Renal Adaptation to a Low Phosphate Diet in Rats: *BLOCKADE BY ACTINOMYCIN D*

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BLOCKADE BY ACTINOMYCIN D

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ABSTRACT The major renal adaptive changes in response to selective dietary phosphate restriction are a marked reduction in urinary excretion of phosphate and an increased urinary excretion of calcium; at the cellular level, there is selective increase in renal cortical brush border membrane phosphate uptake and increase in specific activity of alkaline phosphatase. In the present study we examined whether these functional and biochemical adaptive changes could be blocked by drugs known to inhibit protein synthesis.

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In separate experiments, using the same dietary protocol as above, brush border membrane fraction (vesicles) was prepared from renal cortex of rats sacrificed at the end of the experiment. In this preparation Na^+-dependent ^{32}Pi and D-[^{3}H]glucose uptake and activities of brush border enzymes membrane were determined. Brush border membrane vesicles prepared from rats fed a low phosphate diet showed significantly higher Na^+-dependent ^{32}Pi uptake compared with rats fed a normal phosphate diet. This increase in ^{32}Pi uptake was completely prevented when rats on a low phosphate diet were simultaneously treated with actinomycin D. These differences were specific for ^{32}Pi transport as no differences were observed in D-[^{3}H]glucose uptake among the three groups. There was a positive correlation ($r = 0.82$, $P < 0.01$) between ^{32}Pi uptake and specific activity of alkaline phosphatase measured in aliquots of the same brush border membranes, whereas no such correlation was observed with two other brush border membrane enzymes γ-glutamyltransferase and leucine aminopeptidase.

These observations show that actinomycin D prevents both the functional and cellular renal adaptive changes induced by a low phosphate diet. Taken together, these observations suggest that renal adaptation to a low phosphate diet could be prevented by inhibition of *de novo* protein synthesis.

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INTRODUCTION

The kidney responds to the decrease in dietary phosphorus (P)[1] intake by precipitous decline in the urinary excretion of phosphate ($U_{Pi}V$) as well as by a marked increase in urinary excretion of calcium ($U_{Ca}V$) (1, 2). These adaptive changes in response to a low P diet cannot be accounted for by changes in the filtered load of inorganic phosphate (Pi) or calcium (Ca) and are most likely the result of increased tubular reabsorption of Pi (3–6) and decreased tubular reabsorption of Ca (1, 2, 5). Renal adaptation to a low P diet occurs in thyroparathyroidectomized (3–5) and vitamin-D deficient (5, 7) animals, thus indicating that parathyroid hormone (PTH), vitamin D, calcitonin, and thyroid hormones are not primarily responsible for the adaptation. Similarly, the adaptation is apparently not the result of changes in extracellular volume (4, 8), serum Ca, and/or urine acidification (4, 8).

Some recent observations indicate that at least a part of tubular adaptation, namely, the increase in the Pi reabsorption, occurs at the luminal brush border membrane (BBM) of proximal tubules (6, 9, 10). In animals stabilized on a low P diet, the Na^+-dependent Pi transport across renal cortical BBM is specifically increased (9, 10), whereas in animals fed a high P diet, Pi transport is depressed (10) compared with animals fed a normal P diet. These observations suggest that in the proximal tubule, luminal BBM uptake may be the single most important determinant of Pi reabsorption in response to variations in P content of diet.

In several biological systems P deprivation is accompanied by an increase in alkaline phosphatase (AlPase) activity. In P-deprived microorganisms AlPase is induced (11, 12); an increase in AlPase activity was also observed in kidneys of rats placed on a low P diet (10, 13, 14). This increase in renal AlPase in rats was specific compared with a number of other renal enzymes (10, 13, 14), and BBM activity of AlPase was positively correlated with the rate of Na^+-dependent ^{32}Pi uptake across renal BBM (10).

Several observations reported in the literature suggest that *de novo* protein synthesis may be important for both functional and biochemical aspects of the adaptation to P deprivation. Cellular Pi uptake increases markedly when microorganisms, *Chlorella pyrenoidosa* (15) and *Neurospora crassa* (16), are grown in media with low Pi content, and this increased Pi uptake can be prevented by cycloheximide (15, 16), a protein synthesis inhibitor. Further, the increase in renal AlPase in rats fed a low P diet is also prevented by protein synthesis inhibitors (13).

In the present study, we examined whether renal functional (decreased $U_{Pi}V$, increased $U_{Ca}V$), and BBM-adaptive (Na^+-dependent Pi uptake across BBM and increased AlPase activity) changes in rats placed on a low P diet could be prevented by drugs that inhibit *de novo* protein synthesis.

METHODS

Adult male Sprague-Dawley rats weighing about 200 g were housed in metabolic cages and allowed ad lib. intake of distilled water; the body weight and total urine output were recorded daily. In the course of experiments, rats were fed (as described in detail in Results) the following diets: regular rat diet (Purina Laboratory Rat Chow, Ralston Purina Co., St. Louis, Mo.) containing 0.7% Pi and 1.2% Ca used as "normal P diet"; alternatively (as specified in Results), normal P diet was prepared from the low P diet (ICN Pharmaceuticals Inc., Life Sciences Group, Cleveland, Ohio) supplemented with a mixture of sodium and potassium phosphate (Na:K ratio 1:1.2 and using a ratio of monobasic:dibasic salts of 1:4 to maintain neutral pH) to a final content of 0.6% Pi. "Low P diet" (ICN Pharmaceuticals, Inc.) contained 0.07% Pi and 0.4% Ca.[2]

General experimental design. For the first 3 d of the experiment, rats were fed a normal P diet, and based on the observation in this period, animals were divided into groups having similar $U_{Pi}V$ and body weight. In the following days of the experiment, rats were either continued on the same diet or switched to a low P diet (as specified in Results); from the 3rd d on, "experimental" animals received daily intraperitoneal injections of the tested drugs, whereas "control" animals received solvents without drugs (placebo). It should be emphasized that both control and experimental (drug-treated) rats received the same diet during the course of the experiment (see Fig. 1). Some rats treated with actinomycin D or cycloheximide developed diarrhea, one animal in the actinomycin D-treated group died, but in general, rats tolerated the drug treatment well. Drug-treated rats consumed the same quantity of food as controls and, although they tended to lose weight in the course of the treatment, control and experimental rats did not differ significantly in body weight either at the beginning or at the end of the experiments. At the end of the experiments venous blood was drawn under ether anesthesia; rats were then sacrificed, kidneys removed and immediately chilled by immersion in ice-cold 0.9% NaCl solution. After decapsulation a small piece of kidney cortex was cut with a razor blade and frozen in liquid nitrogen; 5-μm sections of this tissue were cut in cryostat for histologic examination. Sections were stained using standard hematoxylin-eosin and periodic acid-Schiff methods. The rest of the cortical tissue was dissected from the medulla and used for preparations either of total crude membrane (total particulate) fraction or the BBM fraction. Total crude membrane fraction (washed 100,000 *g* pellet of homogenate) was prepared separately for

[1] *Abbreviations used in this paper*: AlPase, alkaline phosphatase; BBM, brush border membrane; b wt, body weight; Ca, calcium; Cr, creatinine; P, phosphorus; Pi, inorganic phosphate; PTH, parathyroid hormone; $U_{Ca}V$, urinary excretion of calcium; $U_{Cr}V$, urinary excretion of creatinine; $U_{Pi}V$, urinary excretion of phosphate.

[2] Content of Pi and Ca in all diets was determined in our laboratory after solubilization of samples either with the use of hot nitric acid or, alternatively, by boiling the dried diet for 1 h in a mixture of concentrated nitric acid and perchloric acid (3:1, vol/vol). Results of our analysis were close to values claimed by the manufacturer (claimed values, in grams per 100 g. diet, are in parentheses); (a) ICN low P diet: Ca, 0.4 (0.41); Pi $\leqq$ 0.07 (<0.07); (b) Purina normal P diet; Ca, 1.21 (1.24); Pi, 0.71 (0.76).

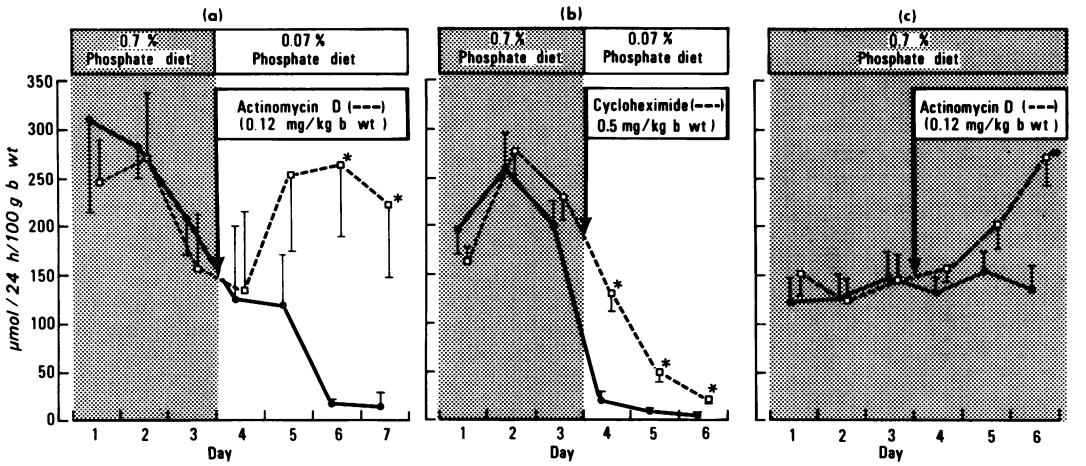

FIGURE 1 Effect of actinomycin D and cycloheximide on $U_{Pi}V$. Three separate experiments (a–c) are shown. During the course of each experiment, both control and experimental group received identical diets. In all three experiments, for the first 3 d animals were fed normal P diet (0.7% P). Then rats were switched to low P diet and received daily intraperitoneal injections of actinomycin D (panel a) or cycloheximide (panel b) while controls also on low P diet received placebo injections. In one experiment (depicted on panel c) rats were continued on normal P diet and the experimental group was treated with actinomycin D (on days 4–6) while controls received placebo injections. Shaded areas, period on normal P diet; clear areas, period on low P diet. *, Significantly differs from controls on the same day at $P < 0.05$ or higher level of significance; *t* test. ●, Control group; □, experimental (drug-treated group): (a) control, $n = 8$; experimental, $n = 7$; (b) control, $n = 9$; experimental, $n = 18$; (c) control, $n = 8$; experimental, $n = 9$.

each individual rat. For preparation of BBM fraction, cortical tissues from three to four rats were pooled together. All the preparations of fractions from cortical homogenates were conducted at 0°–4°C.

Crude membrane fraction. The cortical tissue was homogenized in isotonic medium (1:10 wt/vol) containing 0.25 M sucrose, 5 mM Tris–HCl, 3 mM $MgCl_2$, 1 mM EDTA, pH 7.4, using a motor-driven glass-Teflon homogenizer (E. I. Du Pont de Nemours & Co., Inc., Wilmington, Del). The homogenate was centrifuged at 100,000 *g* for 1 h. The pellet was resuspended in a hypotonic buffer (volume equal to original homogenate) containing 5 mM Tris–HCl, 3 mM $MgCl_2$, 1 mM EDTA, pH 7.4, and centrifuged again at 100,000 *g* for 60 min. The resulting pellet (containing all sedimentable particles) was resuspended in a small volume of the hypotonic buffer, divided into several aliquots and frozen rapidly in dry ice and stored at −80°C. This fraction contains all sedimentable particles, including all membrane sedimentable by ultracentrifugation, and is referred to in the text as "crude membrane fraction."

Preparation of a BBM fraction. BBM fraction was isolated from homogenized renal cortex basically as described by Beck and Sacktor for rabbit kidney (17), and used in our previous study in rats (10). This BBM fraction which reseals spontaneously into vesicles was used for studies on Pi and D-glucose transport in vitro as well as for measurement of specific BBM enzyme activities (10). Briefly, renal cortex was homogenized by Polytron probe PT-10-ST, (Brinkmann Instruments, Inc., Westbury, N. Y.) with three pulses, each 30 s, in a medium (1:20 wt/vol) containing 50 mM mannitol, buffered with 2 mM Tris-Hepes, pH 7 (mannitol-tris-Hepes buffer). 1 M $CaCl_2$ solution was added to the homogenate to a final concentration of 10 mM $CaCl_2$, and after stirring on ice for 20 min, the homogenate was centrifuged at 1,500 *g* for 10 min to remove calcium-aggregated particles. The supernate was collected and centrifuged at 35,000 *g* for 20 min. The pellet—BBM vesicle fraction—obtained at this step was suspended in 300 mM mannitol, buffered with 5 mM Hepes-Tris (pH 8.5), and centrifuged again at 35,000 *g* for 15 min. This procedure was repeated three times. Finally, this washed BBM vesicle fraction was used immediately for measurement of Na^+-dependent uptake of ^{32}P and of D-[^{3}H]glucose; aliquots of the same BBM preparation were frozen rapidly in dry ice and stored at −80°C for measurement of enzyme activities.

Identity and purity of BBM fraction was assessed by comparison of "marker enzymes" in BBM fraction to original renal cortical homogenate. This BBM:homogenate ratio for typical BBM enzymes γ-glutamyltransferase and maltase was 25.0 and 18.0, respectively; for basolateral plasma membrane marker (Na^+-K^+) ATPase, the ratio was 0.5; for mitochondrial marker enzymes cytochrome *c* oxidase and succinate dehydrogenase, the ratio was 0.07 and 0.06, respectively. These results are similar to those obtained by others (18–20) and indicate high purity of BBM fraction.

To exclude the possibility that renal cortical membranes from low P diet rats have different behavior in the course of fractionation, in a control experiment, recoveries of AlPase between low P diet rats and normal P rats were compared. Recovery of AlPase in BBM fraction from kidneys of low P rats (22±3%) did not differ significantly from kidneys of normal P rats (20±2%), values comparable to those reported by Kinne (21). Moreover, the total AlPase activity was significantly higher only in BBM fraction, but not in the other fractions of the homogenate of the renal cortex, from low P rats compared with controls. Electron microscope examination of pellet (kindly performed by Dr. R. E. Scott, Department of Pathology and Anatomy, Mayo Clinic) confirmed the vesicular nature of BBM particles in this fraction.

Enzyme assays. In each experiment, the enzyme activities of both the control and experimental groups were assayed at the same time in order to eliminate interassay variation. AlPase was assayed in 0.1 M glycine–NaOH buffer (pH 10.4), containing 5.6 mM *p*-nitrophenyl phosphate as a substrate; the total volume of the reaction mixture was 1.6 ml. After incubation at room temperature, the reaction was stopped by the addition of 30 μl of 6 N NaOH and the extinction at 405 nm was determined. Because *p*-nitrophenyl phosphate may be hydrolyzed by other phosphohydrolases, the specificity of the hydrolysis at pH 10.4 was ascertained by the use of specific inhibitors of AlPase: levamisole (22–24), *l*-bromotetramisole (25), and L-homoarginine (26). Addition of 1 mM levamisole, 0.1 mM *l*-bromotetramisole, or 14 mM homoarginine caused almost complete (>90%) inhibition of AlPase activity.

The activity of γ-glutamyltransferase was determined at pH 8.2 in the presence of 4 mM glycylglycine (27) and the activity of maltase by the method of Dahlqvist (28). Ouabain-sensitive (Na^+-K^+)-ATPase was assayed as described by Post and Sen (29). Activities of succinate dehydrogenase, cytochrome *c* oxidase, and leucine aminopeptidase were assayed as described in previous studies from our laboratory (30, 31). All measurements of enzyme activities were carried out under conditions in which the reaction rate was linear with respect to both time and protein content. These conditions were established in preliminary experiments.

Transport measurements. Uptake of ^{32}P-phosphate and D-[^{3}H]glucose by isolated BBM vesicles were measured by the Millipore filtration technique (Millipore Corp., Bedford, Mass.) described in detail by Beck and Sacktor (17) and used also by Hoffman et al. (32), and in our previous study (10). The final composition of the incubation medium used for measurement of Pi uptake was 100 mM mannitol, 100 mM NaCl, 5 mM Hepes-Tris, pH 8.5, 0.1 mM $K_2H\ ^{32}PO_4$ (≅3 x 10^5 cpm/tube). Concentration of ^{32}Pi selected for transport studies (0.1 mM), the same as in our other study (10) and by other investigators (33–35), is lower than in plasma Pi and closer to Pi concentrations expected in proximal tubular fluid. Since it has been shown (9) that only V_{max} (maximal velocity) but not K_m of renal BBM uptake of ^{32}Pi is altered by variation in P diet, differences in the Pi transport rate are detectable in a wide range of ^{32}Pi concentrations employed (9). The incubation medium for experiments on D-glucose uptake was identical except that instead of ^{32}Pi, 0.05 mM D-[6-^{3}H]glucose (≅10^6 cpm/tube) was included. All solutions used for the preparation of BBM fractions and for transport measurements were filtered through a 0.45-μm Millipore filter on the day of use. Transport of Pi and glucose at different time periods was measured in triplicate, and mean±SE was entered as $n = 1$. Uptake by BBM vesicles from the three compared groups of rats (see Results) was always measured on the same day.

Uptake of both ^{32}Pi and D-[^{3}H]glucose in the presence of NaCl increased rapidly and reached a peak in the case of ^{32}Pi within 1–2 min, and in the case of D-glucose within 0.5–1.0 min, so-called "overshoot" (10, 32, 36, 37), and then gradually declined to a much lower level of "equilibrium point" at 120 min. This time interval for equilibrium point was chosen, as in our previous study (10), at 120 min primarily because of slow equilibration of Pi across the BBM (32). The initial overshoot was abolished when 5 mM sodium arsenate, a competitive inhibitor of Pi transport (10, 32), was added. Replacement of 100 mM NaCl by 100 mM KCl abolished the initial overshoot and reduced ^{32}Pi uptake at 1 min by >90% (Fig. 2). The initial overshoot of glucose was eliminated by addition of 1 mM phlorizin, a specific competitive inhibitor of D-glucose transport (10, 36, 37), or by replacement of NaCl with KCl. Uptake of D-[^{3}H]glucose at 120 min in the presence of NaCl was identical to the uptake in the presence of KCL, indicating complete equilibration (Fig. 2). On the other hand, in the same BBM vesicle preparation, in the presence of NaCl even at 120 min the ^{32}Pi uptake was still slightly higher (≅16% of the peak value) than in the presence of KCl (Fig. 2), indicating incomplete equilibration, due perhaps to some degree of "trapping" of ^{32}Pi in the intravesicular space (19). When osmolality of the medium was varied by adding sucrose (32, 37), the extent of ^{32}Pi or D-[^{3}H]glucose uptake (either in the presence of NaCl or KCl) was inversely proportional to osmolality. In

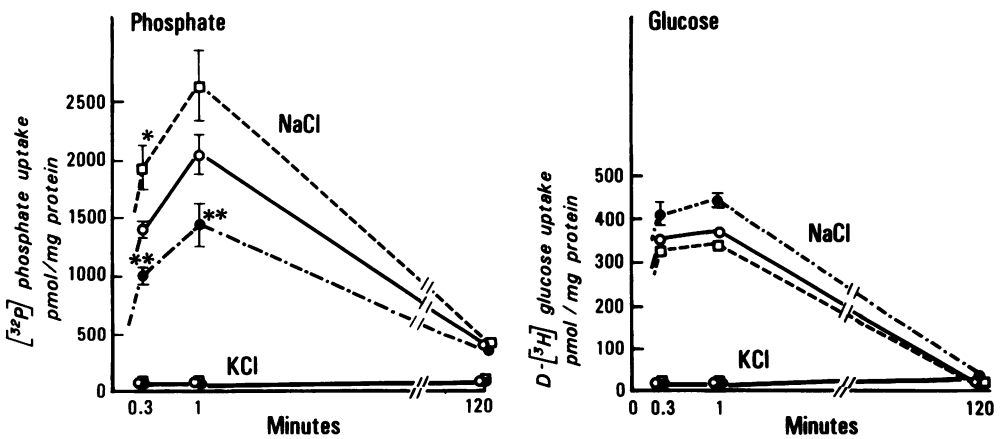

FIGURE 2 Na^+-dependent ^{32}Pi (left panel) and D-[^{3}H]glucose uptake (right panel) by BBM vesicles from renal cortex. Three separate preparations (each from three to four rats) from three different groups (see Methods) were studied. ^{32}Pi and D-[^{3}H]glucose uptakes were studied separately in aliquots of the same BBM vesicle preparation. ○, Normal P diet rats; □, low P diet rats; ●, low P diet rats treated with actinomycin D; *, $P < 0.05$ (or higher significance) compared with normal P diet rats; *t* test; **, $P < 0.05$ (or higher significance) compared with low P diet rats; *t* test. NaCl denotes uptake in the presence of sodium gradient. KCl denotes uptake when NaCl was replaced by KCl.

the case of D-[^{3}H]glucose, uptake at infinite medium osmolarity projected completely to zero. With ^{32}Pi, a small uptake at projected infinite osmolality was observed, and it can be calculated that at an osmolarity of ≅300 mM (final osmolarity in our measurements) ≅15% of ^{32}Pi remained in BBM vesicles, suggesting that unlike D-[^{3}H]glucose, slight BBM trapping of ^{32}Pi occurs, likely because of binding (19) in intravesicular BBM space. Taken together, these observations indicate that uptake reflects true transmembrane movement and did not reflect adsorption or trapping of D-[^{3}H]glucose, and only quantitatively minor trapping of ^{32}Pi was detected. These observations indicate that the majority of the vesicles were intact (32, 37, 38), and the radiolabeled material retained on filters represented compounds taken up into the intravesicular space and were not simply a result of binding to the vesicle exterior. Based on these preliminary experiments and previous studies (10), the ^{32}Pi transport was measured at 20 s, a time point representing "uphill" phase of the uptake, at 60 s, a time point representing maximum or near maximum (peak) of the overshoot, and at 120 min chosen approximately as "equilibrium time" point.

Clearance studies. Rats were anesthetized with pentobarbital (50 mg/kg) and tracheostomy performed. Polyethylene catheters PE-50 (Clay Adams, Parsippany, N. J.) were inserted in jugular veins for infusions and in the carotid artery for blood sampling and blood pressure monitoring (31). Urine was collected from a polyethylene catheter PE-100 inserted into the bladder. Body temperature was maintained at 37°C with a thermal regulated table.

Rats were primed with 0.5 ml of 4% insulin in saline, and the rate of infusion was kept constant at 0.02 ml/min. With a second infusion pump, rats were also infused with 3–5 ml of normal saline for 60 min to expand extracellular volume by ≅10%, as estimated from their body weight, and thereafter saline infusion rate was adjusted to match the urinary output. First, three control periods (10 min each) were collected and then a priming dose of actinomycin D (0.12 mg/kg) was given. Assuming distribution of the drug in total body water compartment, actinomycin D was infused continuously over the next 30 min at a rate matched to urinary output. Three experimental periods, 10 min each, were collected after the priming dose of actinomycin D. Time control experiments were conducted in exactly the same way except that actinomycin D was omitted from infusion fluid in the experimental periods. For both control and experimental periods, the mean of the three periods was used for calculations. The clearance of inulin was calculated by the standard formulas as measure of glomerular filtration rate and for calculation of fractional excretion of Pi (31).

Analytical methods. Plasma and urinary Pi were measured by the method of Chen et al. (39), plasma creatinine was measured colorimetrically (40). Urinary proteins were measured by the biuret method (41). The presence of glucose in the urine was checked by Clinitest (Ames Co., Inc., Elkhart, Ind.). The presence of amino-aciduria was evaluated with use of high-voltage electrophoresis (42); calcium in plasma and urine samples was determined by atomic absorption spectrometry (Jarrell-Ash Division of Fisher Scientific Co., Waltham, Mass.). Inulin concentrations in plasma and urine were determined by the colorimetric method of Walser et al. (43). Urinary urea nitrogen was measured by a colorimetric method based on Fearson reaction (44) using Harleco (Harleco, Co., Philadelphia, Pa.) urea nitrogen kits, urinary uric acid by modification of the method of Liddle et al. (45). Protein in tissue fractions was determined by the method of Lowry et al. (46) after solubilization of the samples in 1% sodium lauryl sulfate, as in our previous studies (30, 31). All analyses were done in duplicate or triplicate.

The results were evaluated statistically by the Student *t* test for group comparisons. Values of $P > 0.05$ were considered not significant.

Materials. Actinomycin D (Cosmegen) was purchased from Merck, Sharp and Dohme Co. (West Point, Pa.) and cycloheximide from Sigma Chemical Co. (St. Louis, Mo.). Carrier-free ^{32}P (NEX-054) was purchased from New England Nuclear (Boston, Mass.) and D-[^{3}H]glucose from Amersham Corp. (Arlington Heights, Ill.). *p*-Nitrophenyl phosphate and other enzyme substrates were purchased from Sigma Chemical Co. *l*-2,3,5,6-Tetrahydro-6-phenyl-imidazo (2,1-6)-thiazole hydrochloride (TRAMISOL, *l*-tetramisole hydrochloride or levamisole hydrochloride) was a gift from Dr. R. H. Schenkel, American Cyanamid Co. (Princeton, N. J.). *l*-*p*-Bromotetramisole (*p*-bromolevamisole) was a gift from Dr. M. Borgers, Janssen Pharmaceutica (Beerse, Belgium). L-Homoarginine was purchased from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.). Low P diet was purchased from ICN Pharmaceuticals, Inc.; regular rat diet—Purina Laboratory Rat Chow—was purchased from Ralston Purina Co. All other drugs, chemicals, and biochemicals were of highest purity grades and purchased from standard suppliers.

RESULTS

For the first 3 d rats were fed normal P diet[3] and then switched to the low P diet. In the period on the low P diet (days 4–7), one group of animals was treated with intraperitoneal injection of actinomycin D (in a dose of 0.12 mg/kg body weight [b wt] per d), whereas another control group received only injections of the solvent. As shown in Fig. 1, control rats, when placed on a low P diet, showed sharply reduced $U_{Pi}V$ (Fig. 1, panel a). On the other hand, animals also on a low P diet treated with actinomycin D maintained the $U_{Pi}V$ during days 4–7 at the level comparable to the initial period on normal P diet. In addition, the $U_{Ca}V$, which was not different between the two groups of rats in the control period on normal P diet, increased markedly on a low P diet in control animals, but no such increase in $U_{Ca}V$ occurred in the actinomycin D-treated group (Table I*a*).; $U_{Ca}V$ in actinomycin D-treated rats was markedly lower than in controls (Table I*a*).

Mean daily food intake was not different between control and actinomycin D-treated rats. At the end of the experiment, actinomycin D-treated animals did not differ from controls in plasma levels of Ca, Pi, or Cr, and there was also no difference in urinary excretion of creatinine ($U_{Cr}V$) (Table I*a*). The two groups did not differ in the total urinary excretion of the urea nitrogen (control rats: 83±4 mg/24 h per 100 g b wt±SEM; actinomycin D-treated rats: 99±32 mg/24 h per 100 g b wt±SEM), or in the total urinary excretion of uric acid (control rats: 0.54±0.2 mg/24 h per 100 g b wt ±SEM; actinomycin D-treated rats: 0.79±0.2 mg/24 h

[3] Normal P diet, unless specified otherwise, indicates Purina Laboratory Rat Chow containing 0.7% P (wt/wt). Low P diet indicates diet containing 0.07% P (wt/wt); see Methods for details.

TABLE I

Effect of Actinomycin D and Cycloheximide on Renal Adaptation to Low P Diet
Urine and Plasma Values, and Specific Activities of Renal Cortical Enzymes

	Urine values					Plasma values			Specific activities of renal cortical enzymes		
	$U_{Pi}V$		$U_{Ca}V$								
	(A)	(B)	(A)	(B)	$U_{Cr}V$	Ca	P_i	Cr	Alkaline phosphatase	γ-Glutamyl-transferase	Leucine aminopeptidase
	μmol/24 h/100 g b wt				*mg/24 h/100 g b wt*	*mM*	*mM*	*mg/dl*	*μmol/h/mg protein*		
a. Control (8)*	207 ±37‡	16.0 ±12.0	3.6 ±1.1	29.8 ±3.8	6.6 ±2.6	2.32 ±0.07	1.33 ±0.20	1.03 ±0.12	11.3 ±0.8	32.2 ±4.2	0.4 ±0.03
Actinomycin D (7)	156 ±56	220 ±76	2.6 ±0.6	5.2 ±1.2	5.4 ±7.6	2.22 ±0.24	1.73 ±0.12	0.99 ±0.07	8.3 ±0.9	39.6 ±3.1	0.5 ±0.04
P value§	NS	<0.02	NS	<0.001	NS	NS	NS	NS	<0.05	NS	NS
b. Control (9)	200 ±23	4 ±1	6.8 ±1.3	41.8 ±17.6	5.8 ±7.8	2.60 ±0.10	1.67 ±0.14	0.58 ±0.04	13.0 ±0.7	62.1 ±3.8	0.5 ±0.02
Cycloheximide (18)	231 ±26	19 ±4	5.3 ±0.7	8.6 ±2.1	4.8 ±3.0	2.24 ±0.03	1.47 ±0.09	0.58 ±0.05	9.8 ±0.4	55.8 ±3.5	0.5 ±0.02
P value	NS	<0.02	NS	<0.02	NS	<0.001	NS	NS	<0.001	NS	NS
c. Control (8)	145 ±29	138 ±25	4.0 ±0.8	2.9 ±0.5	3.9 ±0.1	2.40 ±0.05	2.28 ±0.05	0.68 ±0.02	10.0 ±0.7	49.4 ±3.2	1.5 ±0.20
Actinomycin D (9)	141 ±24	275 ±30	3.8 ±0.8	4.2 ±0.9	4.0 ±0.2	2.28 ±0.12	2.32 ±0.11	0.72 ±0.07	8.1 ±0.4	51.8 ±4.1	1.5 ±0.17
P value	NS	<0.005	NS	NS	NS	NS	NS	NS	$0.05 < P < 0.1$	NS	NS

Values are from collections at the end of experiment (see Methods), except for $U_{Pi}V$ and $U_{Ca}V$, when values at 3rd (A) and the last day (B) are included. See Methods and Fig. 1 for details of the three experiments *a*, *b*, and *c*.

* Number of animals in parentheses.

‡ Mean±SE.

§ Differences between control and drug-treated rats; group *t* test.

per 100 g b wt±SEM) measured at the last day of the experiment.

In the crude membrane fraction of renal cortical homogenate, the specific activity of AlPase was significantly lower in the actinomycin D-treated animals, whereas activities of γ-glutamyltransferase and leucine aminopeptidase were not different between the two groups (Table I*a*).

The effect of cycloheximide, another inhibitor of protein synthesis (47, 48), was examined with use of the similar experimental design as in the experiment described above (Fig. 1, panel b). Rats switched from the normal P to low P diet treated with cycloheximide (0.5 mg/kg b wt/d) decreased $U_{Pi}V$, namely, on days 4 and 5 (Fig. 1, panel b); however, the decrease was significantly less than in the placebo-treated control group. At the end of the experiment (day 6) $U_{Pi}V$ was markedly declined, but still significantly higher than in controls (Fig. 1, panel b; Table I*b*). As in the experiment with actinomycin D, cycloheximide-treated rats did not increase $U_{Ca}V$ compared with placebo-treated controls (Table I*b*). Control- and cycloheximide-treated rats consumed the same quantity of food. Cycloheximide-treated animals had slightly lower plasma Ca; plasma levels of Pi and Cr were not different between cycloheximide-treated and control groups. Likewise, the two groups did not differ in $U_{Cr}V$ (Table I*b*). The AlPase activity in crude membrane fraction of renal cortical homogenate from cycloheximide-treated rat was significantly lower compared with controls, although the two groups did not differ in the specific activities of γ-glutamyltransferase and leucine aminopeptidase (Table I*b*).

In actinomycin D- and cycloheximide-treated animals no evidence of tubular damage was found on light microscopic examination of renal cortical tissue. In

addition, the actinomycin D- or cycloheximide-treated animals showed no signs of glycosuria, amino-aciduria, or proteinuria.

In addition to the effect on renal adaptation to low P diet, the effect of actinomycin D on renal handling of Pi and Ca as well as on the renal enzymes was also examined in animals maintained on the normal P diet through the whole time span of the experiment. Experimental design was similar to the experiments described above with experimental rats being injected with actinomycin D (in the same dose and route as in the first experiment), and controls with placebo (Fig. 1, panel c). Actinomycin D-treated animals did not differ from controls in $U_{Pi}V$ in days 1–5; only at the last day did actinomycin D-treated rats have a significantly higher $U_{Pi}V$ compared with control rats treated with placebo, although actinomycin D treatment had no effect on $U_{Ca}V$ (Fig. 1, panel c; Table Ic). At the end of the experiment, the actinomycin D-treated and control groups did not differ in plasma levels of Ca, Pi, Cr, or $U_{Cr}V$. The two groups did not differ in the activities of leucine aminopeptidase and γ-glutamyltransferase; specific activity of AlPase tended to be lower (difference was of borderline statistical significance) in actinomycin D-treated animals (Table Ic).

To examine whether the observed effects of actinomycin D may be a result of a direct acute effect on the tubular handling of the phosphate, effects of this drug were examined in clearance experiments. Animals were fed a low P diet for 3 d, then fasted overnight and prepared for the clearance experiments as described in Methods. Results summarized in Table II indicate that infusion of actinomycin D had no effect on fractional excretion of phosphate or on the glomerular filtration rate.

The decrease in $U_{Pi}V$ in response to a low P diet is likely the result, at least in part, of increased P reabsorption in the proximal tubule (6). In addition, an increase in Pi uptake by BBM vesicles from rats on a low P diet has been reported (9, 10). In view of this, effects of low P diet alone or with actinomycin D treatment on BBM transport of Pi and glucose were examined in BBM vesicles prepared from the renal cortex. For the initial 3 d animals were kept on the normal P diet (low P diet supplemented with Na and K phosphate to a final phosphate content of 0.6% wt/wt; for details see Methods). On the 3rd d animals were divided into three groups having similar $U_{Pi}V$ and body weight. One group was continued on the same normal P diet for the next 3 d. Two other groups were switched to the low P diet (in which Na and K phosphate were replaced by an equivalent amount of NaCl and KCl); one group was injected with actinomycin D (0.12 mg/kg b wt/d, i.p.), and the other with solvent without the drug. As observed in the preceding experiments (Fig. 1), animals switched from the normal P diet to low P diet reduced $U_{Pi}V$ and increased $U_{Ca}V$ compared with animals continued on the normal P diet (Table III). As in our previous experiment (Fig. 1, panel a), rats switched to the low P diet but treated with actinomycin D had markedly higher $U_{Pi}V$ and lower $U_{Ca}V$ compared with animals fed the same diet but not treated with the drug (Table III). The three groups did not differ in plasma levels of Pi, Ca, or Cr (data not shown here).

At the end of the experiment rats were sacrificed and BBM fraction from renal cortices were prepared as described in Methods. As shown in Fig. 2, ^{32}Pi uptake by BBM vesicles in the initial rapid uphill phase (20 s) and peak (1 min) was higher in animals fed a low P diet compared with animals on a normal P diet. On the other hand, in BBM vesicles from animals also fed a low P diet but treated simultaneously with actinomycin D, the initial ^{32}Pi uptake was even lower than in the BBM vesicles from control rats fed normal P diet, and markedly lower than in animals fed a low P diet treated with placebo (Fig. 2). In contrast to the changes in ^{32}Pi transport, no significant differences in D-[6-^{3}H]glucose transport were observed; if anything,

TABLE II
Acute Effects of Actinomycin D on Fractional Excretion of Phosphate

	FE_{Pi}*		GFR‡		*Blood pressure*	
	C§	E‖	C	E	C	E
	(phosphate clearance/inulin clearance) × 100		*ml/min*		*mm Hg*	
Actinomycin D (n = 4)	0.09±0.01¶	0.10±0.02	2.15±0.25	2.21±0.73	113±6	116±5
Time control (n = 4)	0.11±0.01	0.09±0.03	2.65±0.14	3.20±0.20	117±5	114±5

* FE_{pi}, fractional excretion of phosphate.
‡ GFR, inulin clearance.
§ C, mean of control clearance periods.
‖ E, mean of clearance periods after actinomycin D infusion.
¶ Mean±SEM.

TABLE III

Effect of Actinomycin D on Renal Adaptation to Low P Diet: Urinary Excretions and Specific Activities of Renal Cortical Enzymes Measured in Isolated BBM Fraction

		Urine values					Specific activities of enzymes in BBM fraction		
		$U_{Pi}V$		$U_{Ca}V$			Alkaline phosphatase	γ-Glutamyltransferase	Leucine aminopeptidase
		(A)	(B)	(A)	(B)				
		μmol/24 h/100 g b wt					*μmol/h/mg protein*		
a. Normal P diet	(9)*	965 ±114§	632 ±56	2.3 ±0.4	1.0 ±0.1	(3)‡	36.7 ±4.8	410.5 ±4.3	13.5 ±1.9
b. Low P diet	(9)	1,063 ±74	26 ±14	1.4 ±0.2	38.0 ±9.1	(3)	49.0 ±3.4	372.0 ±19.1	13.6 ±1.2
c. Low P diet + actinomycin D	(11)	1,139 ±115	488 ±33	1.6 ±0.1	0.7 ±0.1	(3)	30.8 ±4.3	369.1 ±48.4	12.1 ±0.5
P value‖									
A↔B		NS	<0.001	NS	<0.005		NS	NS	NS
B↔C		NS	<0.001	NS	<0.001		<0.05	NS	NS
A↔C		NS	<0.05	NS	<0.05		NS	NS	NS

Urinary values are from day 3 (A) and the last day (B) of experiments, and enzyme values from the last day of experiments. For details see Methods.

* Number of observations.

‡ Renal cortices from three to four animals were pooled to obtain one preparation of BBM fraction in which enzymes were measured in triplicate and entered as one value, $n = 1$.

§ Mean±SEM.

‖ *P* value calculated from *t* test using unpaired comparisons.

the differences in D-[6-^{3}H]glucose uptake among the three groups in the initial uphill phase tended to be in an opposite direction from those in ^{32}Pi uptake (Fig. 2). At the late time period of 120 min—an equilibrium time point—there were no differences in the ^{32}Pi or D-[6-^{3}H]glucose uptake among the three experimental groups of animals, indicating that differences in ^{32}Pi uptake were not due to differences in BBM vesicle size. When NaCl was replaced by KCl in the incubation, the rate of ^{32}Pi uptake was not different among the three groups. Uptake of ^{32}Pi in the presence of NaCl at 120 min was slightly higher than in the presence of KCl, likely reflecting small residual trapping of ^{32}Pi within BBM vesicles (see Methods), but was of the same extent in all three groups. There were no significant differences in activities of γ-glutamyltransferase and leucine aminopeptidase in BBM fraction among the three groups of animals. On the other hand, the activity of AlPase in the actinomycin D-treated animals fed the low P diet was significantly lower than in the low P diet controls injected with placebo (Table III). The rate of ^{32}Pi uptake in the initial uphill phase of transport (20-s interval) was positively correlated ($r = 0.82$, $n = 9$, $P < 0.01$) with the activity of AlPase measured in the same BBM fraction (Fig. 3), but there was no significant correlation of ^{32}Pi uptake with activities of the other two tested BBM enzymes.

DISCUSSION

Dietary P deprivation results in a marked reduction in $U_{Pi}V$ and also an increase in $U_{Ca}V$ (1, 2). Micropuncture studies indicate that at least part of the increased Pi reabsorption in low P diet occurs in the proximal tubule (6). In the proximal tubule, the initial step in Pi transport is entry into tubular cells across the luminal BBM. Enhanced Na^+-dependent Pi uptake by isolated BBM vesicles from rats on low P diet (9, 10) indicates that transport across BBM is an important step in the adaptive phenomenon at least in proximal tubule. The only early biochemical change in BBM that accompanies P deprivation known at the present time is an increase in renal cortical AlPase activity (13, 14) located in the BBM fraction (10, 14). Melani et al. (13, 49) have shown that the increase in AlPase activity is a result of increased synthesis of this renal cortical enzyme.

In the present study we examined the effect of actinomycin D, an inhibitor of *de novo* protein synthesis at the transcription level (47, 48), on the functional and biochemical adaptation. We elected to study actino-

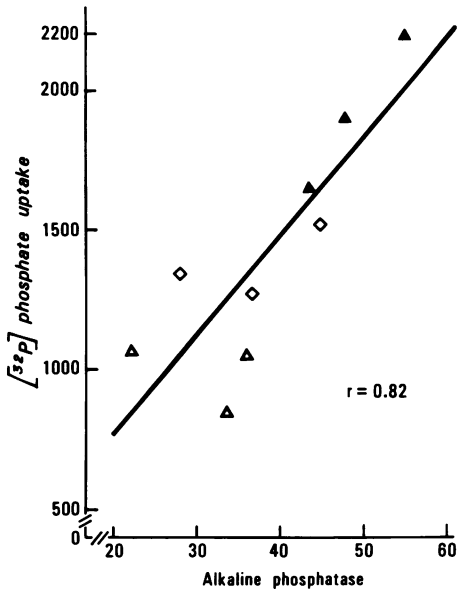

FIGURE 3 Correlations between Na^+-dependent ^{32}Pi uptake (pmol/mg/min; ordinate) at 20-s periods, and alkaline phosphatase activity (μmol/mg/h; abscissa) measured in the same BBM preparations. Each point represents one BBM preparation; correlation coefficient ($r \pm SE$) was $+0.82 \pm 0.03$, $P < 0.01$, $n = 9$. Nine pairs ($n = 9$) (three from each group) were used to calculate correlation coefficient. For details see legend to Fig. 2 or Methods. (◇, BBM preparation from normal P diet rats; ▲, BBM preparation from low P diet rats; △, BBM preparation from low P diet rats treated with actinomycin D (for details see Methods).

mycin D because Melani and associates have previously shown that the biochemical change, namely, increase in renal AlPase in response to low P diet, can be prevented by actinomycin D (13, 49), and the dose required for this effect is well tolerated by rats. Effects of cycloheximide, another inhibitor of protein synthesis that has a different mechanism of action (47, 48), on renal adaptation were also examined in the initial experiments.

The general procedure was to feed both experimental and control groups a regular diet (normal P diet) and then switch both groups to a low P diet to elicit renal functional and biochemical adaptive changes. Thus, both experimental and control groups received exactly the same diet. Although the commercially available normal P and low P diets differ not only in the content of P but also in the content of some other minerals, these differences do not seem to play a major role in adaptation to dietary P deprivation. Marked reduction in $U_{Pi}V$ and rise in $U_{Ca}V$ were observed (Table III) when rats were fed a normal P diet (Pi added to commercial low P diet), and then switched to a low P diet. This observation was similar to that found in our other experiments (Fig. 1; Table I) and with reports from other laboratories (1, 2).

Treatment with actinomycin D or with cycloheximide prevented or delayed the reduction in $U_{Pi}V$ and the increase in $U_{Ca}V$. Comparison of plasma levels of Pi, Ca, and Cr with $U_{Pi}V$, $U_{Ca}V$, and UcrV suggests that differences in renal excretion of Pi and Ca between drug-treated and control animals are solely or predominantly a result of the differences in renal tubular reabsorption rather than because of the changes in the filtered loads of these ions. Cycloheximide only incompletely prevented a decrease in $U_{Pi}V$. Namely, the difference in $U_{Pi}V$ at the last day (day 6) of the experiment was quantitatively small compared with the effects of actinomycin D (Fig. 1, panels a and b), although changes produced by both drugs were in the same direction. This may be a result of different pharmacokinetics of both drugs or a number of other factors. Higher doses of cycloheximide could not be used in the present experimental setting because, according to our preliminary experiments, they would have been prohibitively toxic. Decrease in tubular reabsorption of Pi in actinomycin D-treated and in cycloheximide-treated low P diet rats, resulting in higher $U_{Pi}V$, is unlikely to be a consequence of generalized and nonspecific renal tubular injury. Neither actinomycin D- nor cycloheximide-treated animals showed any signs of general tubular dysfunctions such as glycosuria, animo-aciduria, proteinuria, and no histologic tubular damage was found in microscopic examination of renal tissues. Furthermore, general tubular damage would not account for low $U_{Ca}V$ in the drug-treated animals.

Unlike selective reduction of P in the diet, lack of P intake because of total fasting does not evoke antiphosphaturic response (50–52). It should be considered whether actinomycin D and cycloheximide might have caused a state of starvation although this mechanism of action is unlikely. Even though drug-treated animals tended to lose weight, they consumed normal amounts of food. In a control experiment we found that only total fasting prevents the antiphosphaturia, but even with 75% reduction of total food intake (which leads to loss of body weight) rats adapt to low P diet by precipitous reduction in $U_{Pi}V$[4].

[4] In a control experiment rats were fed with a normal P diet or low P diet for 3 d either consuming unrestricted amounts of food (13–14 g/24 h) or allowed only 3 g/24 h which caused loss of body weight. Rats fed 3 g of low P diet showed antiphosphaturic response (reduction of $U_{Pi}V$ from $1,349 \pm 62$ to 43 ± 20 μmol Pi/24 h per 100 g b wt) similar to rats fed an unrestricted amount of low P diet (reduction of $U_{Pi}V$ from $1,445 \pm 86$ to 2 ± 0.2 μmol Pi/24 h per 100 g b wt). On the other hand, rats completely fasted for 3 d maintained relatively high $U_{Pi}V$ (440 ± 40 μmol Pi/24 h per 100 g b wt) at the end of the experiment. Complete documentation will be supplied with reprint requests.

A survey of several BBM enzymes even in relatively crude total membrane fraction indicates that in kidneys of animals in which antiphosphaturic response to low P diet was blocked by administration of actinomycin D, or diminished by cycloheximide, the specific activity of AlPase but not other BBM-associated enzymes (γ-glutamyltransferase and leucine aminopeptidase) was significantly lower in drug-treated rats. This suggests that an increase in AlPase occurring in the response to low P diet (10, 13, 14) was prevented with drug treatment. Thus, actinomycin D and cycloheximide had a similar, although not quantitatively identical, effect on adaptive change both in the renal excretory parameters and the AlPase activity measured in the total membrane fraction.

It seems unlikely that the effect of actinomycin D and cycloheximide to prevent the decrease in $U_{Pi}V$, the increase in $U_{Ca}V$, and the increase in renal cortical AlPase activity is the result of direct acute effects of these drugs. In vitro addition of actinomycin D or cycloheximide directly to renal cortical membrane fraction did not influence AlPase (data not shown here). Further, in clearance experiments, actinomycin D administered acutely to rats adapted to low P diet did not increase fractional excretion of Pi, showing that in rats already adapted to low P diet actinomycin D has no direct effect on tubular handling of Pi. It is also unlikely that increased $U_{Pi}V$ in the actinomycin D- and cycloheximide-treated rats could be a result of the phosphaturic effect of increased levels of endogenous PTH. There is no indication that the use of actinomycin D or cycloheximide would promote PTH formation or release; on the contrary, various protein synthesis inhibitors were found to inhibit protein biosynthesis in parathyroid glands (53) and, specifically, synthesis of PTH (54). According to a recent report (51), the well-known resistance to phosphaturic effects of PTH (55) occurs after 3 d of low P diet (51)—a period used in our studies. Inasmuch as actinomycin D treatment tended to lower total plasma Ca (Table I*a* and *c*; ionized Ca was not measured), in a control experiment we examined the effect of actinomycin D in thyroparathyroidectomized rats, using design analogous to that in the experiment depicted in Fig. 1, panel 1*a*, and Table I*a*. Rats thyroparathyroidectomized 14 d before experiment adapted to low P diet by a sharp drop in $U_{Pi}V$, but this drop was later prevented by actinomycin D treatment.[5] This control experiment affirms that actinomycin D action to prevent antiphosphaturia is not a result of its effect on PTH release and action.

To locate the effect of actinomycin D on adaptive changes in BBM of proximal tubules, in the present study we examined whether actinomycin D could prevent both the enhanced ^{32}Pi transport and increase in AlPase in BBM vesicles prepared from rats on a low P diet. ^{32}Pi uptake by BBM vesicles from renal cortices of animals fed a low P diet compared to those fed a normal P diet was significantly higher in the active uphill phase of the transport process. This finding is similar to other reported studies (9, 10). In contrast, rats on a low P diet, but treated with actinomycin D, showed marked reduction in ^{32}Pi transport. This is unlikely to be the direct effect of actinomycin D because addition of this drug directly to BBM vesicles does not inhibit ^{32}Pi uptake (data not shown). Lack of differences in D-glucose transport in three groups emphasizes that the observed differences in ^{32}Pi uptake were not the result of general inhibition of Na^+-dependent transport by actinomycin D. According to basic observations on crude membrane cortical fraction, where the differences cannot be well quantified, the specific activity of AlPase in BBM fraction was higher in rats on low P diet than in rats on the same diet but treated with actinomycin D. In addition, correlation between the uphill phase of ^{32}Pi uptake and AlPase activity in BBM (Fig. 3), and lack of similar correlation with other BBM enzymes, further suggest a specific association between Pi transport and AlPase.

Our results show that actinomycin D and, to a lesser degree, cycloheximide prevent the functional and biochemical adaptation in rats to a low P diet. These observations suggest that tested drugs, namely, actinomycin D, prevented induction of synthesis of proteins involved in Pi transport and in AlPase activity of BBM.

Experiments using general inhibitors of protein synthesis such as actinomycin D and cycloheximide should be interpreted with caution, given their inherent limitations. Actinomycin D and cycloheximide are known to inhibit *de novo* protein synthesis; however, the effect of these inhibitors administered to the whole organisms depends on the drug distribution in various tissues as well as on the turnover rate of each individual protein. AlPase has been shown in other studies (13, 49) to have a rather high turnover rate, and it was also shown that administration of actinomycin D prevents the increase in AlPase activity in response to low P diet, whereas a number of other enzyme activities, mostly of glycolytic pathway, are not influenced (13). Considering the similar principles, it could be inferred that protein(s) involved in one or more components of Pi uptake in BBM have a higher turnover rate than other BBM proteins such as those involved in Na^+-dependent glucose transport or proteins related to other BBM enzymes such as γ-glutamyltransferase or leucine aminopeptidase. Regardless of the mechanism involved, this selectivity of actinomycin D effect on Pi transport and AlPase in BBM strongly suggests that the ability of this drug to prevent functional renal adaptation is not a nonspecific, generalized toxic action. If actinomycin D inhibits synthesis of BBM protein(s) related to Pi transport and AlPase which have a high turnover rate

[5] Data not shown. Complete documentation will be supplied with reprint requests.

as discussed above, this could also explain increased Pi excretion on the last day in normal P diet rats treated with actinomycin D (Fig. 1, panel c; Table Ic) and be consistent with a decrease in AlPase observed in the same animals. It thus appears that actinomycin D not only prevents induction of protein(s) related to Pi transport and AlPase in rats on low P diet, but also reduces turnover of Pi transport-related protein(s) in rats in normal P balance.

Although association between AlPase and Pi transport in kidney (10, 14, 31) and intestine (56) has been observed both in previous studies and in the present study, it does not constitute a direct evidence that these two BBM components are causally related. It is of interest to note, however, that in intestinal epithelium, which in many ways is similar to renal brush border, Moog and Glazier (56) showed that the Pi transport varied in parallel with AlPase activity and that agents inhibiting AlPase activity resulted in the inhibition of Pi transport (56). Also, according to a recent preliminary report, levamisol, a specific AlPase inhibitor (22–24), decreased tubular Pi reabsorption in a dog kidney (57).

In conclusion, our results show that actinomycin D prevents both the renal functional as well as BBM transport and biochemical adaptation to low P diet in rats. The general pattern of how the adaptive changes in response to a low P diet are blocked by actinomycin D, and to a lesser degree by cycloheximide, resembles the blocking effect of actinomycin D and cycloheximide on the renal tubular adaptation of Na^+ transport elicited by aldosterone (58, 59) also involving stimulation of *de novo* synthesis of renal proteins.

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