Effect of a rickets-inducing diet and vitamin D metabolites [25-OH-D_3 and 1,25$(OH)_2D_3$] on transmembrane transport systems in rat kidney

Maria Stio, Teresa Iantomasi, Fabio Favilli, Maria Teresa Vincenzini, and Cristina Treves

Department of Biochemical Sciences, University of Florence, Florence, Italy

We previously demonstrated that feeding rats Steenbock and Black's rickets-inducing diet (deficient in vitamin D and with an altered Ca:P ratio) produces remarkable changes in the metabolic picture of the intestinal mucosa, kidney, and liver and in the transmembrane transport systems of D-glucose in intestinal and renal brush-border membrane vesicles. We have now investigated the effect of both 25-hydroxyvitamin D_3 and 1,25-dihydroxyvitamin D_3 on Na^+-dependent D-glucose and citrate transport and on calcium, phosphorus, and citrate content in rat serum and kidney. Na^+-dependent D-glucose uptake, which decreased in rachitic rat jejunum brush-border membrane vesicles, returned to control values after 25-hydroxyvitamin D_3 or 1,25-dihydroxyvitamin D_3 administration, while in kidney normal D-glucose transport was restored only after 1,25-dihydroxyvitamin D_3 treatment. Na^+-dependent citrate uptake was lowered in rachitic rat renal brush-border membrane vesicles and both 25-hydroxyvitamin D_3 and 1,25-dihydroxyvitamin D_3 proved to be ineffective in restoring to normal values. The in vitro addition to vesicle preparations of calcium or phosphate, citrate, 1,25-dihydroxyvitamin D_3, or valinomycin did not show a selective influence on D-glucose and citrate transport.

Keywords: rickets-inducing diet; 25-hydroxyvitamin D_3; 1,25-dihydroxyvitamin D_3; membrane transport; rat kidney

Introduction

It is well known that vitamin D, through its polar metabolite, 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], plays a primary role in calcium and phosphorus homeostasis.[1] Furthermore, some of the actions of 1,25$(OH)_2D_3$ may be mediated by mechanisms other than the steroid-receptor interaction. One example of this is the effect of vitamin D on membrane structure and architecture, with special reference to its lipid components,[2-6] although the relationship of these phospholipid effects to membrane functions remains to be clearly established. In previous studies we reported the consequences of feeding rats the Steenbock and Black rickets-inducing diet[7] ie, deficient in vitamin D and with an altered Ca:P ratio (high in calcium and low in available phosphorus). Under those conditions serum phosphorus is low; serum calcium is slightly elevated; and citrate levels are increased in the kidney and intestinal mucosa parallel to an increase of citrate synthase and a decrease of NAD^+- and $NADP^+$-dependent isocitrate dehydrogenase.[8-10]

In addition, we observed a drastic reduction in Na^+-dependent D-glucose uptake in brush-border membrane vesicles (BBMV) that had been prepared from rachitic rat jejunum and kidney. Subsequent 25-hydroxyvitamin D_3 (25-OH-D_3) administration to rats fed the rickets-inducing diet restored Na^+-dependent D-glucose uptake to normal in jejunum alone.[11,12] These findings agree with those of Peterlik et al.,[13] who reported that vitamin D stimulates absorption of D-glucose in chick jejunum and ileum by increasing the flux of the carrier and/or the number of carrier sites without significantly changing the affinity of the transport system for D-glucose.

Address reprint requests to Prof. Cristina Treves, Department of Biochemical Sciences, University of Florence, Viale Morgagni, 50, 50134 Florence Italy.
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To examine the possible changes related to experimental rickets in the rat, we investigated the effect of 25-OH-D_3 (orally supplied to rats fed the rickets-inducing diet) and of 1,25$(OH)_2D_3$ (injected intraperitoneally in control and rachitic rats) on Na^+-dependent D-glucose and citrate transport and on calcium, phosphorus, and citrate content in serum and kidney. In particular we studied D-glucose uptake in BBMV prepared from control and treated rat jejunum and kidney, and citrate uptake in renal BBMV. We also determined Na^+-dependent succinate transport in control and rachitic rat renal BBMV, as this substance is correlated with citrate metabolism. Moreover, as we previously demonstrated,[11] the uptake of phenylalanine in jejunum-ileum BBMV of control and rachitic rats showed no appreciable difference between the two groups, we sought to verify that a similar behavior occurred with renal BBMV also. The influence of the in vitro addition of calcium, phosphate, citrate, or 1,25$(OH)_2D_3$ on D-glucose and citrate uptake in renal BBMV was explored, and the effect of K^+ and valinomycin was determined. We report K_m value for D-glucose and citrate uptake in control and treated rat renal BBMV.

Methods and materials

Chemicals and Biochemicals

All enzymes, coenzymes, and substrates were from Boehringer und Soehne (Mannheim, Germany). 25-OH-D_3 in 1,2 propylenglycol was a pharmacologic product from Roussel Maestretti S.p.A., Milano, Italy. 1,25$(OH)_2D_3$ was the generous gift of Dr. H. Gutmann, Dr. W. Meier, Dr. A. Kaiser, and Dr. U. Fischer (F. Hoffmann-La Roche Inc., Nutley, NJ, USA). D-[U-^{14}C]-glucose was purchased from Amersham Radiochemical Centre, Ltd., Buchs, Switzerland. [1,5-^{14}C]-citric acid, L-[^{14}C]-phenylalanine and [2,3-^{14}C]-succinic acid were from New England Nuclear Corp. (Boston, MA, USA). Valinomycin was from Sigma Chemical Company (St. Louis, MO, USA). All other chemicals were the highest grade available from E. Merck (Darmstadt, Germany).

Animals

The subjects were 3-week-old albino Wistar rats whose average weight at the beginning of treatment was 50 g. The rats were divided into the following five groups: (1) animals fed a complete standard diet ad libitum (control rats); (2) animals fed a complete standard diet ad libitum and injected intraperitoneally with 300 ng/100 g body weight of 1,25$(OH)_2D_3$ in 0.1 mL ethanol 24 hr before killing (control plus 1,25$(OH)_2D_3$ rats). This dose, as reported by Armbrecht,[14] was found to produce maximal changes in the intestine; (3) animals kept in constant darkness and fed the rickets-inducing diet of Steenbock-Black ad libitum[7] for 25–30 days (rachitic rats); (4) animals kept in constant darkness and fed the rickets-inducing diet of Steenbock-Black ad libitum for 25–30 days; at three-day intervals they received 5 μg of 25-OH-D_3 orally for 25–30 days (rachitic plus 25-OH-D_3 rats); (5) animals kept in constant darkness and fed the rickets-inducing diet of Steenbock-Black ad libitum for 25–30 days and injected intraperitoneally with 300 ng/100 g body weight of 1,25$(OH)_2D_3$ in 0.1 mL ethanol at 24 hr before killing (rachitic plus 1.25$(OH)_2D_3$ rats).

The standard diet contained about 1.20% calcium and 0.95% phosphorus; 1400 IU of vitamin D was present for each kg of diet. The rickets-inducing diet, consisting of 76% yellow corn, 20% wheat gluten, 3% $CaCO_3$, and 1% NaCl was free of vitamin D and contained a Ca:P ratio greater than 3.5. In the two diets all the components, with the exception of calcium, phosphorus, and vitamin D, were the same and at about the same concentration. Immediately before the experiments the rats were killed by a blow to the head followed by cervical dislocation.

Determination of calcium, phosphorus, and citrate in serum and kidney homogenates

To determine calcium and phosphorus content in serum and kidney, the procedure described by Treves et al.[9] was followed. Citrate concentration in serum and kidney was assayed as reported by Möllering.[15]

Alkaline phosphatase determination

Serum alkaline phosphatase was assayed using the Merckotest purchased from E. Merck.

Protein determination

Protein concentration was determined by Bradford's method,[16] using bovine serum albumin as standard.

Preparation of brush-border membrane vesicles (BBMV)

BBMV were isolated from rat jejunum enterocytes by the method of Kessler et al.[17] and from kidney cortex by the method of Biber et al.[18] The final intestinal and renal pellets were resuspended in 300 mM mannitol, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)/tris(hydroxymethyl)aminomethane (Tris), 0.1 mM $MgSO_4$, pH 7.1 (uptake buffer). BBMV were used to measure solute transport, enzyme activities, and protein concentration. The purity of the membranes was assessed by the determination of alkaline phosphatase, leucine aminopeptidase, and γ-glutamyltranspeptidase, as previously reported.[11,12]

Transport measurements

The uptake of radiolabeled glucose into intestinal membrane vesicles and of radiolabeled glucose, citrate, phenylalanine, and succinate into renal membrane vesicles was determined by the millipore filtration technique, as described by Hopfer et al.[19] All transport studies were performed at room temperature. The uptake of glucose in intestinal and renal BBMV was carried out as previously described.[11,12] For the experiments with citrate, phenylalanine, and succinate, renal BBMV (approximately 70 μg protein in 10 μL) suspended in the uptake buffer were rapidly mixed with 20 μL of the uptake buffer. The uptake buffer contained radiolabeled compounds, 100 mM NaCl and 10 mM HEPES/Tris pH 7.1. Incubation times ranged from 15 sec to 60 min and the procedure was the same as previously described.[11,12] To determine the effect of calcium, phosphate, or citrate (i.e., $CaCl_2$, KH_2PO_4, sodium citrate) on uptake, each substance, diluted in the uptake buffer, was added to the renal BBMV (at a final, respective concentration of 5 mM) immediately prior to uptake measurement. We prepared a parallel experiment that involved the addition to the vesicles of the uptake buffer alone.

Likewise, to determine the effect of 1,25$(OH)_2D_3$ on uptake, the vitamin, diluted in ethanol, was added to renal BBMV, at 15 μM final concentration, immediately prior to uptake measurement. We performed a parallel experiment that involved the

Table 1 Calcium, phosphorus, citrate, and alkaline phosphatase in serum of control and treated rats

	Groups				
	C	C+1,25D	R	R+25D	R+1,25D
Ca (mg/100mL)	9.5 ± 0.3	10.2 ± 0.5	11.0 ± 0.5[a]	10.5 ± 0.4[a]	11.5 ± 0.6[a]
P_i (mg/100mL)	9.2 ± 0.8	9.4 ± 0.7	3.4 ± 0.3[a,b]	8.2 ± 0.9	7.0 ± 0.5[a]
Citrate (μmol/mL)	0.26 ± 0.03	0.27 ± 0.05	0.26 ± 0.05	0.26 ± 0.02	0.27 ± 0.06
Alk.Pase (μmol/min/mL)	0.081 ± 0.006	0.091 ± 0.008	0.172 ± 0.01[a,c]	0.124 ± 0.01[a]	0.124 ± 0.01[a]

Values are the mean ± SD of four determinations, each one performed (in duplicate) on a pool of four rats/group. Means were compared using Student's *t* test.
[a] Significantly different from C group ($P < 0.01$).
[b] Significantly different from R+25D and R+1,25D groups ($P < 0.01$).
[c] Significantly different from R+25D and R+1,25D groups ($P < 0.05$). Otherwise not significantly different.
Abbreviations: C, control rats, C+1,25D, control plus $1,25(OH)_2D_3$ rats; R, rachitic rats; R+25D, rachitic plus 25-OH-D_3 rats; R+1,25D, rachitic plus $1,25(OH)_2D_3$ rats.

Table 2 Calcium, phosphorus, and citrate content in kidney of control and treated rats

	Groups				
	C	C+1,25D	R	R+25D	R+1,25D
Ca (mg/g fresh weight)	0.041 ± 0.001	0.055 ± 0.004[a]	0.066 ± 0.003[a]	0.063 ± 0.001[a]	0.065 ± 0.006[a]
P_i (μmol/g fresh weight)	6.4 ± 0.1	6.9 ± 0.1[a]	5.3 ± 0.6[b]	5.2 ± 0.1[a]	5.6 ± 0.4[b]
Citrate (μmol/g fresh weight)	0.092 ± 0.006	0.098 ± 0.004	0.170 ± 0.008[a]	0.156 ± 0.008[a]	0.160 ± 0.006[a]

Values are the mean ± SD of four determinations, each one performed (in duplicate) on a pool of four rats/group. Means were compared using Student's *t* test.
[a] Significantly different from C group ($P < 0.01$).
[b] Significantly different from C group ($P < 0.05$). Otherwise not significantly different.
Abbreviations: C, control rats; C+1,25D, control plus $1,25(OH)_2D_3$ rats; R, rachitic rats; R+25D, rachitic plus 25-OH-D_3 rats; R+1,25D, rachitic plus $1,25(OH)_2D_3$ rats.

addition to the vesicles of ethanol alone at the same concentration used above.

The influence of electrical potential difference across the renal membrane on D-glucose and citrate uptake was studied with K^+ and valinomycin.[20] BBMV were pre-equilibrated for 30 min in a solution that contained 100 mM KCl, 100 mM mannitol, 10 mM HEPES/Tris pH 7.1, and 15 μg valinomycin/mg protein. Valinomycin was dissolved in 0.5% ethanol. We performed a parallel experiment that involved the addition to the vesicles of an appropriate amount of 0.5% ethanol alone. Transport experiments were terminated by the addition of 3 mL ice-cold stop-solution. The stop-solution for glucose contained 150 mM NaCl buffered with 10 mM HEPES/Tris pH 7.1; for phenylalanine, 100 mM mannitol and 100 mM NaCl buffered with 10 mM HEPES/Tris pH 7.1; for citrate, 100 mM mannitol and 50 mM citrate buffered with 10 mM HEPES/Tris pH 7.1; for succinate, 300 mM mannitol buffered with 10 mM HEPES/Tris pH 7.1. The stop solution used for the experiments with K^+ and valinomycin contained 250 mM NaCl buffered with 10 mM HEPES/Tris pH 7.1. The mixture was quickly filtered through a wet cellulose nitrate microfilter and washed with 3 + 3 mL stop solution. The filter was then placed in a scintillation vial and radioactivity was measured on a scintillation counter. All experiments, performed in triplicate, were repeated at least three times.

Results

The appearance of the characteristic signs of rickets in rats (assessed by the assay of serum alkaline phosphatase and by X-ray) occurs after 20–25 days of treatment; our experiments were therefore carried out on animals that had been fed on the rickets-inducing diet for at least 25 days. Also, Tanaka and DeLuca[21] reported that weanling male rats fed a vitamin D-deficient diet that contained either 1.2% calcium-0.1% phosphorus and 18% casein + 0.2% cystine, or 0.47% calcium-0.016% phosphorus and 18% egg white instead of casein for two weeks developed severe rickets. Furthermore, DeLuca et al.[22] observed that weanling male albino rats fed a high calcium, low phosphorus, vitamin D-deficient diet for three weeks, were vitamin D deficient and severely rachitic. The quantity of 25-OH-D_3 and $1,25(OH)_2D_3$ given to our rat groups was that considered to be a replacement dose, as reported by many authors.[4,14,21,23-25] The Steenbock-Black rickets-inducing diet is high in calcium and low in available phosphorus in addition to being vitamin D deficient. To visualize calcium and phosphorus content in the five groups of rats, the values previously reported[9,12] were compared with those determined in the animals that received $1,25(OH)_2D_3$. We also decided to assay citrate content in serum and kidney, because we had previously demonstrated that rats fed on this diet show increasing citrate levels in the kidney and intestinal mucosa.[8,9] The differences between the published data and those reported here are generally modest and due to the fact that we again assayed calcium, phosphorus, citrate, and alkaline phosphatase in all the animal groups. *Tables 1 and 2* report calcium, phosphorus, and citrate content, as

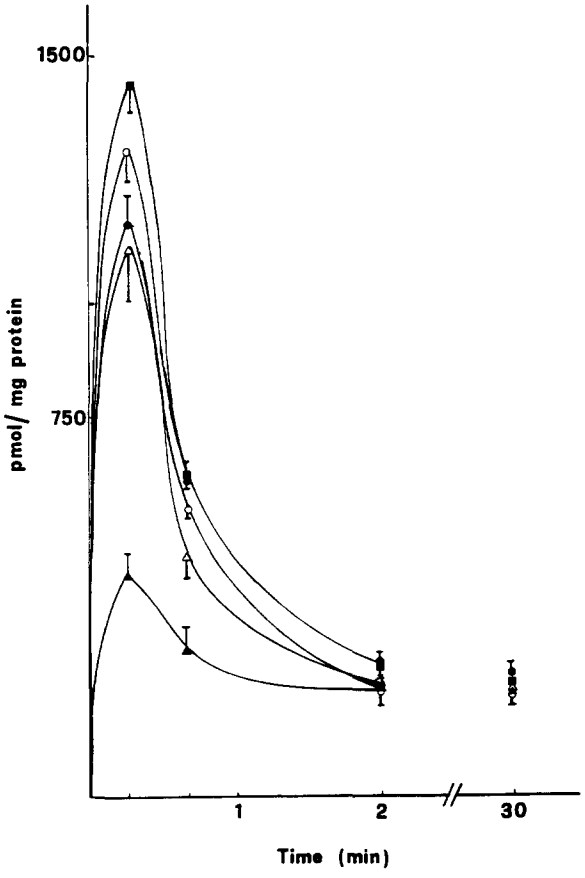

Figure 1 Time course of Na^+-dependent D-glucose uptake by brush-border membrane vesicles from jejunum of control and treated rats. The reaction, carried out at room temperature, was started by the addition of 10 μL of vesicles to 20 μL of the uptake buffer (see Materials and methods) containing 110 μM D-[^{14}C]-glucose and an initial NaSCN gradient (100 mM out, 0 in). ●–●, control. ○–○, control treated with 1,25$(OH)_2D_3$. ▲–▲, rachitic. △–△, fed on the rickets-inducing diet plus 25-OH-D_3. ■–■, rachitic treated with 1,25$(OH)_2D_3$. Means ± SD of triplicate determinations in four different membrane preparations are shown.

determined in serum and kidney homogenate. In *Table 1* serum alkaline phosphatase activity is also presented. The group of rachitic rats that received 1,25$(OH)_2D_3$ showed the highest calcium concentration in the serum, while serum P_i, which is particularly low in rachitic animals (an indication that this kind of rickets is a low-phosphorus disease) rose, but not to normal, after 25-OH-D_3 and 1,25$(OH)_2D_3$ treatment. Citrate content in rachitic kidney was considerably higher than in the control group and was not altered by 25-OH-D_3 or 1,25$(OH)_2D_3$ treatment. Alkaline phosphatase activity was higher in rachitic rat serum than control (a typical sign of rickets) and did not return to normal after 25-OH-D_3 or 1,25$(OH)_2D_3$ treatment.

In the transport experiments we used isolated BBMV that had been prepared from the jejunum and the kidney cortex of the five rat groups to study concentrative compound uptake by right-side-out vesicles, energized by an extravesicular > intravesicular Na^+-gradient.[19] The uptake is demonstrated by the characteristic overshoot phenomenon. *Figure 1* shows the time courses of Na^+-dependent D-glucose uptake into BBMV prepared from rat jejunum. We demonstrated that treatment with 1,25$(OH)_2D_3$ caused an increase in Na^+-dependent D-glucose uptake in the jejunum of controls but the effect was particularly large in rachitic rats. In the last case, the maximum uptake value is much higher than in the control. As reported in a previous paper,[12] we showed a decrease in Na^+-dependent D-glucose uptake in the renal BBMV of rachitic rats, 25-OH-D_3 administration to treated rats proving to be ineffective. On the contrary, after the treatment of rachitic rats with 1,25$(OH)_2D_3$, Na^+-dependent D-glucose uptake exceeded control values (*Figure 2*).

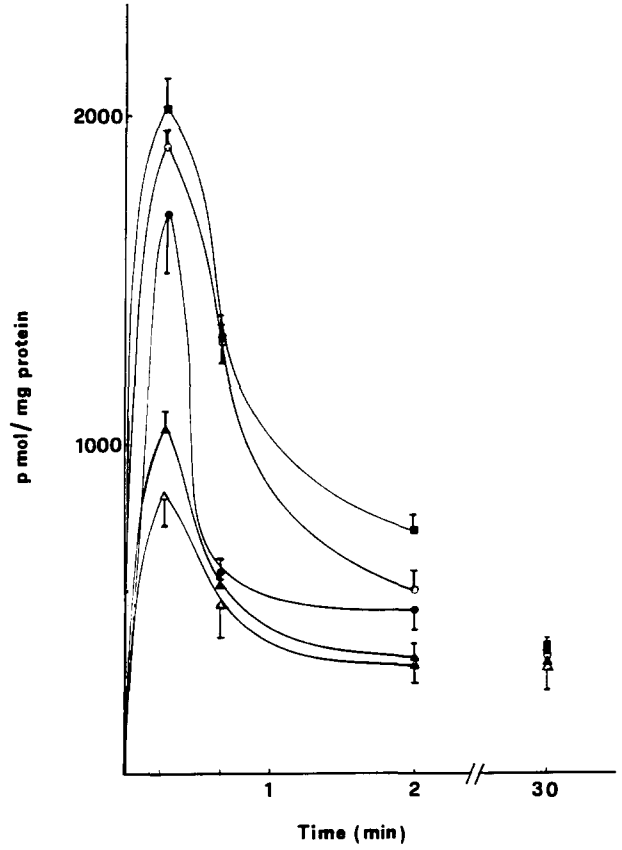

Figure 2 Time course of Na^+-dependent D-glucose uptake by brush-border membrane vesicles from kidney cortex of control and treated rats. The reaction, carried out at room temperature, was started by the addition of 10 μL of vesicles to 20 μL of the uptake buffer (se Materials and methods) containing 110 μM D-[^{14}C]-glucose and an initial NaSCN gradient (100 mM out, 0 in). ●–●, control. ○–○, control treated with 1,25$(OH)_2D_3$. ▲–▲, rachitic. △–△, fed on the rickets-inducing diet plus 25-OH-D_3. ■–■, rachitic treated with 1,25$(OH)_2D_3$. Means ± SD of triplicate determinations in five different membrane preparations are shown.

Figure 3 shows the time courses of Na^+-dependent citrate uptake by renal BBMV. In the rachitic animals, the overshoot decreased and it was not restored to normal by the administration of either 25-OH-D_3 or 1,25$(OH)_2D_3$. No appreciable difference between control and rachitic rats in Na^+-dependent phenylalanine uptake in renal vesicles was demonstrated (*Figure 4A*). The content and the uptake of succinate were determined in the kidney of control and rachitic rats. Neither succinate concentration in kidney (control, 1.1 ± 0.09; rachitic, 0.94 ± 0.09. The values ± SD represent the μmol of succinate/g fresh weight) nor Na^+-dependent succinate uptake in renal BBMV showed significant variation in rachitic rats in comparison with the control values (*Figure 4B*).

The K_m values for Na^+-dependent D-glucose and citrate uptake determined in the renal BBMV, at 5 sec and 30 sec, respectively, are reported in *Table 3*. The

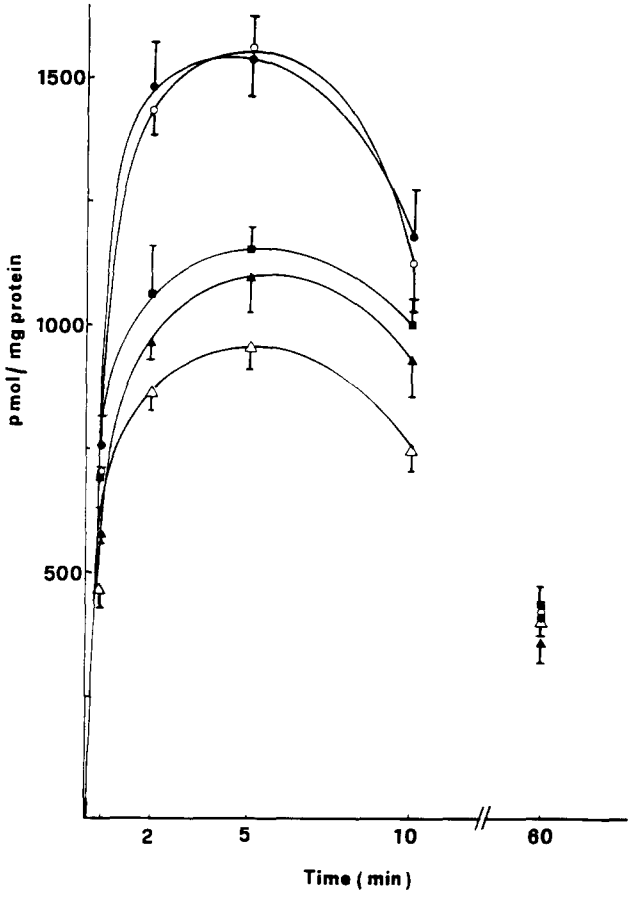

Figure 3 Time course of Na^+-dependent citrate uptake by brush-border membrane vesicles from kidney cortex of control and treated rats. The reaction, carried out at room temperature, was started by the addition of 10 μL of vesicles to 20 μL of the uptake buffer (see Materials and methods) containing 100 μM [^{14}C]-citrate and an initial NaSCN gradient (100 mM out, 0 in.) ●–●, control. ○–○, control treated with 1,25$(OH)_2D_3$. ▲–▲, rachitic. △–△, fed on the rickets-inducing diet plus 25-OH-D_3. ■–■, rachitic treated with 1,25$(OH)_2D_3$. Means ± SD of triplicate determinations in five different membrane preparations are shown.

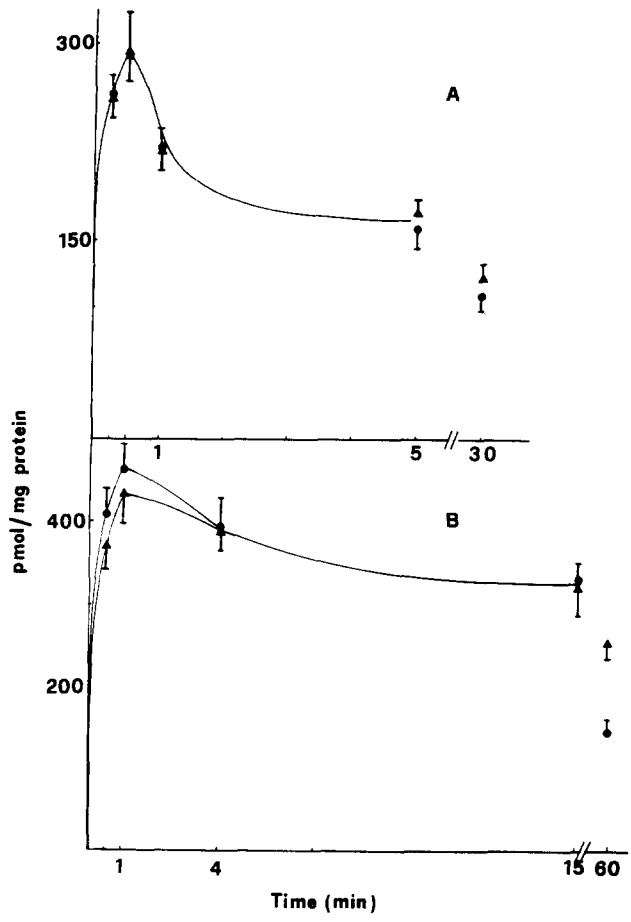

Figure 4 (A) Time course of Na^+-dependent L-phenylalanine uptake by brush-border membrane vesicles from kidney cortex of control and rachitic rats. The reaction, carried out at room temperature, was started by the addition of 10 μL of vesicles to 20 μL of the uptake buffer (see Materials and methods) containing 33 μM L-[^{14}C]phenylalanine and an initial NaSCN gradient (100 mM out, 0 in. ●–●, control. ▲–▲, rachitic. Means ± SD of triplicate determinations in three different membrane preparations are shown.
(B) Time course of Na^+-dependent succinate uptake by brush-border membrane vesicles from kidney cortex of control and rachitic rats. The reaction, carried out at room temperature, was started by the addition of 10 μL of vesicles to 20 μL of the uptake buffer (see Materials and methods) containing 100 μM [^{14}C]-succinic acid and an initial NaSCN gradient (100 mM out, 0 in). ●–●, control. ▲–▲, rachitic. Means ± SD of triplicate determinations in three different membrane preparations are shown.

K_m value for D-glucose, which was higher in the rachitic animals in comparison with the controls, returned to the control value after 1,25$(OH)_2D_3$ administration. The K_m value for citrate did not differ significantly among the five experimental groups.

By reviewing calcium, phosphorus, and citrate content changes in the kidney of the animals supplied with the rickets-inducing diet in comparison with the controls, we sought to establish whether the in vitro addition of these metabolites to renal membrane vesicles could directly influence Na^+-dependent D-glucose up-

Table 3 Comparison of the K_m values of Na^+-dependent D-glucose and citrate uptake by brush-border membrane vesicles prepared from kidney cortex of control and treated rats

	Groups			
	C	R	R+25D	R+1,25D
D-glucose (K_m mM)	0.18 ± 0.03	0.32 ± 0.06[a,c]	0.28 ± 0.04[a]	0.18 ± 0.05
Citrate (K_m mM)	0.23 ± 0.03	0.28 ± 0.05[b]	0.25 ± 0.01	0.25 ± 0.02

Uptakes of D-glucose and citrate were determined at 5 sec and 30 sec, respectively.
Values are the mean ± SD of three determinations, each one performed (in triplicate) on a pool of six rats/group. Means were compared using Student's *t* test.
[a] Significantly different from C group ($P < 0.01$).
[b] Significantly different from C group ($P < 0.05$).
[c] Significantly different from R+1,25 group ($P < 0.01$). Otherwise not significantly different.
Abbreviations: C, control rats; R, rachitic rats; R+25D, rachitic plus 25-OH-D_3 rats; R+1,25D, rachitic plus 1,25$(OH)_2D_3$ rats.

Table 4 Effect of in vitro addition of Ca^{2+}, phosphate, or citrate on D-glucose uptake (determined at 15 sec) by brush-border membrane vesicles prepared from kidney cortex of control and treated rats

	Groups				
	C	C+1,25D	R	R+25D	R+1,25D
Without addition	100	100	100	100	100
+ 5 mM Ca^{2+}	78.1 ± 2	80.4 ± 6	77.2 ± 6	79.2 ± 5	79.5 ± 3
+ 5 mM P	78.7 ± 5	76.4 ± 6	79.2 ± 7	83.0 ± 2	73.0 ± 3
+ 5 mM citrate	74.7 ± 7	44.1 ± 2[a]	73.6 ± 4	72.3 ± 4	60.7 ± 3[a,b]

Calcium, phosphate, or citrate were added to renal BBMV immediately before the uptake determinations (see Methods and materials). D-glucose uptake is expressed as percent of a control without additions. Values are the mean ± SD of five determinations, each one performed (in duplicate) on a pool of six rats/group. Means were compared using Student's *t* test.
All the observed values significantly different from 100 (without addition) ($P < 0.01$).
[a] Significantly different from C group ($P < 0.01$).
[b] Significantly different from R group ($P < 0.01$). Otherwise not significantly different.
Abbreviations: C, control rats; C+1,25D, control plus 1,25$(OH)_2D_3$ rats; R, rachitic rats; R+25D, rachitic plus 25-OH-D_3 rats; R+1,25D, rachitic plus 1,25$(OH)_2D_3$ rats.

Table 5 Effect of in vitro addition of Ca^{2+} or phosphate on citrate uptake (determined at 5 min) by brush-border membrane vesicles prepared from kidney cortex of control and treated rats

	Groups				
	C	C+1,25D	R	R+25D	R+1,25D
Without addition	100	100	100	100	100
+ 5 mM Ca^{2+}	61.2 ± 5	71.8 ± 6[a]	61.1 ± 1	61.3 ± 1	61.5 ± 7
+ 5 mM P	80.3 ± 3	79.9 ± 3	76.3 ± 4	80.8 ± 2	78.1 ± 6

Calcium or phosphate were added to renal BBMV immediately before the uptake determinations (see Methods and materials). Citrate uptake is expressed as percent of a control without additions. Values are the mean ± SD of five determinations, each one performed (in duplicate) on a pool of six rats/group. Means were compared using Student's *t* test.
All the observed values significantly different from 100 (without addition) ($P < 0.01$).
[a] Significantly different from C group ($P < 0.05$). Otherwise not significantly different.
Abbreviations: C, control rats; C+1,25D, control plus 1,25$(OH)_2D_3$ rats; R, rachitic rats; R+25D, rachitic plus 25-OH-D_3 rats; R+1,25D, rachitic plus 1,25$(OH)_2D_3$ rats.

take. Renal BBMV of the five rat groups were incubated before glucose uptake with either Ca^{2+}, phosphate, or citrate (5 mM final concentration). D-glucose uptake decreased 20%–30%; a higher inhibition occurred in those experiments in which citrate was added to vesicles prepared from rats treated with 1,25$(OH)_2D_3$ (*Table 4*).

Likewise, Na^+-dependent citrate uptake was studied after the incubation of the vesicles with either Ca^{2+} or phosphate (5mM final concentration). The results are reported in *Table 5*. Citrate uptake was negatively affected and the inhibition was higher in the presence of Ca^{2+} (30%–40%) than of phosphate (20%–25%).

We determined the Na^+-dependent D-glucose and citrate uptake (at the maximum overshoot, 15 sec and 5 min, respectively) in the renal BBMV of four groups of rats (ie, excluding the group to which 25-OH-D_3 was supplied) after adding 1,25$(OH)_2D_3$ (15 μM final

Table 6 Effect of in vitro addition of $1,25(OH)_2D_3$ on D-glucose and citrate uptake (determined at 15 sec and 5 min, respectively) by brush-border membrane vesicles prepared from kidney cortex of control and treated rats

	Groups			
	C	C + 1,25D	R	R + 1,25D
Without addition	100	100	100	100
D-glucose	96.5 ± 9	86.6 ± 6[a]	95.1 ± 2	99.8 ± 4
Citrate	73.1 ± 5[a]	79.6 ± 7[a]	81.8 ± 10[a]	79.1 ± 8[a]

$1,25(OH)_2D_3$ final concentration was 15 μM. D-glucose and citrate uptakes are expressed as percent of a control without $1,25(OH)_2D_3$ addition. Values are the mean ± SD of three determinations, each one performed on a pool of six rats/group. Means were compared using Student's *t* test.
[a] Significantly different from 100 (without addition) ($P < 0.01$). Otherwise not significantly different.
Abbreviations: C, control rats; C + 1,25D, control plus $1,25(OH)_2D_3$ rats; R, rachitic rats; R + 1,25D, rachitic plus $1,25(OH)_2D_3$ rats.

Table 7 Effect of in vitro addition of valinomycin on D-glucose and citrate uptake (determined at 15 sec and 5 min, respectively) by brush-border membrane vescicles prepared from kidney cortex of control and treated rats

	Groups			
	C	C + 1,25D	R	R + 1,25D
Without addition	100	100	100	100
D-glucose	77.1 ± 2	72.5 ± 1	72.9 ± 2	76.1 ± 1
Citrate	79.5 ± 5	76.2 ± 4	78.5 ± 6	77.4 ± 6

D-glucose and citrate uptakes are expressed as percent of a control without valinomycin addition. Values are the mean ± SD of three determinations, each one performed (in duplicate) on a pool of six rats/group. Means were compared using Student's *t* test.
All the observed values significantly different from 100 (without addition) ($P < 0.01$).
Abbreviations: C, control rats; C + 1,25D, control plus $1,25(OH)_2D_3$ rats; R, rachitic rats; R + 1,25D, rachitic plus $1,25(OH)_2D_3$ rats.

concentration) to the vesicles. We carried out these experiments to ascertain whether a direct and selective effect of the vitamin on the D-glucose and citrate transport systems could be demonstrated. D-glucose uptake was not affected by the in vitro addition of $1,25(OH)_2D_3$. Citrate transport was inhibited by about 20%, higher inhibition (approximately 27%) was observed in the control group (*Table 6*).

We then studied the effect of valinomycin on Na^+-dependent D-glucose and citrate uptake into the renal BBMV of the same four groups of rats. The results, shown in *Table 7*, indicate that in the presence of K^+ and valinomycin, overshoots decreased in the four groups by about the same percentage (20%—28%).

Discussion

Our studies report both the metabolic consequences of feeding rats on the Steenbock and Black rickets-inducing diet and the significant reduction of Na^+-dependent D-glucose and citrate transport in BBMV.

The increase of calcium content, compared to control values, in serum of controls plus $1,25(OH)_2D_3$ is probably a consequence of $1,25(OH)_2D_3$ administration, as reported by other authors[23] and for the other groups of rats, of the unbalanced diet (high Ca:P ratio). In the kidney 25-OH-D_3 or $1,25(OH)_2D_3$ administration proved to be ineffective in restoring calcium content to the control values. The behavior of serum and renal P_i is in agreement with the finding of Lee et al.[24] and with the results of Brautbar et al.,[25] who demonstrated that the administration of vitamin D_3 or $1,25(OH)_2D_3$ to P-depleted rats leaves the renal retention of P_i unaltered despite a significant increase in serum P_i.

The high levels of citrate, demonstrated in the kidney of treated rats in comparison with the controls, indicate that the function of this metabolite could possibly be connected to the regulation of calcium influx and efflux mechanisms, the increase in citrate appearing to counter-balance the high calcium levels of the kidney. The fact that serum alkaline phosphatase activity was higher in rachitic rats and did not return to the control value after either 25-OH-D_3 or $1,25(OH)_2D_3$ administration confirms the importance of the altered Ca:P ratio of the diet.

The results of our experiments demonstrate that in rate jejunum vitamin D directly affects Na^+-dependent D-glucose transport. Different behavior was observed studying Na^+-dependent D-glucose uptake in renal BBMV. The different response as provoked by the administration of 25-OH-D_3 and $1,25(OH)_2D_3$, both of which act selectively in this situation, is not surprising given that a direct role for 25-OH-D_3, without conversion to $1,25(OH)_2D_3$, is possible, so that 25-OH-D_3 and $1,25(OH)_2D_3$ may be regarded as two metabolically active forms of vitamin D_3.[26,27]

It is well known that vitamin D also exerts its effects on membrane structure and architecture, with special reference to its lipid components,[28] suggesting that the action of $1,25(OH)_2D_3$ is in part produced through direct effects on plasma membrane

phospholipids.[2-4,6,29] Consequently, the decrease in D-glucose uptake in rachitic rat jejunum and kidney BBMV corrected by $1,25(OH)_2D_3$ administration could arguably reflect the action of this hormone on plasma membrane phospholipids. It must be supposed however, that the changes observed in the transport systems that we studied may be related not only to vitamin D deficiency, but also to the metabolic modifications produced by rickets. In particular, considering that P_i content, very low in serum of rachitic rat if compared to control values, increases after 25-OH-D_3 or $1,25(OH)_2D_3$ administration (*Table 1*), we can state that serum phosphorus may have had a major effect on the transport systems studied. In fact, Kurnik and Hruska,[23] using a model of partial vitamin D depletion developed by feeding weanling rats a vitamin D-deficient diet containing 1.8% calcium and 1.2% phosphorus, demonstrated that there were no significant differences in glucose uptake between any of the groups of rats studied, which, however, did not include rachitic animals, as our research did. The decrease in citrate uptake in rachitic rats, not corrected by 25-OH-D_3 or $1,25(OH)_2D_3$ administration could be due to the high citrate levels in the kidney. Simpson[30] reported that changes in cytoplasmic citrate levels, acting on the citrate carrier, could alter the rate of citrate reabsorption from the tubular fluid. On the contrary, succinate content in the kidney is not affected by rickets and succinate uptake in renal BBMV of control and rachitic rats remains unchanged. The fact that phenylalanine and succinate uptake in renal BBMV did not change in rachitic rats excludes consideration of serious structural alterations in renal membranes and supports the hypothesis that Na^+-dependent transmembrane systems respond selectively to the pathologic alterations caused by rickets. This selectivity has also been reported by Dabbagh et al.[31] Moreover, it may be supposed that some kidney transmembrane systems could possibly be influenced by the remarkable changes in serum P_i levels associated with our rachitic model of vitamin D deficiency. Thus, either vitamin D metabolites or their effect on serum phosphorus could have affected our results on the transport systems studied.

Na^+-dependent D-glucose and citrate uptake was studied in vitro by the addition of calcium, phosphate, or citrate. Under these experimental conditions a generalized decrease in D-glucose and citrate transport in all the groups studied was observed, while no effect was demonstrable by adding the same metabolites at 1 mM final concentration (data not reported). Our results indicate that, very probably, only the in vivo changes of the metabolite levels can explicate a selective action on some transport systems.

The experiments with valinomycin indicate that the role played by the membrane's electrical potential in D-glucose and citrate accumulation within the vesicles was roughly unvarying and was not responsible for the changes observed in the uptake of these metabolites.

In conclusion, our results clearly indicate that the observed changes in some renal transmembrane transport systems could either be due to the direct effect of the vitamin D metabolite or the indirect effect on extracellular and intracellular calcium and phosphorus pools.

Further experiments are therefore in progress to clarify the possible relationship between vitamin D deficiency, metabolic changes, and selected transport phenomena. The specific effects of $1,25(OH)_2D_3$ remain to be elucidated.

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