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pH gradient as an additional driving force in the renal re-absorption of phosphate

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The effects of the Na^+ gradient and pH on phosphate uptake were studied in brush-border membrane vesicles isolated from rat kidney cortex. The initial rates of Na^+-dependent phosphate uptake were measured at pH 6.5, 7.5 and 8.5 in the presence of sodium gluconate. At a constant total phosphate concentration, the transport values at pH 7.5 and 8.5 were similar, but at pH 6.5 the influx was 31% of that at pH 7.5. However, when the concentration of bivalent phosphate was kept constant at all three pH values, the effect of pH was less pronounced: at pH 6.5, phosphate influx was 73% of that measured at pH 7.5. The Na^+-dependent phosphate uptake was also influenced by a transmembrane pH difference: an outwardly directed H^+ gradient stimulated the uptake by 48%, whereas an inwardly directed H^+ gradient inhibited the uptake by 15%. Phosphate on the *trans* (intravesicular) side stimulated the Na^+-gradient-dependent phosphate transport by 59%, 93% and 49%, and the Na^+-gradient-independent phosphate transport by 240%, 280% and 244%, at pH 6.5, 7.5 and 8.5 respectively. However, in both cases, at pH 6.5 the maximal stimulation was seen only when the concentration of bivalent *trans* phosphate was the same as at pH 7.5. In the absence of a Na^+ gradient, but in the presence of Na^+, an outwardly directed H^+ gradient provided the driving force for the transient hyperaccumulation of phosphate. The rate of uptake was dependent on the magnitude of the H^+ gradient. These results indicate that: (1) the bivalent form of phosphate is the form of phosphate recognized by the carrier on both sides of the membrane; (2) protons are both activators and allosteric modulators of the phosphate carrier; (3) the combined action of both the Na^+ (out/in) and H^+ (in/out) gradients on the phosphate carrier contribute to regulate efficiently the re-absorption of phosphate.

INTRODUCTION

Renal phosphate re-absorption occurs mainly in the proximal tubule [1,2] by an active process which involves a Na^+-dependent transport system located on the luminal side of the cells [3]. During the last decade, the use of brush-border membrane vesicles to study transport properties has brought a considerable progress to our knowledge of phosphate re-absorption and its regulation [4–9].

One of the most debated questions remains the nature of the transported anion, either univalent or bivalent, and the influence of pH on the transport system itself. An increase in the pH of the incubation medium from 6.0 to 8.0 was found to increase the rate of phosphate transport [6,10–12], thus suggesting that the bivalent anion is the preferentially transported substrate. pH had no effect on the apparent affinity of the co-transport system when calculated on the basis of bivalent phosphate concentrations [12]. It was also reported that a transmembrane pH difference affects the rate of phosphate transport [13]. The effect of pH is strongly influenced by the concentration of Na^+ in the incubation medium [14]. This observation led Amstutz *et al.* [14] to suggest that the major effect of pH is not related to the preferential transport of one given ion species, but rather to an alteration of the properties of the transport system. Another study suggested that pH affected the phosphate-binding site of the carrier rather than its Na^+-binding site [15].

In the present work, the combined effects of Na^+ and H^+ gradients were studied. We present evidence that the effect of pH on phosphate transport is related to a preferential transport of bivalent phosphate ions, to a modulation of phosphate transport by an H^+ gradient which can act as a driving force, and to an allosteric modulation of transport by protonation of the carrier itself. Our previously published kinetic model for phosphate transport [4] was modified to account for these pH effects.

MATERIALS AND METHODS

Preparation of brush-border membrane vesicles

The studies were performed on male Sprague–Dawley rats (250–300 g), fed on a normal phosphate diet (0.8% phosphate). Animals were killed by decapitation, the kidneys were perfused *in situ* with 0.85% NaCl via the abdominal aorta, and the cortex was sliced. Brush-border membrane vesicles were prepared by a Mg^{2+}-precipitation procedure [16]. The purified membrane vesicles were resuspended in 300 mM-mannitol/20 mM-Hepes/Tris, pH 7.5, to a final protein concentration of 20 μg/μl and stored in liquid N_2 for up to 7 days. The storage of vesicles in liquid N_2 for 1 week had no effect on their transport capacity [4]. The enrichments for alkaline phosphatase and Na^+/K^+-dependent ATPase were 13.1 ± 1.0- and 0.23 ± 0.09-fold respectively.

Transport studies

To preload the vesicles with a medium containing different salts and buffers (see legends of Figures), the vesicle suspensions (100 μl) were diluted with 10 ml of the appropriate medium and homogenized with a glass/Teflon homogenizer. The membranes were centrifuged for 30 min at 33000 *g*, resuspended in the same medium and passed 10 times through a fine needle ($27\frac{1}{2}$ gauge). The membranes were finally allowed to equilibrate for 1 h at 25 °C. This procedure was sufficient to replace the intravesicular medium by a medium of desired composition [17]. The transport rates were measured by a rapid filtration technique [18].

Abbreviation used: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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Transport measurements, performed in triplicate at 25 °C, were initiated by mixing 5 μl of vesicle suspension (80–100 μg of membrane protein) with 25 μl of an appropriate incubation medium containing 4 μCi of [^{32}P]P_i or 1 μCi of D-[^{3}H]glucose. All incubation conditions are given in the Figure legends. When bivalent phosphate on the *trans* side was to be maintained constant at 8.33 mM at pH *trans* of 6.5, 7.5 or 8.5, total phosphate was 25, 10 or 8.5 mM respectively, according to calculations based on a pK_a of 6.8. When *cis* bivalent phosphate was held constant, 500, 200 or 170 μM total phosphate was used, at pH 6.5, 7.5 or 8.5 respectively, to give a bivalent phosphate concentration of 167 μM. The reaction was terminated by addition of 1 ml of ice-cold stop solution containing 150 mM-KCl and 5 mM-Hepes/Tris, pH 7.5. The suspension was applied to 0.45 μm-pore-size Millipore filters under vacuum. Filters were washed with 8 × 1 ml of ice-cold stop solution and processed for liquid-scintillation counting. Transport values were corrected for non-specific binding by vesicles and filters in a separate assay where incubation medium and vesicles were added directly to the stop solution. Non-specific absorption never exceeded 5% of normal transport values.

Na^+-dependent transport was calculated as the difference between transport values measured in the presence of Na^+ and K^+ in the incubation media. When not indicated, S.D. values were less than 10% of the mean. Statistical analyses were made by an unpaired Student's *t* test. Data are means ± S.D. of 3–5 experiments done in triplicate.

RESULTS

We have studied the influx of phosphate at pH 6.5, 7.5 and 8.5 by incubating the vesicles in the presence of sodium gluconate. As shown in Fig. 1, the highest values of Na^+-dependent phosphate influx were obtained at pH 7.5. When total phosphate was maintained constant, the influx at pH 6.5 was only 31% of that at pH 7.5. The effect of pH was less pronounced when bivalent phosphate was maintained constant. At pH 6.5, phosphate influx was 73% of that at pH 7.5. At pH 8.5, influx was 86% of the influx measured at pH 7.5.

In addition, the Na^+-dependent phosphate influx was inhibited by an inwardly directed proton gradient (Fig. 2). When the pH was 6.5 in the extravesicular medium and 7.5 in the intravesicular medium, phosphate influx was 83% with constant total phosphate, and 75% with constant bivalent phosphate, compared with the influx observed when both intravesicular and extravesicular media were at pH 6.5 (Fig. 1). In contrast, an outwardly directed proton gradient stimulated Na^+-dependent phosphate influx. Compared with conditions where the pH of both extravesicular and intravesicular media was 8.5 (Fig. 1), transport at external pH of 8.5 and internal pH of 7.5 was 161% when total phosphate was kept constant, and 115% when bivalent phosphate was kept constant (Fig. 2). There was no difference in Na^+-independent phosphate influx at pH 6.5, 7.5 and 8.5 when total phosphate was maintained constant (Table 1). At pH 6.5, when the bivalent form of phosphate was maintained constant, the Na^+-independent phosphate influx was about 2.2 times that measured with total phosphate constant. This result is expected if it is assumed that Na^+-independent phosphate influx represents the passive permeability of the membrane for phosphate and that, at pH 6.5, the total phosphate necessary to obtain the same bivalent phosphate concentration is 2.5 times that at pH 7.5. The proton gradient did not affect the Na^+-independent phosphate influx.

To determine which form of phosphate (bivalent or univalent) is recognized by the transporter on the cytosolic side of the membrane, we have studied the effect of *trans* pH on the

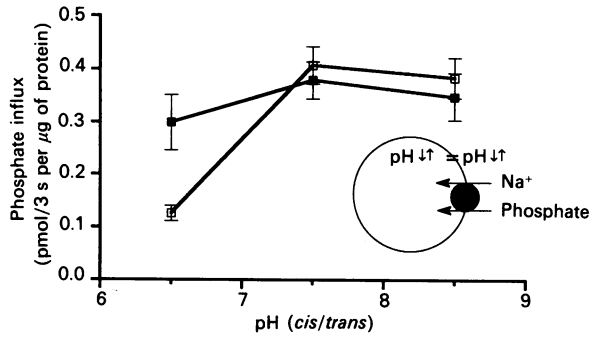


Fig. 1. Effect of pH on Na^+-gradient-dependent phosphate influx

Membrane vesicles were pre-equilibrated in 300 mM-mannitol and 20 mM-Mes/Tris, pH 6.5, -Hepes/Tris, pH 7.5, or -Tris/Hepes, pH 8.5. Uptake of [^{32}P]K_2HPO_4/KH_2PO_4 was measured in a medium containing 100 mM-mannitol, 100 mM-sodium gluconate and 20 mM-Mes/Tris, pH 6.5, -Hepes/Tris, pH 7.5, or -Tris/Hepes, pH 8.5. Phosphate concentration was 0.2 mM when total phosphate was maintained constant (□). The bivalent form of phosphate was at a concentration of 0.167 mM when constant (■). Data are means ± S.D. of at least three experiments.

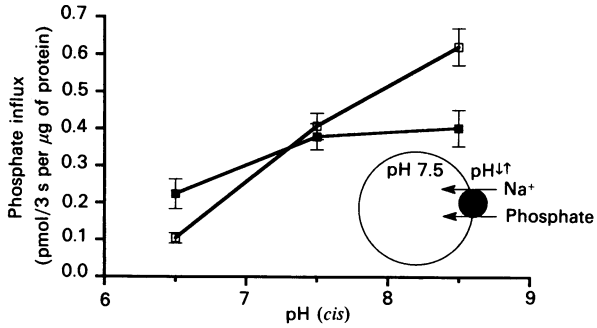


Fig. 2. Effect of *cis* pH on Na^+-gradient-dependent phosphate influx

Membrane vesicles were pre-equilibrated in 300 mM-mannitol/20 mM-Hepes/Tris, pH 7.5. Uptake of [^{32}P]K_2PO_4/KH_2PO_4 was measured in a medium containing 100 mM-mannitol, 100 mM-sodium gluconate and 20 mM-Mes/Tris, pH 6.5, -Hepes/Tris, pH 7.5, or -Tris/Hepes, pH 8.5. Phosphate concentration was 0.2 mM when total phosphate was maintained constant (□). The bivalent form of phosphate was at a concentration of 0.167 mM when constant (■). Data are means ± S.D. of at least three experiments.

stimulation of Na^+-gradient-dependent phosphate influx by *trans* phosphate (Fig. 3). The influx was measured with a 200 mM-Na^+ gradient (zero *trans*) at pH *trans* of 6.5, 7.5 and 8.5 and pH *cis* of 7.5. Unlabelled phosphate was added on the *trans* side by preincubation of the vesicles. Electrical potential was shunted by use of the same concentration of the highly permeant NO_3^- ion on both sides of the membrane. In the absence of *trans* phosphate, an inside acidic pH stimulated the initial rate of influx by 48% (Fig. 3) and an inside alkaline pH produced an inhibition of 15%. When the same concentration of total phosphate was added on the *trans* side, the Na^+-gradient-dependent influx was stimulated only at pH *trans* 7.5 and 8.5. This stimulation was 93% and 54% at pH *trans* 7.5 and 8.5 respectively. On the other hand, when the same concentration of bivalent phosphate was added on the *trans* side, the Na^+-gradient-dependent phosphate influx was stimulated at all *trans* pH values studied: 59, 93 and 49% stimulation at pH *trans* 6.5, 7.5 and 8.5 respectively. These results indicate that bivalent phosphate is the form recognized by the phosphate transporter on the cytosolic side of the membrane.

Table 1. Effect of pH on Na^+-independent phosphate influx when concentrations of the total phosphate or the bivalent form of phosphate are maintained constant

Membrane vesicles were pre-equilibrated in 300 mM-mannitol and 20 mM-Mes/Tris, pH 6.5, -Hepes/Tris, pH 7.5, or -Tris/Hepes, pH 8.5. Uptake of $[^{32}P]K_2HPO_4/KH_2PO_4$ was measured in a medium containing 100 mM-mannitol, 100 mM-potassium gluconate and 20 mM-Mes/Tris, pH 6.5, -Hepes/Tris, pH 7.5, or -Tris/Hepes, pH 8.5. Phosphate concentration was maintained at 0.2 mM when total phosphate was maintained constant. The bivalent form of phosphate was at a concentration of 0.167 mM when maintained constant. Data are means ± S.D. of at least three experiments: NS, not significant.

pH (out/in)	Phosphate influx (pmol/3 s per μg of protein)	
	Constant total phosphate	Constant HPO_4^{2-}
1. 6.5/6.5	0.027 ± 0.009	0.058 ± 0.027
2. 7.5/7.5	0.028 ± 0.007	0.028 ± 0.007
3. 8.5/8.5	0.031 ± 0.018	0.023 ± 0.011
4. 6.5/7.5	0.019 ± 0.008	0.062 ± 0.026
5. 8.5/7.5	0.038 ± 0.017	0.028 ± 0.022
Student's *t*-test analysis		
1 versus 2	NS	$P < 0.0001$
1 versus 3	NS	$P < 0.0001$
1 versus 4	NS	NS
1 versus 5	NS	$P < 0.001$
2 versus 3	NS	NS
2 versus 4	NS	$P < 0.005$
2 versus 5	NS	NS
3 versus 4	NS	$P < 0.0001$
3 versus 5	NS	NS
4 versus 5	NS	$P < 0.005$

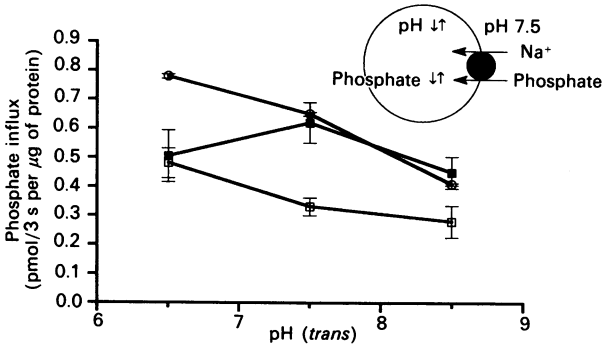


Fig. 3. Effect of *trans* pH on the stimulation of Na^+-gradient-dependent phosphate influx by *trans* phosphate

Membrane vesicles were pre-equilibrated with 200 mM-KNO_3, 20 mM-Mes/Tris, pH 6.5, -Hepes/Tris pH 7.5, or -Tris/Hepes, pH 8.5, and no (control) or 10 mM-K_2HPO_4/KH_2PO_4 (total *trans* phosphate constant) or 8.5 mM, 10 mM- and 25 mM-K_2HPO_4/KH_2PO_4 (bivalent *trans* phosphate constant). Osmolarity was maintained with K_2SO_4. Vesicles (5 μl) were diluted in 620 μl of 100 mM-mannitol/200 mM-$NaNO_3$/20 mM-Hepes/Tris, pH 7.5, plus enough $[^{32}P]K_2HPO_4/KH_2PO_4$ to obtain a final concentration of 0.2 mM. Data are means ± S.D. of three experiments. □, Control; ■, total *trans* phosphate constant; ○, bivalent *trans* phosphate constant.

Fig. 4 shows the effect of pH *trans* on the Na^+-gradient-independent phosphate influx. The influx was measured with 200 mM-$NaNO_3$ on both sides of the membrane and a pH *cis* of 7.5. The Na^+-gradient-independent phosphate influx was stimulated by an outwardly directed proton gradient and inhibited by

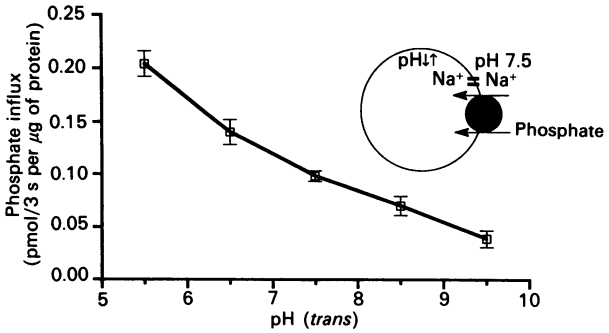


Fig. 4. Effect of *trans* pH on Na^+-gradient-independent phosphate influx

Membrane vesicles were pre-equilibrated with 100 mM-mannitol, 200 mM-$NaNO_3$, 1 mM-EDTA and 20 mM-Mes/Tris, pH 5.5, -Mes/Tris, pH 6.5, -Hepes/Tris, pH 7.5, -Tris/Hepes, pH 8.5, or -CAPSO/Tris, pH 9.5. Uptake of 0.2 mM-$[^{32}P]K_2HPO_4/KH_2PO_4$ was measured in a medium containing 100 mM-mannitol, 200 mM-$NaNO_3$, 20 mM-Hepes/Tris, pH 7.5, and 1 mM-EDTA. Data are means ± S.D. of three experiments.

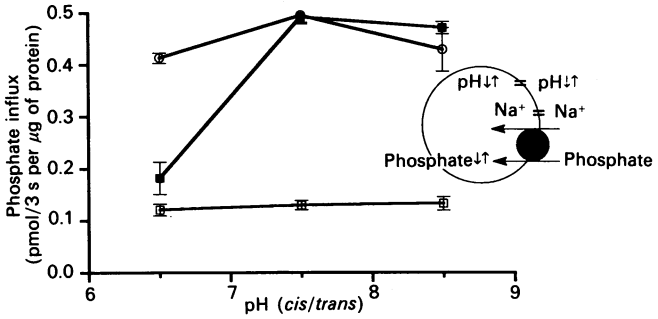


Fig. 5. Effect of pH on the stimulation of Na^+-gradient-independent phosphate influx by *trans* phosphate

Membrane vesicles were pre-equilibrated as described in Fig. 3, with $NaNO_3$ replacing KNO_3. Vesicles were diluted in 100 mM-mannitol/200 mM-$NaNO_3$ containing 20 mM-Mes/Tris, pH 6.5, -Hepes/Tris, pH 7.5, or -Tris/Hepes, pH 8.5, and enough $[^{32}P]K_2HPO_4/KH_2PO_4$ to maintain bivalent *cis* phosphate at 0.167 mM. Data are means ± S.D. of three experiments done in triplicate. □, Control; ■, total *trans* phosphate constant; ○, bivalent *trans* phosphate constant.

an inwardly directed proton gradient. The degree of stimulation or inhibition was a function of the magnitude of the pH gradient. The rates of Na^+-gradient-independent phosphate transport were stimulated by 43% and 108% when transport was measured with an outwardly directed proton gradient of 1 and 2 pH units respectively. In contrast, an inwardly directed proton gradient of 1 and 2 pH units inhibited the transport by 29% and 60% respectively.

We also studied the effect of pH on the *trans* stimulation by phosphate of Na^+-gradient-independent phosphate influx (Fig. 5). In these experiments, pH *cis* and *trans* were varied simultaneously. The influx was measured with the same *cis* bivalent phosphate concentration at all pH values. In the absence of *trans* phosphate, no difference was observed between the phosphate influx measured at pH 6.5, 7.5 and 8.5. When the same concentration of total phosphate was added on the *trans* side, the Na^+-gradient-independent influx was stimulated by 280 and 244% at pH 7.5 and 8.5 respectively. A very small stimulation was observed at pH 6.5, but, when the same concentration of bivalent phosphate was added, we observed a stimulation of 240% at pH 6.5. This is another indication that bivalent phosphate is the

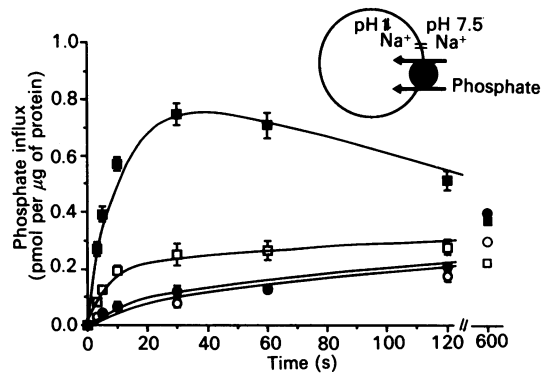


Fig. 6. Time course of Na^+-gradient-independent phosphate uptake

Membrane vesicles were pre-equilibrated with 100 mM-mannitol, 200 mM-NaCl (□, ■) or -KCl (○, ●), 1 mM-EDTA and 20 mM-Mes/Tris, pH 5.5 (■, ●), or -Hepes/Tris, pH 7.5 (□, ○). Uptake of 0.2 mM-[^{32}P]K_2HPO_4/KH_2PO_4 was measured in a medium containing 100 mM-mannitol, 200 mM-NaCl (□, ■) or -KCl (○, ●), 1 mM-EDTA and 20 mM-Hepes/Tris, pH 7.5. Data are means ± S.D. of four experiments.

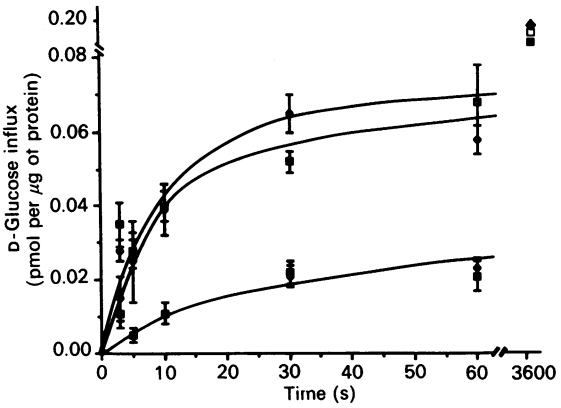


Fig. 7. Time course of Na^+-gradient-independent glucose uptake

Membrane vesicles were pre-equilibrated with 100 mM-mannitol, 200 mM-NaCl (□, ◆) or -KCl (■, ◇), 1 mM-EDTA and 20 mM-Mes/Tris, pH 5.5 (◆, ◇), or -Hepes/Tris, pH 7.5 (□, ■). Uptake of 50 μM-D-[^{3}H]glucose was measured in a medium containing 100 mM-mannitol, 200 mM-NaCl (□, ◆) or -KCl (■, ◇), 1 mM-EDTA and 20 mM-Hepes/Tris, pH 7.5. Data are means ± S.D. of three experiments.

phosphate form which is recognized by the transporter on the cytosolic side of the membrane.

Fig. 6 shows that, in the absence of a Na^+ gradient as the driving force, an outwardly directed H^+ gradient can produce a transient hyperaccumulation of phosphate. When pH *trans* was 5.5 and pH *cis* was 7.5, the initial rate of uptake, measured at 3 s, was about 3–4 times that when both pH *cis* and *trans* were 7.5. Maximal uptake occurred at 30 s. The equilibrium uptake at 10 min was identical with or without an H^+ gradient. The stimulation of phosphate influx by an outwardly directed proton gradient was observed only in the presence of Na^+. The action of the H^+ gradient was specific for the uptake of phosphate, since glucose transport was not affected by an H^+ gradient (Fig. 7).

Additional aspects of the Na^+-gradient-independent phosphate uptake were investigated. As shown in Table 2, phosphate uptake was about 3.2-fold higher in the presence of a proton gradient. The Na^+-gradient-independent phosphate uptake was not influenced by the presence of amiloride in the absence or in the presence of a proton gradient (Table 2). Therefore, in the absence of a Na^+ gradient the Na^+/H^+ exchanger did not produce

Table 2. Effect of pH gradient, amiloride, CCCP and monensin on phosphate uptake, in the absence of a Na^+ gradient

Membrane vesicles were pre-equilibrated with 100 mM-mannitol containing 200 mM-NaCl, 20 mM-Hepes/Tris, pH 7.5, or -Mes/Tris, pH 5.5, and 1 mM-EDTA. Uptake at 30 s was measured in 100 mM-mannitol/200 mM-NaCl/1 mM-EDTA/20 mM-Hepes/Tris (pH 7.5)/0.2 mM-[^{32}P]KH_2PO_4/K_2HPO_4, containing 1 mM-amiloride, 20 μM-CCCP, 6 μg of monensin/mg of protein or 1% ethanol (control). Data are means ± S.D. for three experiments.

Conditions pH (in/out)...	Phosphate uptake (pmol/30 s per μg of protein) 7.5/7.5	5.5/7.5
Control	0.23 ± 0.025	0.73 ± 0.092
Amiloride	0.20 ± 0.011	0.67 ± 0.084
CCCP	0.24 ± 0.026	0.28 ± 0.094
Monensin	0.21 ± 0.025	0.20 ± 0.041

Table 3. Effect of pH gradient, amiloride, CCCP and monensin on glucose uptake in the absence of Na^+ gradient

Membrane vesicles were pre-equilibrated with 100 mM-mannitol containing 200 mM-NaCl, 1 mM-EDTA and 20 mM-Hepes/Tris, pH 7.5, or -Mes/Tris, pH 5.5. Uptake at 30 s was measured in 100 mM-mannitol/200 mM-NaCl/1 mM-EDTA/20 mM-Hepes/Tris (pH 7.5)/50 μM-D-[^{3}H]glucose, containing 1 mM-amiloride, 20 μM-CCCP, 6 μg of monensin/mg of protein or 1% ethanol (control). Data are means ± S.E.M. of the experiments.

Conditions pH (in/out)...	Glucose uptake (pmol/30 s per μg of protein) 7.5/7.5	5.5/7.5
Control	0.053 ± 0.006	0.048 ± 0.005
Amiloride	0.052 ± 0.005	0.053 ± 0.004
CCCP	0.049 ± 0.003	0.063 ± 0.007
Monensin	0.046 ± 0.006	0.056 ± 0.004

a significant efflux of H^+, and the electrochemical gradient of H^+ could sustain the stimulation of phosphate transport. In the absence of a H^+ gradient, no difference of uptake was seen between control and CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) conditions. In the presence of a proton gradient, CCCP caused an inhibition (65%) of phosphate influx compared with the control. This can be explained by the fact that CCCP dissipates the H^+ gradient which acts as the driving force. When both pH *cis* and *trans* were 7.5, the uptake of phosphate was not affected by monensin (Table 2). In the presence of an H^+ gradient, the addition of monensin decreased phosphate uptake by 74% (Table 2). This is because monensin produced the exchange of internal H^+ for external Na^+, thus abolishing the proton gradient and the driving force for phosphate uptake.

We have also studied the characteristics of the Na^+-gradient-independent glucose uptake. In contrast with phosphate uptake, glucose uptake was not stimulated by a proton gradient (Table 3). Also, glucose uptake remained identical in the absence or in the presence of amiloride, even in the presence of a proton gradient. In the presence or absence of an H^+ gradient, glucose influx remained unaffected by CCCP (Table 3). This lack of effect is expected, since the pH gradient had no stimulatory effect on glucose influx. The creation of an inside-negative potential was prevented by the presence of a high concentration of chloride on

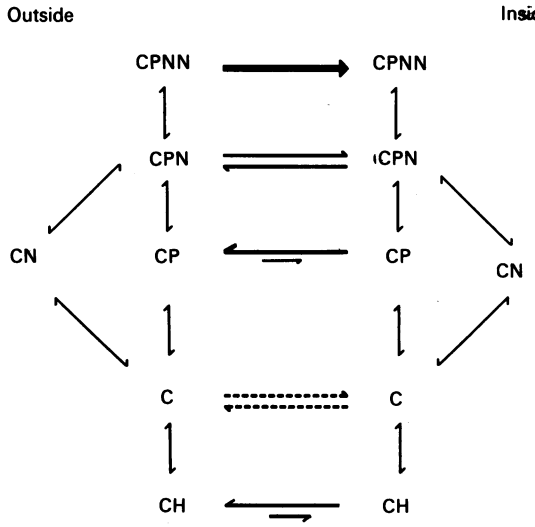
both sides of the membrane. Independently of the presence or absence of a pH gradient, monensin by itself had no effect on the Na^+-gradient-independent influx of glucose.

DISCUSSION

The effects of the Na^+ gradient, H^+ gradient, $[HPO_4^{2-}]$ and $[H_2PO_4^-]$ on the transport of phosphate by the renal brush-border membrane were investigated. Three different effects of H^+ are reported. The first one is on the identity of the phosphate ion (univalent or bivalent) interacting with the carrier protein. *Trans* (intravesicular) phosphate is a strong stimulator of the influx of phosphate (Fig. 3). At pH 7.5 and 8.5, the stimulation is identical whether total phosphate or bivalent phosphate is considered. At an internal pH of 6.5, the apparent lack of stimulation by 10 mM total phosphate contrasts with the stimulation observed when the phosphate concentration is adjusted to 25 mM, to give the concentration of internal bivalent phosphate (8.33 mM) that was used at pH 7.5 and 8.5. This indicates that the form of phosphate interacting with the carrier, on the intravesicular side, to cause *trans*-stimulation, is bivalent. This conclusion was confirmed by the similar stimulation of phosphate transport by bivalent phosphate at pH 6.5, 7.5 and 8.5 in the presence of Na^+, but in the absence of Na^+ and H^+ gradients (Fig. 5). On the extravesicular side, bivalent phosphate also appears to be the favoured substrate. As shown in Fig. 1, the transport at pH 6.5 was increased from 31 to 73% of the value measured at pH 7.5 by adjusting the bivalent phosphate concentration at pH 6.5 to that at pH 7.5.

The second effect of pH is an allosteric modulation of the transport protein itself. The evidence for allostery comes from the experiments performed in the presence of identical bivalent phosphate concentrations and shown in Figs. 1 and 5. In the absence of a proton gradient (Fig. 1), phosphate transport shows a maximum at pH 7.5. These results are in agreement with those showing a modulation of the affinity for Na^+ by external pH [14]. This allosteric modulation is also evident in Fig. 5, where in the absence of Na^+ and H^+ gradients, but in the presence of *trans* phosphate, the transport shows a maximum at pH 7.5. This allosteric modulation does not occur in the absence of internal phosphate, even when internal Na^+ is present (Fig. 5, control), indicating that the allosteric modulation is on the binary complex CP_i and not on the free carrier C or on the binary complex CNa^+. The allosteric protonation of the carrier would then favour its interaction with HPO_4^{2-} on the cytosolic side of the membrane. The larger stimulation by *trans* phosphate at pH 7.5 (*trans*) than at pH 6.5 or 8.5, in these two conditions, indicates a protonation–deprotonation of the carrier molecule itself. These results are in agreement with those of Bindels *et al.* [15], who showed that the $V_{max.}$ for phosphate transport was maximal between pH 7.1 and 7.4, suggesting that this pH range should correspond to the pH optimum of the transport protein itself. This allosteric modulation at the physiological pH of 7.5 would contribute to optimize phosphate re-absorption by allowing faster recycling of the carrier to the extracellular side.

The third effect of H^+ is a stimulation of phosphate transport by an acidic intravesicular pH. Two sets of evidence indicate that an H^+ gradient is also a driving force for the transport of phosphate, in addition to the Na^+ gradient. First, under Na^+-equilibrium conditions, there is a marked overshoot of phosphate transport, twice the equilibrium value (Fig. 6). This stimulation was specific for phosphate transport: glucose transport remained unaffected by an acidic intravesicular pH (Fig. 7). The stimulation of phosphate transport by an acidic intravesicular pH was also evident under Na^+-gradient conditions (Fig. 3). Secondly, CCCP strongly decreases phosphate transport driven by the acidic



Scheme 1. Kinetic model of phosphate transport by the renal phosphate transporter

The thin arrows represent the rapid equilibrium steps (binding or unbinding of Na^+, HPO_4^{2-} or H^+ to or from the carrier). The translocation of the quaternary complex (CPNN) indicated by the thickest arrow is the fastest step of the overall process. Broken arrows represent the overall rate-limiting steps of the complete cycle, i.e. the return of the free carrier (C). The carrier could exist in a protonated form (CH) which would allow a faster recycling of the carrier, compared with its free form (C). Abbreviations: N, Na^+; C, carrier; P, phosphate; H, proton.

intravesicular pH. This confirms that the pH gradient is a driving force, because its destruction by CCCP causes the inhibition of transport. Since internal H^+ is able to stimulate phosphate transport, in the absence of internal Na^+ or phosphate (Fig. 3), we have to postulate the existence of a protonated carrier (CH), on the internal face, that would recycle faster than the free carrier (C) (Scheme 1). The form CPH was not included in the model, for three reasons. (1) In the complete absence of Na^+, phosphate uptake is independent of the pH (Table 1), suggesting the absence of an external CPH. (2) Na^+-independent phosphate uptake is totally unaffected by a pH gradient (in > out) (Fig. 6). (3) The stimulation by intravesicular phosphate (Fig. 3) is similar at acidic and alkaline intravesicular pH, suggesting that the form CPH either is not present or does not significantly accelerate the recycling (in/out) of the carrier. Similarly, the CPNH or CNH forms do not appear to exist because the stimulation caused by internal bivalent phosphate, in the presence of internal Na^+, is similar at acidic and alkaline pH values (Fig. 5).

An acidic micro-environment on the cytosolic side of the luminal membrane could make this additional driving force play a major role in the re-absorption of phosphate from the glomerular filtrate. Micro-compartmentation can be restricted to a very small region. Micro-acidification at the catalytic site where the carrier could get protonated would be sufficient to activate the transporter. The increase in proton concentration could simply come from the deprotonation of sites in physical proximity of the carrier. The structural constraint imposed by the lipid bilayer on the membrane proteins could favour such a precise alignment of these acid–base sites. The role of H^+-buffering domains in the membrane phase, in relation to energy coupling, is well established in other systems such as the thylakoid membrane [19], where micro-compartmentation of protons was shown to be implicated in the energy-transduction processes performed by this membrane. Heterogeneity of pH in the aqueous cytoplasm was also previously reported [20], in renal proximal-tubule cells, suggesting that the micro-environment inside the cell plays an

important role in the energy metabolism. A pH-gradient-stimulated phosphate-transport activity from medullary brush-border membrane vesicles was described recently [21]. This carrier transports preferentially the acidic form of phosphate by a facilitated diffusion mechanism. It differs from the carrier studied in the present paper since it is located in the outer medulla and is totally Na^+-independent. An additional Na^+-dependent transport system has been reported, in renal brush-border membrane vesicles [12,15,22]. In the rat, however, this system operates at a much higher temperature (35 °C) than that used in the present paper. Since all experiments were performed at 25 °C, we think that the second system did not contribute to the effects reported here. More importantly, the affinity of the system that appears at 35 °C is much lower than that at 25 °C: 1.6 mM compared with 80 μM. Since our experiments were performed at low phosphate concentrations (200 μM), the contribution of the low-affinity system would be negligible.

In conclusion, bivalent phosphate is the form of phosphate interacting with the carrier molecule on both sides of the membrane. Internal pH modulates allosterically the recycling of the carrier molecule. Phosphate re-absorption is modulated by two distinct driving forces: the Na^+ gradient (out/in) and an H^+ gradient (in/out). The co-ordination of these forces contributes to optimize re-absorption of phosphate from the glomerular filtrate.

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