bands was difficult due to the diffuse nature of these bands). Additionally, immunohistochemical analysis exemplified the specificity of the antiserum for the transporter as only apical staining was apparent in the kidney cortex. This technique exemplified the increased ability of the weanling rat to upregulate protein levels in response to dietary $P_i$ deprivation.

Previous studies by Lelievre-Pegorier *et al.* have examined the effect of early weaning (16 days) in rats and found a decrease in Na-dependent phosphate uptake in kidney BBMV when compared to controls (14–21 days) (15). These authors postulated that the difference may be due to enhanced phosphate supply. The results of this study are not directly comparable to the current investigation since weanling rats used in this study were in the presence of the maternal rat. Using thyroparathyroidectomized rats at four different age groups (3–4, 5–6, 10–14, and 52 weeks) for their experiments, Haramati *et al.* have reported that the



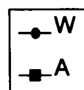
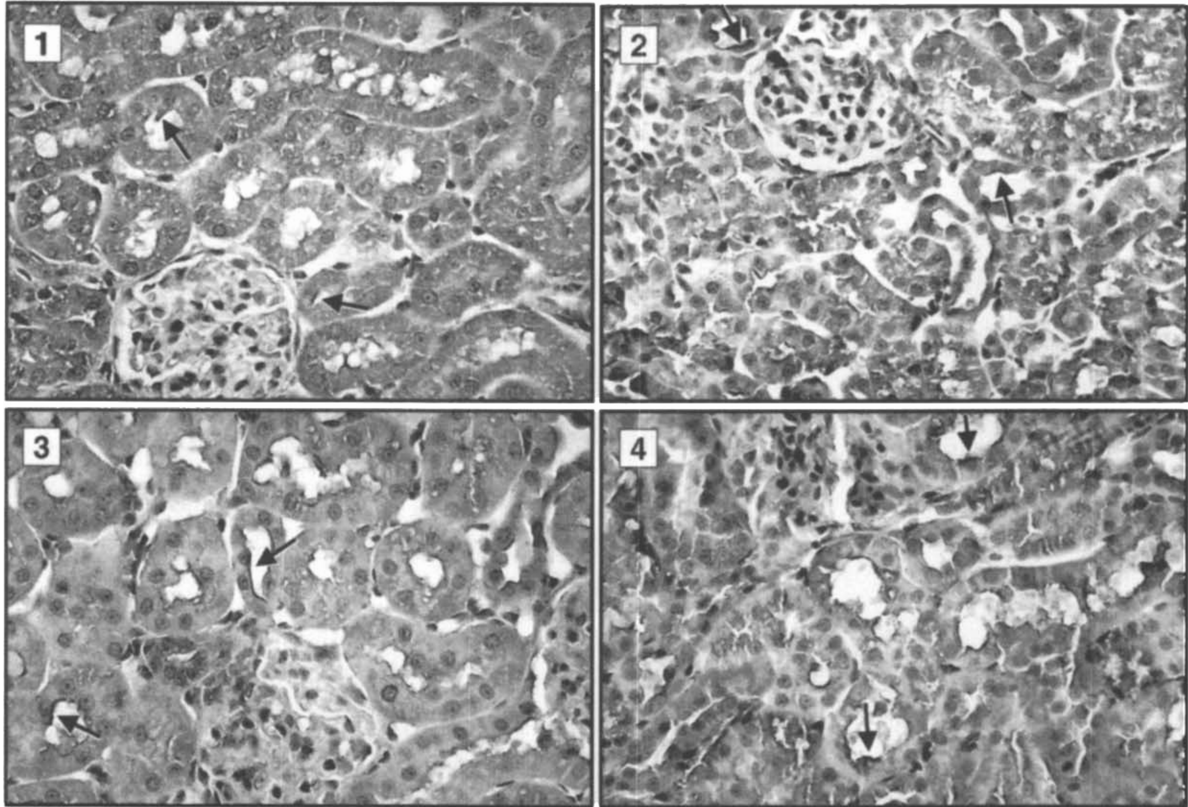




**Figure 5.** Interaction between developmental stage and diet for the immunoreactive protein levels from Western blot analysis. Developmental stages shown are weanling (W) male rats (21 days old) and adolescent (A) male rats (42 days old). CPD, control-phosphate diet; LPD, low-phosphate diet. The figure indicates significant age and diet interaction. The two-way ANOVA is shown on Table IV.



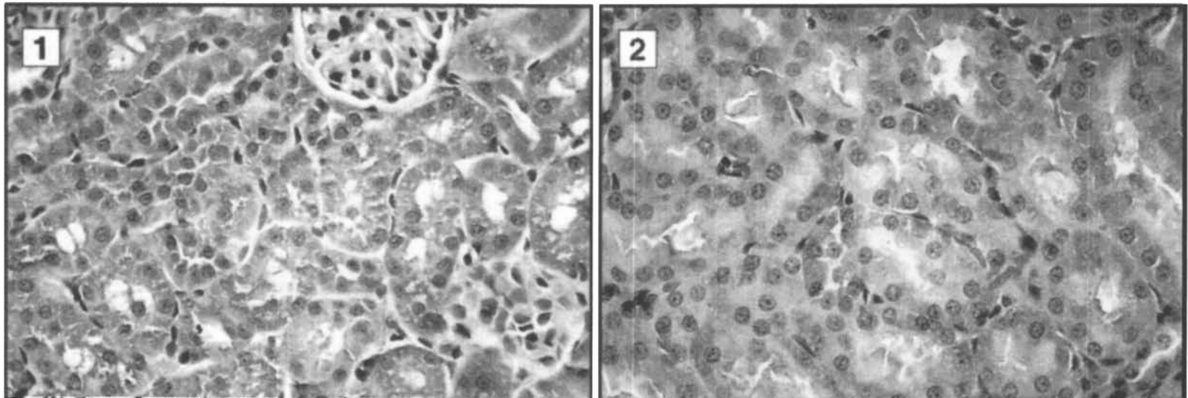




**Figure 6.** Immunohistochemical analysis of rat kidney cortex. Kidney cortical sections were taken from 3- and 6-week rats on CPD or LPD for 1 week. Tissue was fixed, embedded in paraffin, and slices were taken and affixed to slides. Tissue sections were processed with polyclonal antiserum specific for the $Na^+/P_i$ transporter and an immunoperoxidase staining system. (A) Sections reacted with untreated antiserum. (B) Reaction with antigenic peptide blocked antiserum (400 µg/ml for 16 hr at 10°C). Panel A: 1, 3-week CPD; 2, 3-week LPD; 3, 6-week CPD; 4, 6-week LPD. Panel B: 1, 3-week LPD; 2, 6-week LPD. Magnification: ×360. Arrows in Panel A indicate areas of specific staining.

maximum capacity of phosphate reabsorption was highest in the 3- to 4-week rats compared with other age groups (16). However, their investigation did not look at the effect of dietary phosphate deprivation. This study contrasts the results of the present investigation, which shows near equivalent activity of the transporter in 3-week rats as compared with adolescent rats at 6 weeks. The discrepancies could be due to experimental techniques, dietary $P_i$ levels, or other intangible factors. Caverzasio *et al.* reported that $Na^+$-dependent phosphate uptake in BBMV was higher in young growing (8-week) rats than in adult (8- to 9-month) rats fed a low phosphate diet, but differences were abolished when rats were fed a high-phosphate diet (HPD); 0.8% $P_i$ (17). Direct comparison of this study with the current results is difficult since adult rats were not studied, and dietary $P_i$ levels were different. Using renal BBMV from guinea pigs,

Neiberger *et al.* have demonstrated that low dietary $P_i$ did not significantly affect the $V_{max}$ of $Na^+/P_i$ transport in the newborn (3–14 days), but increased it in the older (>57 days) animals (18). Once again, direct comparison between the current study and this one is difficult since the ages and the experimental species were not the same. Werner *et al.* demonstrated a 2-fold decrease in the $V_{max}$ of $Na^+/P_i$ transport in uptake studies performed on renal BBMV from 200- to 250-g thyroparathyroidectomized rats placed on a LPD when compared with rats on a HPD with 1.3% $P_i$ (2). Biber *et al.* have reported similar results on rabbits fed a LPD when compared with HPD (3). It therefore becomes apparent that previous investigations are difficult to reconcile with the present results due to the fact that different age groups, as well as different species, have been used. Additionally, in the current and previous studies, variable dietary $P_i$ levels have been studied, and this further complicates data comparison.

Previous studies on the rabbit kidney cortex have shown no differences in the activity of brush border membrane $Na^+/P_i$-1 transporter between animals on a high- or low-phosphate diet (3). Levi *et al.* reported that in the rat kidney cortex there was an increased abundance of $Na^+/P_i$-2–related protein when animals were fed a low- compared with a high-phosphate diet (19). This study postulates that the increase in transporter protein activity represents a change in membrane composition due to recruitment of a related regulatory protein. One recent investigation showed a significant increase in mouse renal $Na^+/P_i$ transporter message, and immunoreactive and functional protein levels in response to dietary phosphate deprivation (7). Animals of different ages were not examined in this investigation. Another recent study examined the effect of low-$P_i$ diet in 3- and >12-week rats, and showed increased $Na^+/P_i$ transporter message, and immunoreactive protein levels in both age groups (20).

The present studies suggest that weanling rats have a greater ability to upregulate the renal $Na^+/P_i$ transporter upon chronic $P_i$ deprivation than adolescent rats. This is exemplified by the two-way ANOVA showing significant interaction between developmental stage and diet in regard to $V_{max}$ and immunoreactive protein. The data suggest increased *de novo* synthesis of the $Na^+/P_i$ transporter mRNA which leads to corresponding changes in immunoreactive protein levels. Immunoreactive protein levels may seem disproportionately increased due to differences in the sensitivity and quantification capabilities of the techniques used to assess mRNA and immunoreactive protein levels [similar problems associated with quantifying mRNA and immunoreactive protein level changes associated with diet induced changes in expression of this transporter have been documented previously (13)]. Or alternatively, increased mRNA translation or stability may play a role in these observations. However, these differences do not obscure the fact that the overall trend seen here strongly suggests an increased capability of the weanling rats to adapt to $P_i$ deprivation. A previous investigation also showed discrepancies between transporter activity, message levels, and immunoreactive protein levels (21). In this study, 7 days of low-$P_i$ diet increased BBMV uptake 3.4-fold, mRNA levels by 2.2-fold, and immunoreactive protein levels by 4.9-fold.

To analyze further the interaction between the developmental stage and diet, the data was subjected to two-way ANOVA. As seen in Tables II and IV, and in Figures 2 and 5, a significant synergistic effect was noted between the developmental stage and low-phosphate diet with regard to $V_{max}$ and protein expression levels. This synergism was not found with the mRNA levels, suggesting that differences in mRNA stability or translational efficiency may play a role in the observed differences in immunoreactive and functional protein levels between the two age groups studied.

In conclusion, this is the first study to examine the effect of chronic $P_i$ deprivation on the activity, mRNA, and immunoreactive protein expression of the renal $Na^+/P_i$ transporter across the developmental stages of weaning and adolescence in rats. Although previous studies investigating the functional upregulation of the $Na^+/P_i$ transporter with relation to ontogenic stage and chronic phosphate deprivation have not been in agreement, the present study shows very clear results. Overall, the data presented here suggest an increased adaptive capability to dietary phosphate deprivation of weanling rats as compared with the response of adolescent rats. These data further confirm previous suggestions that more rapidly developing animals have increased capabilities to adapt to chronic $P_i$ deprivation.

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