A pH gradient (intravesicular > extravesicular), in the absence of a Na\(^+\) gradient (extravesicular > intravesicular) stimulates phosphate uptake by renal brush border membrane vesicles and provided the driving force to effect the transient accumulation of phosphate against its concentration gradient. The pH gradient-dependent uptake of phosphate had an absolute requirement for Na\(^+\). The rates of uptake and peak accumulation were functions of the ΔpH and the concentration of H\(^+\) in the intravesicular medium. The pH gradient-energized Na\(^+\)-phosphate cotransport system was not affected by valinomycin- or carbonyl cyanide p-fluoromethoxyphenylhydrazone-induced ion diffusion potentials. Therefore, it was independent of the membrane potential, i.e., an electroneutral process. Amiloride, which inhibited the H\(^+\)-Na\(^+\) exchange reaction and prevented the efflux of H\(^+\) from the intravesicular medium, enhanced the uptake of phosphate. A model is proposed by which the H\(^+\) gradient mediates the uphill transport of phosphate. It is suggested that a similar model may operate in more physiologically intact preparations and may provide one mechanism by which acid-base balance regulates renal phosphate transport.

Previous studies have shown that phosphate reabsorption by the kidney takes place predominantly, although not exclusively, in the proximal tubule (1), is highly dependent on the presence of Na\(^+\) in the tubular fluid (2), and is transported actively against an electrochemical gradient (3). These physiological results become explicable with the demonstration of a Na\(^+\)-phosphate cotransport system in the proximal tubule brush border membranes and with the finding that a Na\(^+\) gradient, extravesicular > intravesicular ([Na\(^+\)\(_{iv}\)] > [Na\(^+\)\(_{ev}\)]), can energize the transient concentrative accumulation of phosphate into the membrane vesicles (4–8). Regulation of the renal reabsorption of phosphate has been found to be mediated by various physiological-pathological factors, including hormones, diet, metabolism, and acid-base status (1, 9). The effect of pH on renal phosphate clearance is seemingly conflicting, however. Alkalization of the filtrate has been reported to decrease phosphate reabsorption by the tubule and increase phosphate excretion (10). On the other hand, microperfusion studies indicate that an acidic and not an alkaline pH inhibits phosphate transport (11). Investigations of the effect of pH on phosphate uptake by membrane vesicles tend to support the latter conclusion (4, 8). When the pH of the intravesicular and extravesicular media is increased from 6.0 to 8.5, a 10-fold increase is found in the Na\(^+\) gradient-dependent rate of phosphate uptake (8). This enhancement is due to the pH dependency of the transport system per se and to an increase in the proportion of phosphate anions that are in the divalent form relative to those that are in the monovalent form (8). In this paper, the effect of pH on phosphate uptake is examined further and evidence is presented showing that a pH gradient, [H\(^+\)\(_{iv}\)] > [H\(^+\)\(_{ev}\)], stimulates phosphate transport and, in fact, can provide the driving force for the uphill uptake of phosphate in the absence of a Na\(^+\) gradient. A preliminary account of part of this work has been presented in abstract form (12).

EXPERIMENTAL PROCEDURES

Rabbit renal brush border membrane vesicles were prepared by the method previously reported (13). In experiments in which the intravesicular medium was varied, the vesicles were preloaded by resuspending and washing the 35,000 × g pellet initially in 300 mM mannitol buffered with 5 mM Hepes/Tris, pH 7.3, followed by carrying out the remainder of the washing procedure in the described medium (13). The efficacy of the preloading procedure to vary the intravesicular medium, thereby to establish gradients of Na\(^+\), K\(^+\), and H\(^+\) across the intravesicular and extravesicular media or to equalize the distribution of these ions in the two media, was ascertained previously (8, 13, 14). The quality of the membrane preparations, evaluated by specific activities of enzyme markers, was the same as reported earlier (13).

Uptakes of \([^{32}\text{P}]\)phosphate and \([^{3}H]\)glucose were measured by a Millipore filtration technique (15, 16), using 0.65 μm filters. Incubations were carried out in triplicate with freshly prepared membranes. Each experiment was repeated at least three times with different membrane preparations. The results are expressed as the mean ± SE. Membrane protein was assayed by a standard method (17) using bovine serum albumin as the reference protein. [\(^{32}\text{P}]\)Phosphoric acid (carrier free) and [\(^{3}H]\)glucose (30 Ci/mmol) were obtained from New England Nuclear. Valinomycin was purchased from Sigma. FCCP was obtained from Pierce Chemical Co. 5-Adenosine was donated by Eli Lilly Laboratories. Amiloride was obtained from Merck and Co.

RESULTS

We previously reported that an imposed Na\(^+\) gradient, [Na\(^+\)\(_{iv}\)] > [Na\(^+\)\(_{ev}\)], provided the driving force to effect the transient uptake of phosphate into renal brush border membrane vesicles against its concentration gradient (8). If the vesicles were preloaded with Na\(^+\) so that [Na\(^+\)\(_{iv}\)] = [Na\(^+\)\(_{ev}\)], the overshoot was abolished (8). The experiments illustrated in

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Fig. 1 now show that a H⁺ gradient, \([H^+]_i > [H^+]_o\), in the absence of a Na⁺ driving force, also affected the transient concentrative uptake of phosphate. When the intravesicular medium was pH 5.5 and the extravesicular medium was 7.3, the initial rate (the 10-s uptake value was taken to represent an approximation of initial velocity) was increased about 5 times compared to the uptake rate when both media were pH 7.3. Accumulation was maximal in 1 min. Thereafter, the amount of phosphate in the vesicles decreased, indicating efflux. The level of uptake in the presence and absence of a H⁺ gradient was identical after about 15 min, indicating that equilibrium had been achieved and the average intravesicular volumes of the vesicles in the presence and absence of the pH gradient were the same (16). Fig. 1 also shows that when Na⁺ was omitted from the incubation media the intravesicular > extravesicular H⁺ gradient had no effect. Thus, the increased uptake of phosphate due to a H⁺ gradient had an absolute requirement for Na⁺.

The action of the H⁺ gradient on phosphate uptake was specific for the anion. Na⁺-D-glucose cotransport was only slightly affected by the intravesicular > extravesicular H⁺ gradient (Fig. 1). This minimal effect could be explained by the generation of a small membrane potential (inside negative) concomitant with the movement of H⁺ down its electrochemical gradient, from the intravesicular to extravesicular medium (15). The inside negative membrane potential would enhance the electrogenic Na⁺-D-glucose cotransport, even in the absence of a Na⁺ gradient (16). This small hyperpolarization of the membrane would not influence Na⁺-phosphate cotransport, however, since the Na⁺-phosphate cotransport system was found to be electroneutral and thus independent of the membrane potential (8). Moreover, in other experiments (not illustrated) in which Na⁺ was added to the media in the form of NaCl rather than Na-gluconate (Fig. 1), the slight H⁺ gradient stimulation of D-glucose was not observed. Since the brush border membrane was found to be more permeable to Cl⁻ than to gluconate (14), Cl⁻ could cross the membrane to compensate for the inside negative potential; thus, the development of a potential-induced uptake of D-glucose would be precluded. On the other hand, the substitution of NaCl for Na-gluconate did not affect the H⁺ gradient-dependent uptake of phosphate (data not shown).

The H⁺ gradient-dependent overshoot of phosphate uptake was completely inhibited by monensin (Fig. 2). The ionophore, which increased the electroneutral exchange of H⁺ for Na⁺, presumably dissipated the H⁺ gradient, thereby obviating the driving force for the concentrative uptake.

Increases in the H⁺ gradient-dependent uptake of phosphate were found to be a function of the ΔpH. As shown in Fig. 3, both the initial rate of uptake and the accumulation of phosphate at the peak of the overshoot were increased as the intravesicular pH was decreased from 7.3 to 5.0 while the pH of the extravesicular medium was maintained at 7.3. The same correlation with H⁺ gradient was seen when the pH of the intravesicular medium was kept at 5.5 and the pH of the extravesicular medium was varied from 6.5 to 8.0 (Fig. 4). In addition to the ΔpH, the actual concentration of H⁺ in the intravesicular medium was of importance to the uptake of phosphate. Table I shows that although the ΔpH was kept the same at 1.5 pH units, i.e. intravesicular pH of 5.5 and extravesicular pH of 7.0 compared to intravesicular pH of 7.0 and extravesicular pH of 8.5, the greater the internal [H⁺], the more rapid was the initial rate of phosphate uptake and the greater was the accumulation at 1 min.

The kinetic effect of the H⁺ gradient is shown in Fig. 5. A \(K_v\) value of 126 μM was calculated, when the intravesicular pH was 5.5 and the extravesicular pH was 7.3. This was virtually identical with a \(K_v\) value of 131 μM, when the pH of both intravesicular and extravesicular media was 7.3. The \(V_{max}\) of the phosphate uptake system was greatly increased in the presence of the H⁺ gradient.

Since it was reported previously that Na⁺ gradient-dependent phosphate uptake, in the absence of a H⁺ gradient, was an electroneutral cotransport process (8), i.e. not associated with the net transfer of electrical charge, the question was posed as to whether the same was true for the H⁺ gradient-dependent system. To resolve this question, the effects of imposed membrane potentials were determined. Fig. 6 shows the effect of a valinomycin-induced K⁺ diffusion potential (interior negative) on the H⁺ gradient-dependent uptake of phosphate in an experiment in which \([Na^+]_i = [Na^+]_o\). The membrane potential had no significant effect on phosphate uptake. In contrast, as reported previously (13), valinomycin greatly enhanced the overshoot when D-glucose was the transported solute. The ionophore presumably induced the efflux of K⁺ down its electrochemical gradient with concomitant generation of a membrane potential, interior negative, and, in the case of D-glucose uptake, the development of this potential accelerated the influx into the vesicles of the positively charged Na⁺ coupled to the transport of the uncharged sugar, even though initially there was no Na⁺ gradient.

Fig. 7 illustrates how a membrane potential, interior negative, generated by a H⁺ diffusion potential rather than a K⁺ diffusion potential, affected the uptake of phosphate and D-
Fig. 2 (left). The effect of monensin on the \( H^+ \) gradient-dependent uptake of phosphate. The intravesicular medium contained 125 mM mannitol, 100 mM Na-gluconate, 75 mM Mes/Tris, pH 5.5. The extravesicular medium contained 125 mM mannitol, 100 mM Na-glucinate, 75 mM Hepes/Tris, pH 7.3. The concentration of \( K_2H_2PO_4 \) was 25 \( \mu M \). Monensin (6 \( \mu g/mg \) of protein, in ethanol) or ethanol alone (0.9%, final concentration) was added, as indicated.

Fig. 3 (center). The effect of the intravesicular \( H^+ \) concentration on the \( H^+ \) gradient-dependent uptake of phosphate. The intravesicular media were 125 mM mannitol, 100 mM Na-gluconate, 75 mM Mes or 75 mM Hepes, adjusted to the indicated pH with Tris. The extravesicular medium was 125 mM mannitol, 100 mM Na-gluconate, 25 \( \mu M \) \( K_2H_2PO_4 \), and either 75 mM Hepes/Tris, pH 7.0, or 75 mM Tris/Hepes pH 8.5.

Table I

| Incubation condition | Uptake | pmol/mg protein |
|----------------------|--------|-----------------|
| pH 5.5/7.0,          |        |                 |
| pH 7.0/8.5,          |        |                 |
| pH 7.0/7.0,          | 87 ± 2 | 155 ± 3         |
| pH 7.0/8.5,          | 60 ± 3 | 96 ± 1          |

Fig. 4 (right). The effect of the extravesicular \( pH \) on the \( H^+ \) gradient-dependent uptake of phosphate. The intravesicular medium was the same as given in Fig. 2. The extravesicular media were 125 mM mannitol, 100 mM Na-gluconate, 75 mM Hepes, adjusted to the indicated pH with Tris. The concentration of \( K_2H_2PO_4 \) was 25 \( \mu M \).

Fig. 5. The effect of the \( H^+ \) gradient on the kinetics of phosphate uptake. The composition of the intravesicular and extravesicular media as was described in Fig. 1. Uptakes were determined after a 10-s incubation. The inset shows a double reciprocal plot of the data. ○ and □ show the data with and without the \( H^+ \) gradient, respectively.

In this experiment, \( H^+ \) diffusion potentials were induced by the mitochondrial uncoupler, FCCP. Again, \( H^+ \) gradient-dependent Na\(^+\)-phosphate cotransport was affected slightly, if at all, whereas the electrogenic Na\(^+\)-d-glucose co-

transport was greatly stimulated by the interior negative potential, despite the absence of a Na\(^+\) gradient. It was important to note that in these experiments the glutonate anion was used throughout. In the presence of this anion, to which the membrane was relatively electrophoretically impermeable, the FCCP-induced membrane potential (exterior positive) would oppose the efflux of \( H^+ \) down its electrochemical gradient. Thus, in these experiments FCCP would presumably induce the inside negative membrane potential without appreciably dissipating the \( H^+ \) chemical gradient.

The distinction in the effects of \( K^+ \) and \( H^+ \) diffusion potentials on the uptake of phosphate and that of d-glucose suggested that with this experimental condition, \( i.e. [H^+]_i > [H^+]_e, [Na^+]_i = [Na^+]_e \), the \( H^+ \) gradient-driven cotransport of Na\(^+\) and phosphate did not result in the net transport of charge. This electroneutral mechanism would imply that if the \( H^+ \) gradient ([\( H^+ \)_i > [\( H^+ \)_e]-dependent uphill transport of phosphate was mediated by an efflux of \( H^+ \), then there would be the coupled counterflow of Na\(^+\). However, an alternate hypothesis might suggest that the \( H^+ \) gradient driving force for the concentrative uptake of phosphate was not concomitant with the downhill efflux of \( H^+ \). To test this possibility, \( H^+ \) gradient-dependent uptake of phosphate was examined when the movement of \( H^+ \) from the intravesicular to the extravesicular medium was inhibited by amiloride, a drug previously reported to block H\(^+\)-Na\(^+\) exchange in renal brush border membranes (19).

First, it was necessary to show that amiloride would inhibit the exchange reaction when the conditions described here to measure phosphate uptake were employed, namely, the presence of a \( H^+ \) gradient but the absence of a Na\(^+\) gradient and with a high concentration of Na\(^+\) in the intravesicular and extravesicular media. Fig. 8 shows that amiloride decreased the initial rate of \(^{22}\text{Na}\) uptake about 90%. Thus, this finding indicated that amiloride did block the \( H^+\)-Na\(^+\) exchange reaction when driven by a \( H^+ \) gradient.

Next, the \( H^+ \) gradient-dependent uptake of phosphate was examined in the presence of amiloride. As shown in Fig. 9A, uptake was markedly enhanced. This stimulation by amiloride
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FIG. 8. The effect of amiloride on the uptake of \(^{22}\)Na\(^+\). The intravesicular medium was 125 mM mannitol, 50 mM NaCl, 75 mM Mes/Tris, pH 5.5. The extravesicular medium was 125 mM mannitol, 50 mM NaCl, 75 mM Hepes/Tris, pH 7.3, without \(\bigcirc\) and with \(\bigcirc\) 5 mM amiloride.

was dependent on the presence of a H\(^+\) gradient, for, in the absence of H\(^+\) and Na\(^+\) gradients, amiloride had no effect. Thus, the inhibition of the exchange reaction, which presumably prevented efflux of H\(^+\) (19) and sustained a high concen-

FIG. 6. The effect of valinomycin-generated K\(^+\) diffusion potential (interior negative) on the H\(^+\) gradient-dependent uptake of phosphate. The intravesicular medium was 25 mM mannitol, 100 mM Na-glucuronate, 50 mM K-glucuronate, 75 mM Mes/Tris, pH 5.5. The extravesicular medium contained 125 mM mannitol, 100 mM Na-glucuronate, 75 mM Hepes/Tris, pH 7.3, and either valinomycin (VAL) (5 \(\mu\)g/mg of protein, in ethanol \(\bigcirc\)) or 0.9% ethanol \(\bigcirc\). The K\(_2\)HPO\(_4\) and \(\beta\)-[\(^{3}H\)]glucose concentrations were the same as reported in Fig. 1.

FIG. 7. The effect of a FCCP-generated H\(^+\) diffusion potential (interior negative) on the H\(^+\) gradient-dependent uptake of phosphate. The intravesicular and extravesicular media were the same as reported in Fig. 6. The FCCP concentration was 20 \(\mu\)M. Ethanol (0.9%) was included in the minus FCCP controls.

FIG. 9. The effect of amiloride on the H\(^+\) gradient-dependent uptake of phosphate and D-glucose. The intravesicular medium was 125 mM mannitol, 100 mM Na-glucuronate, 75 mM Mes/Tris, pH 5.5 (C, \(\bigcirc\)), or 75 mM Hepes/Tris, pH 7.3 (\(\bigtriangledown\), \(\Delta\)). The extravesicular medium was 125 mM mannitol, 100 mM Na-glucuronate, 75 mM Hepes/Tris, pH 7.3. When indicated, 10 mM amiloride was added. In A, the K\(_2\)HPO\(_4\) concentration was 25 \(\mu\)M and in B, 50 \(\mu\)M D-[\(^{3}H\)]glucose was used.
tration of intravesicular H⁺, augmented the uptake of phosphate. This finding would favor the hypothesis that the H⁺ gradient-dependent phosphate uptake was not obligatorily increased (Fig. 10). In the absence of a Na⁺ gradient, the rate of uptake of phosphate into the membrane vesicle would be dependent, in part, on the relative concentrations of phosphate in the extravesicular and intravesicular media. But, the species of the phosphate anion should be considered, for in isotope experiments with ³²P the anionic forms of phosphate were indistinguishable. In an extravesicular medium of pH 7.3, the predominant anion form of phosphate would be HPO₄²⁻. Additionally, in the presence of both the divalent and monovalent species, HPO₄²⁻ was the probable preferred species for transport (8). Thus, relatively more HPO₄²⁻ would be taken up by the membrane vesicle than H₂PO₄⁻. With an intravesicular pH more acidic than the extravesicular pH, H⁺ would convert HPO₄²⁻ to H₂PO₄⁻. In this way, the extravesicular to intravesicular gradient of HPO₄²⁻ was largely maintained; consequently, HPO₄²⁻ uptake was not appreciably diminished. Intravesicularly, the H₂PO₄⁻ species accumulated, since efflux was considerably slower than influx (8). This resulted in the overshoot, or the transient accumulation of total phosphate in the intravesicular medium.

It is suggested that a similar mechanism may operate in more physiologically intact preparations, even in the presence of a Na⁺ gradient, for any process that results in intracellular acidification will tend to increase the uptake of phosphate from the filtrate of the proximal tubular lumen. Thus, one mechanism by which acid-base balance may regulate renal phosphate transport is implied. In addition, this investigation is consistent with and further strengthens the view that the HPO₄²⁻ anion is preferentially transported relative to the H₂PO₄⁻ anion.

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