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Phosphate Transport in Intestinal Brush-Border Membrane

Soraya P. Shirazi-Beechey,[1,3] Jean-Pierre Gorvel,[2] and R. Brian Beechey[1]

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Abstract

In the small intestine of the rabbit the process of Na^+-dependent uptake of phosphate occurs only at the brush-border of duodenal enterocytes. Li^+ can replace Na^+. The process is activated when either K^+, Cs^+, Rb^+, or choline is present in the intravesicular space. The presence of membrane-permeable anions is essential for maximum rates of phosphate transport. We conclude that the mechanism of the phosphate carrier is electrogenic at pH 6–8, probably two Na^+ moving with each $H_2PO_4^-$. This will lead to the development of a positive charge within the vesicle. The variation of the K_m for $H_2PO_4^-$ with pH is thought to be the consequence of the affinity of the carrier protein for $H_2PO_4^-$ increasing as the pH increases. Polyclonal antibodies against membrane vesicles isolated from rabbit duodenum, jejunum, and ileum were prepared. The antibodies raised against the ileum and jejunum both activated the phosphate transport process, while the anti-duodenum antibody preparation inhibited phosphate transport.

Key Words: Glucose transport; phosphate transport; small intestine; brush-border; vesicles.

Introduction

The uptake of phosphate into cells and its assimilation into cellular material is an essential feature of metabolism. In mammals the process begins by the entry of phosphate into the enterocytes lining the intestinal tract. Mature organisms require the phosphate for the matinenence of the cells, but there is an obligatory requirement for a net uptake of phosphate in pregnant,

[1]Department of Biochemistry, University College of Wales, Penglais, Aberystwyth, Dyfed SY23 3DD, UK.
[2]Centre de Biochimie et de Biologie Moleculaire, CNRS, 31 Chemin Joseph-Aiguier. B.P. 71, 13277 Marseille Cedex 9, France.
[3]To whom correspondence should be addressed.

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lactating, and growing animals. In ruminants the absorption of phosphate in the intestine is an essential aspect of the massive recycling of phosphate that is required to supply nutrient to the rumen organisms and to play a role in buffering the pH of the rumen (Clark *et al.*, 1973; Scott *et al.*, 1985).

The process of phosphate transport at the brush-border membrane of the mammalian intestinal enterocyte is poorly characterized. Much of the experimentation that has been performed has been derived from studies relating to the movement of phosphate across renal epithelial cells (Amstutz *et al.*, 1985; Schali *et al.*, 1986). It should be appreciated that the conditions under which phosphate is transported in the kidney are not subjected to the variations that can exist within the lumen of the gut. In rabbits and in rats the process is known to be facilitated by a Na^+-phosphate symporter (Berner *et al.*, 1976; Shirazi *et al.*, 1981; Danisi *et al.*, 1984; Shirazi-Beechey and Beechey, 1986).

This report sets out to describe some of the important features of the mechanism for the uptake of phosphate in the rabbit intestine. They are intended to serve as a baseline for current studies on the flux of phosphate across the enterocyte, the isolation of the proteins involved, their synthesis and incorporation into the different regions of the plasmalemma membrane, and also the development of the capacity to transport phosphate as a function of the maturity of the enterocyte.

Methods

Preparation of Intestinal Brush-Border Vesicles

These were prepared from the duodenum, mid-jejunum, and the distal ileum of rabbit. Sections of gut were everted and washed with saline. They were cut into 1-cm pieces and dropped into liquid nitrogen. These were either defrosted and used immediately for the preparation of brush-border vesicles or were stored at $-80°C$ until use. The method of Kessler *et al.* (1978) combined with the magnesium precipitation technique of Biber *et al.* (1981) was used. The final suspension of brush-border vesicles in the appropriate buffer was made homogeneous by passing the suspension through a 27-gauge needle 10 times. Routinely the final suspension was made in a medium that contained 300 mM mannitol, 0.1 mM $MgSO_4$, 0.02% NaN_3, and 10 mM Hepes/Tris, pH 7.4. The final protein concentration was approximately 20 mg/ml. The addition of azide is essential to avoid misleading results due to the presence of bacteria.

Storage of the Vesicles

Aliquots, 50 μl, of the suspensions of vesicles were stored in liquid nitrogen. For experimentation the vesicles were removed from the liquid

nitrogen, thawed, and diluted with the final suspension buffer to a protein concentration of 10 mg/ml.

Loading of the Brush-Border Vesicles

The contents of the vesicles were changed using a procedure based on that of Rudnick (1977). A 100-μl suspension of brush-border vesicles (20 mg of protein/ml) was diluted with 1 ml of the medium with which the vesicles were to be loaded. The suspension was made homogeneous by passing through the 27-gauge needle. It was centrifuged at 27,000 ***g*** for 30 min. The pellet was then resuspended using the 27-gauge needle in 200 μl of the medium with which the vesicles were to be loaded. They were then placed in liquid nitrogen for a minimum of 5 min before removal and use.

Assay of Na^+-Dependent Phosphate-Transport Activity

The uptake of phosphate was measured using a filtration stop technique (Shirazi *et al.*, 1981). Routinely the assay was begun by the addition of 100 μl of incubation medium to 5 μl of a suspension of brush-border vesicles, 50 μg of protein. After an appropriate period the uptake was quenched by the addition of 1 ml of an ice-cold stop solution. Incubations were carried out in triplicate, either at 25 or 37°C. The brush-border vesicles were separated from the incubation medium by placing 0.9 ml of the quenched assay medium onto a 0.22-μ cellulose acetate/nitrate filter (Millipore GSWP 02500) and filtering, under vacuum. The filter was washed five times with 1-ml aliquots of stop buffer. The radioactivity retained on the filter was measured using a scintillation counter. The uptake at "zero time" was measured by adding the stop solution prior to the addition of the vesicles.

The incubation medium contained 100 mM NaCl, 100 mM mannitol, 0.02% NaN_3, 10 mM Hepes-Tris, pH 7.4, and 0.1 mM ^{32}P-phosphate (7×10^6 cpm). To assay the passive, non-carrier-mediated, uptake of phosphate by the brush-border vesicles, sodium chloride was replaced by choline chloride. The stop solution contained 150 mM NaCl, 0.02% NaN_3, and 10 mM Hepes/Tris, pH 7.4. The uptake of phosphate into the brush-border vesicles was linear with time over the first 30 s. There was a direct proportionality between the phosphate uptake and the amount of vesicles present, up to 100 μg of protein per assay. Routinely each assay was done at least in triplicate. The variation was no greater than 10% of the mean value.

Assay of Sodium-Dependent Uptake of D-Glucose

The method was similar to that used for the uptake of phosphate save that the reaction medium contained 0.1 mM ^{14}C-D-glucose, 80 Ci/mole, 100 mM NaSCN, 0.1 mM $MgSO_4$, 0.02% NaN_3, 10 mM Hepes/Tris, pH 7.4,

and, where appropriate, 500 μM phlorizin was present. The stop solution was the same as that used for the assay of phosphate transport with the addition of 250 μM phlorizin. Routinely each assay was performed in triplicate. The values were $\pm 4\%$ of the mean.

Protein was assayed by its capacity to bind Coomassie blue. The procedure used was that described by Biorad.

Results and Discussion

Properties of the Brush-Border Vesicles

The activities of sucrase, aminopeptidase N, and alkaline phosphatase were ten- to twelvefold enriched in these vesicles over the original tissue homogenates (Shirazi-Beechey and Beechey, 1986). The recoveries of each of these enzymes in the brush-border vesicle fraction amounted to 40% of the activity in the original homogenate. There was no significant enrichment of K^+-stimulated phosphatase, succinic acid dehydrogenase, NADH-cytochrome *c* reductase, and acid phosphatase activities in the brush-border vesicles. This indicated that there was no significant contamination of the brush-border vesicles with other cell membranes. Detergents had no effect on the level of the sucrase activity. The results of control experiments showed that sucrose could not penetrate the membrane within 1 min, the time of the sucrase assay. From these results we conclude that the majority of the brush-border vesicles had the same orientation as seen in intact cells. The internal volume of the vesicles from the duodenum was 1 μl/mg of protein. This is similar to the values of 0.81 μl/mg of protein, calculated from the data of Danisi *et al.* (1984). The vesicles isolated from the jejunum and ileum had a volume of 0.5 μl/mg of protein. These volumes were calculated from the equilibrium distribution levels of ^{14}C-D-glucose.

The procedures described for the preparation of the vesicles and the conditions used for their storage have proved very reliable. The vesicles have been shown to retain totally their ability to transport both phosphate and D-glucose for periods as long as 8 weeks when stored at the temperature of liquid nitrogen. The initial rate of phosphate uptake was usually in the range 400–460 pmol of phosphate per min per mg of protein. Values of some tenfold greater were obtained with the vesicles isolated from some rabbits; see the legend to Table II.

The Location of Na^+-Dependent Phosphate Transport in the Small Intestine of Rabbit

Vesicles were prepared from the duodenum, the mid-jejunum, and the distal ileum regions of the rabbit small intestine. The abilities of the vesicles

from the different regions to transport both phosphate and D-glucose at 25°C in a Na^+-dependent manner was assayed. The Na^+-dependent, phlorizin-sensitive transport of D-glucose was affected to different extents by the vesicles from the three regions. The time course of the uptake of D-glucose showed that it resulted in a transient increase in the intravesicular concentration of D-glucose to values of 0.75 mM (duodenum), 2.8 mM (jejunum), and 1 mM (ileum). These were greater than the external concentration, 0.1 mM. The intravesicular level of D-glucose then diminished with time to that of the external medium, i.e., typical overshoot behavior. These results indicate that the vesicles from all three regions of the small intestine were physically and functionally intact. The abilities of the vesicles from the different regions of the small intestine to affect the Na^+-dependent transport of phosphate was investigated. The results are illustrated in Fig. 1. In the vesicles from the duodenum the uptake of phosphate increased with time to a point where the intravesicular concentration of phosphate (450 μM) was much higher than that in the external medium (100 μM). This transient accumulation of phosphate diminished with time. When choline ions were substituted for Na^+, the uptake of phosphate was very slow and no overshoot was observed. Phosphate uptake was seen only in the vesicles isolated from the duodenum (Fig. 1). This observation was supported by the results of the investigations of the immunological properties of the vesicles (see later). It

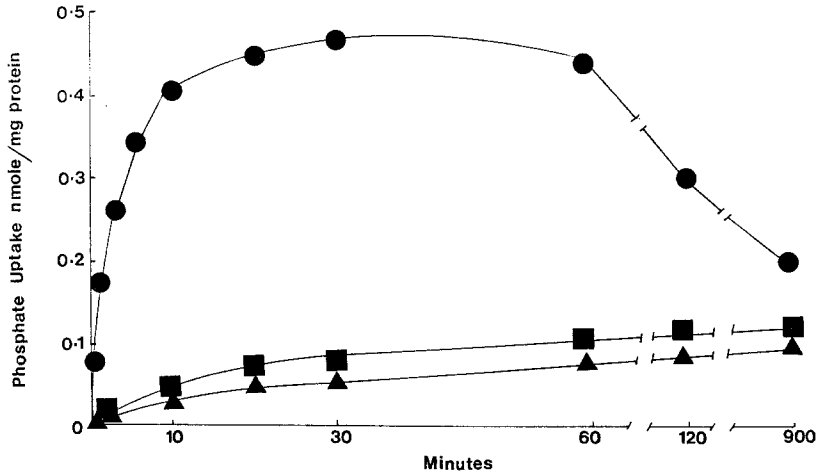

Fig. 1. The Na^+-dependent transport of phosphate in brush-border vesicles isolated from the duodenum, jejunum, and ileum of rabbit. The preparation of vesicles and the assay of phosphate transport activity are described in the Methods section. The temperature was 25°C. Duodenal vesicles, 100 mM NaCl present (●). Jejunal or ileal vesicles, 100 mM NaCl present (■). Duodenal, jejunal, or ileal vesicles, 100 mM choline Cl present (▲). Each assay was performed in triplicate. Each point is the average of five assays.

correlates well with those of Danisi and Straub (1980) who used sections of intact tissues to show that phosphate was transported in a Na^+-dependent manner only in the rabbit duodenum.

Properties of the Na^+-Dependent Phosphate-Transport System

The Effect of Temperature on the Initial Rate and the Time Course of Na^+-Dependent Phosphate Transport. The time courses of Na^+-dependent phosphate transport into brush-border vesicles at 25 and 37°C have been measured at pH 7.4. Typical results are shown in Figs. 1 and 2. It can be seen that the phosphate content at the peak of the overshoot at 25°C has risen from 0.45 to 0.6 nmol/mg of vesicle protein at 37°C, equivalent to

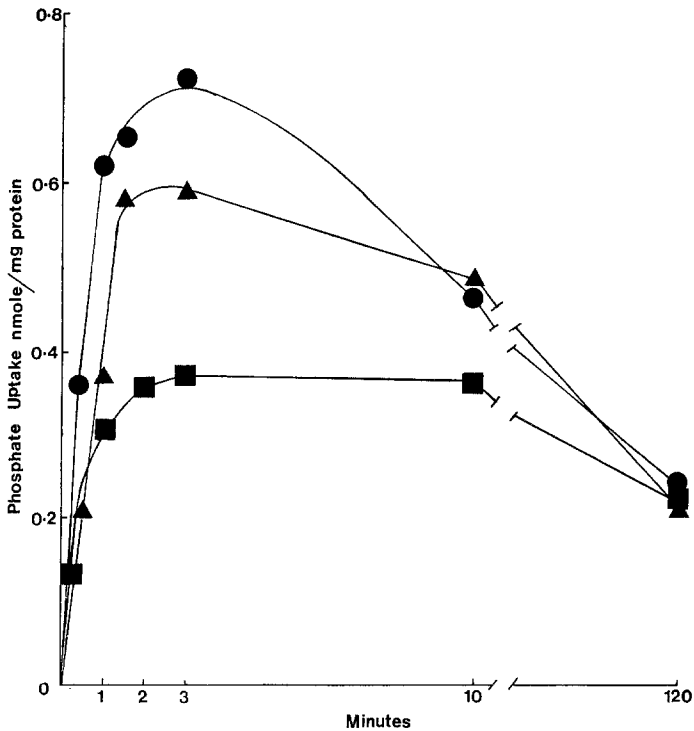

Fig. 2. The effects of pH gradients on the time course of Na^+-dependent phosphate transport. Duodenal brush-border vesicles were loaded with 300 mM mannitol, 0.1 mM $MgSO_4$, 0.02% NaN_3, and either 10 mM MES-Tris, pH 6, or 10 mM Hepes-Tris, pH 7.4 or pH 8. The time course of Na^+-dependent phosphate transport was measured at 37°C using standard incubation media with modified pH. The pH of the stop solution was the same as that of the incubation medium. pH 6 out/pH 8 in (●); pH 7.4 out/pH 7.4 in (▲); pH 8 out/pH 6 in (■). Each assay was performed in triplicate. Each point is the average of three assays.

intravesicular concentrations of phosphate of 0.45 and 0.6 mM respectively. Also, the initial rate of uptake increased from 0.15 to 0.75 nmol of phosphate per minute per mg of protein. This represents a temperature coefficient of almost 5, which may indicate that there is a significant increase in the membrane fluidity as the temperature is increased from 25 to 37°C. Gorvel *et al.* (1984) showed that there were significant increases in the polarization of fluorescence as the temperature of rabbit brush-border membrane was increased. This was interpreted as a change in the physical state of the membrane.

The Effect of Varying Substrate Concentration. The variation of phosphate transport was measured over the phosphate concentration range, 0.0125–2 mM. Transport was measured at 15 s, during the linear phase of uptake. Na^+-dependent uptake was calculated as the difference between the uptake in the presence of 100 mM Na^+ and 100 mM choline.

When the total phosphate concentration, i.e., the sum of the concentrations of mono- and dihydrogen orthophosphate ions, was regarded as the substrate for transport, the K_m (at 37°C) for total phosphate was 0.05–0.1 mM (pH 6–8). This is in agreement with the observations of Danisi *et al.* (1984) for rabbit, K_m = 0.16–0.22 mM (25°C and pH 6–7.6), and Quamme (1985) for chicken duodenal vesicles, K_m = 0.1–0.07 mM (21°C and pH 6.5–7.5). Both Quamme and Danisi *et al.* have interpreted their results in terms of a carrier mechanism that has (1) a stoichiometry that varies, (2) a mechanism which is capable of accepting both the monobasic and dibasic forms of orthophosphate, and (3) an affinity for Na^+ that is sensitive to pH. A simpler model that will account for all the experimental observations is that of a carrier which has a fixed stoichiometry, two Na^+ per dihydrogen orthophosphate ion, $H_2PO_4^-$. The affinity of this carrier for $H_2PO_4^-$ is sensitive to pH. The calculated values for K_m are 0.094 mM at pH 6, 0.020 mM at pH 7.4, and 0.014 mM at pH 8.

Arsenate (1 mM), an analogue of phosphate, completely inhibited the Na^+-dependent uptake of phosphate.

The Effects of Monovalent Cations on Phosphate Transport. The uptake of phosphate by duodenal brush-border vesicles in response to gradients of monovalent cations was measured using reaction media that contained 100 mM concentrations of either Na^+, Li^+, Cs^+, Rb^+ or choline ions. Chloride was the counter ion. The uptake of phosphate was not seen in the presence of gradients of K^+, Rb^+, Cs^+, and choline ions. Only gradients of Na^+ and Li^+ were capable of sustaining the uptake of phosphate, leading to an overshoot. A concentration gradient of 100 mM outside and zero inside of either Na^+ or Li^+ facilitated the transport of phosphate to the same extent. The affinity of the carrier for the two ions was very different, the K_m for Na^+ = 7 mM, and the K_m for Li^+ = 65 mM at pH 7.4 and 37°C. The only

other studies of the effects of cations on intestinal phosphate transport are those of Berner *et al.* (1976) and Shirazi *et al.* (1981). Berner *et al.* showed that in rat-intestinal brush-border vesicles, at pH 6 there was a nonspecific activation by monovalent cations. At pH 7.4 there was a twofold selectivity in favor of Na^+ and Li^+. This latter observation was confirmed by Shirazi *et al.* (1981); these workers found no activation by Rb^+ and Cs^+. The vesicles used by both these groups did not show the overshoot phenomenon.

In the present study the K_m for Na^+ was found to be 7–10 mM. It did not change over the pH range 6–7.4 (sodium azide was not included in the suspension medium and the reaction medium for this experiment). The pH-independence of the K_m is in agreement with the results of Quamme (1985), but in conflict with the data of Danisi *et al.* (1984) where the K_m is reported as 90 mM at pH 6 and 19 mM at pH 7. In the present study it was noted that at concentrations below 2 mM Na^+ there was a marked deviation from Michaelis–Menten kinetics. Similar deviations have been noted by Danisi *et al.* (1984), but at much higher concentrations of Na^+, 10–100 mM, depending on the pH. The calculated values of the Hill coefficients, around 2, have been used as a basis for assuming that more than one Na^+ is transported per phosphate ion (Quamme, 1985; Danisi *et al.*, 1984).

The Effects of Intravesicular Cations. The brush-border vesicles were loaded with 100 mM solutions of K^+, Cs^+, Rb^+, choline, Na^+, or Li^+ ions. The counter ion was chloride. The abilities of these vesicles to transport phosphate in a Na^+-dependent manner, at pH 7.4 (inside and outside), was measured. The results are presented in Table I. It can be seen that when either the permeable cations, K^+, Cs^+, Rb^+ or the impermeable choline cations were present in the intravesicular space, the initial rate of phosphate uptake was stimulated by 40–50%. This effect could be seen at intravesicular

Table I. The Effect of Intravesicular Cations on the Rate of Phosphate-Transport[a]

Intravesicular medium	Phosphate uptake (% control)
Mannitol	100[b]
KCl	146
RbCl	150
CsCl	144
Choline chloride	151

[a] Duodenal brush-border vesicles were loaded as described in the Methods section, with 100 mM solutions of K^+, Rb^+, Cs^+, Li^+, Na^+ and choline chlorides buffered at pH 7.4 with 10 mM Hepes-Tris. The internal osmolarity was maintained at 300 milliosmolar by adjusting the concentration of mannitol. The ability of these loaded vesicles to transport phosphate was assessed as the initial rate of phosphate uptake, measured using the standard assay conditions, 15 s uptake time. Each assay was done in triplicate.

[b] 100% = 460 ± 45 (N = 4) pmol per min per mg protein.

concentrations of K^+ as low as 2 mM. The nature and permeability of the anion did not appear to be important. Similar results were obtained with gluconate (impermeable) and chloride (permeable) ions. Thus the permeability of the ions did not affect the activation. We conclude that the stimulation is not caused by the imposition of membrane potential, but is probably due to a nonspecific activation of the carrier by cations consequent to their interaction with the section of the carrier molecule that faces the interior of the vesicle.

Na^+-Dependent Phosphate Transport in the Absence of a Gradient of Na^+

Two methods were used to equalize the concentration of Na^+ in the intra- and extravesicular media. In the first the vesicles were suspended in a medium containing 100 mM NaSCN and 50 μM monensin. In the second method the loading procedure described in the Methods section was used. Vesicles were loaded with 100 mM Na^+ using both these procedures. Consequently in the phosphate-uptake assay medium the transmembrane Na^+ gradient was zero. Under these conditions the initial rate of the uptake of phosphate was 40% of that seen in the presence of a Na^+ gradient (100 mM outside, 0 mM inside). No overshoot was seen. If Na^+ was replaced by choline there was no significant phosphate uptake over the first 30 s. The final, and maximal, intravesicular concentration of phosphate was close to that in the reaction medium. We ascribe this movement of phosphate as a response to the gradient of phosphate. These results also demonstrate clearly the requirement of a gradient of Na^+ in order to achieve the concentration of phosphate within the vesicles, i.e., an overshoot. The presence of Li^+ inside the vesicles diminished the uptake of phosphate in response to a gradient of Na^+.

Sodium-Ion Dependent Phosphate Exchange

It would be expected that a system which facilitates the Na^+-dependent uptake of phosphate will also allow the Na^+-dependent exchange of phosphate across the membrane in the absence of gradients of both Na^+ and phosphate. Vesicles were loaded with the incubation medium which contained unlabelled phosphate. Phosphate exchange was measured by adding these vesicles to the incubation medium which contained [^{32}P]-phosphate. There was a Na^+-dependent exchange of phosphate between the extra and intravesicular compartments which was complete within a minute. In the absence of Na^+ there was no carrier-mediated exchange. Since there was no gradient of Na^+ across the vesicle membrane there was no overshoot.

Factors Affecting the Time Course of Na^+-Dependent Phosphate Transport

The time course of the uptake of phosphate by duodenal brush-border membrane vesicles was characterized by the so-called overshoot phenomenon

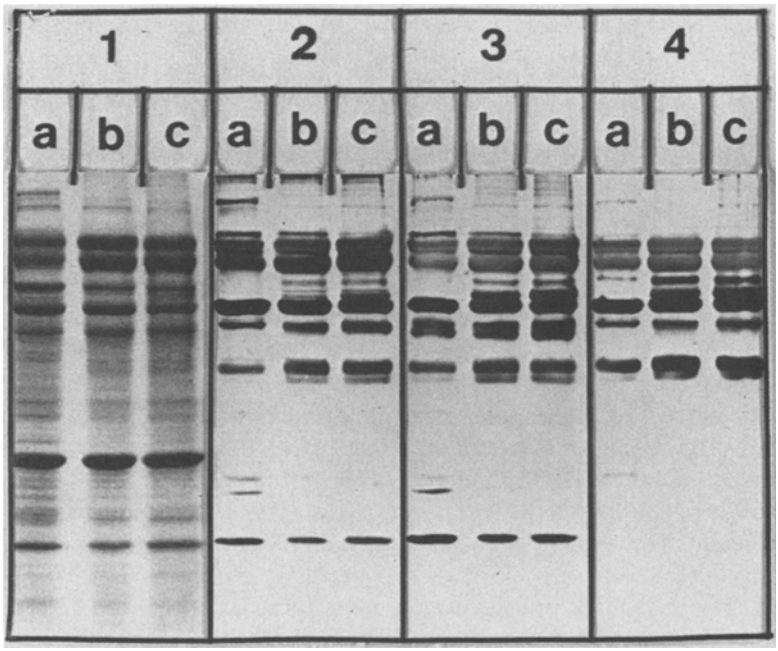

Fig. 3. Analysis of the protein components of the brush-border membrane vesicles and their interaction with antibody preparations. Brush-border vesicles, 6 mg protein, were incubated overnight with 1 ml of a solution which contained 10 mM Tris-Cl, pH 7.4, 0.15 M NaCl, and 2% (v/v) Triton X-100. 50 μl of the Triton-extract, 300 μg of protein, were mixed with 50 μl of a solution that contained 1 M sucrose, 0.01% bromothymol blue, 5 mM EDTA, and 0.2 M Tris-Cl, pH 8.8, and immediately heated with 20 μl of 20% (w/v) sodium dodecyl sulfate and 4 μl of 0.25 M dithiothreitol for 5 min at 95°C. After cooling, 20 μl of 0.5 M iodoacetate was added. Fifteen minutes later, samples containing 6 μg of protein were applied to a gradient polyacrylamide gel, 7.5–15% (Blobel and Dobberstein, 1975). After electrophoresis the proteins were stained with Coomassie blue BL or with Ponceau S after electrotransfer onto nitrocellulose paper. Antigens were detected by immunoprinting (Coudrier *et al.*, 1983). The immunoglobulins directed against brush-border proteins were used at a concentration of 10 mg/ml, and anti-guinea pig immunoglobulin coupled with horseradish peroxidase was used at a concentration of 2 mg/ml. Peroxidase activity was revealed using a solution of 3,3-diamino-benzidinetetrahydrochloride (50 mg in 100 ml of 10 mM phosphate buffer, pH 7.3, 0.15 M NaCl, and 0.025% hydrogen peroxide). Region 1. Polyacrylamide gel electrophoretic analysis of the brush-border membrane proteins. Region 2. Interaction of anti-duodenum antibodies with the proteins of the brush-border membranes. Region 3. Interaction of anti-jejunum antibodies with the proteins of the brush-border membrane. Region 4. Interaction of anti-ileum antibodies with the proteins of the brush-border membrane. Track a, duodenum brush-border membrane proteins. Track b, jejunum brush-border membrane proteins. Track c, ileum brush-border membrane proteins.

(see Figs. 1 and 2). In response to the experimentally imposed Na^+ gradient, 100 mM outside, 0 inside, the uptake of phosphate increased with time to a point where the concentration of phosphate within the vesicles, 0.6 mM at 37°C, was much higher than that outside of vesicles, 0.1 mM. The high internal concentration of phosphate then diminished. The final intravesicular

concentration was equal to the phosphate concentration present in the reaction medium. This lowering of the internal phosphate level has been ascribed in the past to the increased permeability of the vesicle membrane with time. We do not believe that this is an adequate explanation. In our hands the vesicles are stable for many hours. We suggest that the gradual decline of the internal phosphate content of the vesicles is due to the efflux of phosphate via the Na^+ phosphate symporter. To achieve this condition there must be other routes which can lead to the accumulation of Na^+ within the vesicle during the time course of the experiment.

It is known that a Na^+/H^+ antiporter is present in these brush-border membranes (Murer *et al.*, 1976). We suggest that the operation of this antiporter, in response to the experimentally imposed Na^+ gradient, enhances the accumulation of Na^+ within the vesicles. The combined activities of the Na^+-dependent phosphate symporter and the Na^+/H^+ antiporter will lead to a situation where the Na^+ concentration reaches a value that causes the efflux of phosphate.

If this model is apposite, then changes in the intra- and extravesicular $[H^+]$, i.e., the imposition of pH gradients across the vesicle membrane, will modulate the activity of the Na^+/H^+ antiporter. The acidification of the interior of the vesicles will enhance the uptake of Na^+ by the Na^+/H^+ antiporter. This in turn will lead to a reduction in the uptake of phosphate via the Na^+ phosphate symporter. Conversely the alkalinization of the interior of the vesicle should enhance the activity of the phosphate uptake. The results shown in Fig. 2 are from an experiment designed to test these predictions. They show that the magnitude of the overshoot is dependent on a pH gradient across the vesicle membrane in a manner that is totally compatible with the predictions made above. The results of control experiments showed that the magnitude of the overshoot was constant over the pH range 6–8, in the absence of a pH gradient. Thus the results reported are not due to the direct effect of pH on the systems under study.

A further indication that the Na^+/H^+ antiporter is involved in the dissipation of the overshoot is the observation that 1 mM amiloride, an inhibitor of the Na^+/H^+ antiporter (Vigne *et al.*, 1984) stimulates the Na^+-dependent phosphate uptake in the presence of 10 mM Na^+ by 33%.

The Electrogenic Nature of the Na^+-Phosphate Symporter. The Effects of Applied Membrane Potential on the Transport of Phosphate

In the absence of permeable cations, anions such as SCN^-, NO_3^-, and Cl^- can pass through the vesicle membrane, resulting in a potential, negative on the inside, being applied across the vesicle membrane (Kimmich *et al.*, 1985; Wright *et al.*, 1983). The uptake of phosphate into duodenal

Table II. The Effects of Extravesicular Anions on the Transport of Phosphate[a]

Extravesicular anion	Initial rates of phosphate transport (% of rate measured with Cl^-)		
	pH 6 in/out	pH 7 in/out	pH 8 in/out
SCN^-	156	132	163
NO_3^-	126	112	128
Cl^-	100[b]	100[b]	100[b]
Isethionate	67	63	57
Gluconate	28	34	39
Cl^- (choline)	13	5	7

[a]Duodenal brush-border vesicles were loaded with solutions of 300 mM mannitol, 0.1 mM $MgSO_4$, and 0.2% NaN_3 the pH of which had been adjusted to either 8 or 7 with Hepes-Tris or to pH 6 with Mes-Tris. The ability of these vesicles to transport phosphate in a Na^+-dependent manner was assayed as described in the Methods section using a reaction medium that had been modified by (1) changing the counter anion from chloride to that indicated and (2) by adjusting the pH to either 6 or 7 or 8 using the buffers described above. Each assay was done in triplicate. Each point is the mean of three experiments.

[b]100% values: pH 6, 1538 ± 138 (N = 3); pH 7, 1880 ± 189 (N = 3); pH 8, 1220 ± 120 (N = 3) pmol/mg protein/15 sec.

brush-border membrane vesicles was studied in the presence of 100 mM NaCl, $NaNO_3$, NaSCN, sodium isethionate, and sodium gluconate at 37°C. As can be seen in Table II, the initial rate of Na^+-dependent phosphate uptake was affected significantly by the nature of the anion that was present in the assay medium. The variation was seen over the pH range 6–8 (intra- and extravesicular pH values being the same). There is a correlation between the rate of phosphate uptake and the relative permeability of the anions to move through the membrane and establish a membrane potential. It appears that the transport of phosphate responds to the presence of this potential. We conclude that it is probable that the mechanism of the phosphate carrier is electrogenic, leading to the development of a positive charge on the internal face of the vesicular membrane. From this we deduce that there must be at least two Na^+ transported with each phosphate ion. The Murer group claim that the Na^+-dependent transport of phosphate in rabbit duodenum is electrogenic at all pH values (Danisi *et al.*, 1984). However, Amstutz *et al.* (1985) reinterpret the data of Danisi *et al.* as indicating that the carrier is electrogenic only at acid pH.

All the previous results are explicable in terms of an electrogenic Na^+ (Li^+) phosphate symporter being present in the brush-border membrane of duodenal enterocytes. The affinities of this protein for the ions that it carries are sensitive to pH. We propose that the simplest example of such a carrier would be one that transports two Na^+ for each ion of dihydrogen orthophosphate.

The energy required for the flow of phosphate into an enterocyte via a Na^+-linked symporter must be lower than the energy made available by the movement of the Na^+ down the electrochemical potential gradient. This is described by Eq. (1) (Kimmich, 1981). If the process is electroneutral, then the membrane potential has no role in the energetic aspects of the process, and FV can be regarded as zero.

$$RT \ln \frac{[P]_i}{[P]_o} > nRT \ln \frac{[Na^+]_o}{[Na^+]_i} + mFV \tag{1}$$

where R = gas constant, T = temperature, F = Faraday constant, V = membrane potential, $[P]_i$, $[P]_o$ = intra- and extravesicullar concentrations of phosphate, $[Na^+]_i$, $[Na^+]_o$ = intra- and extravesicullar concentrations of Na^+, n = number of Na^+ moved per phosphate transported, and m = net charge moved.

If the movement of phosphate into the cell is mediated by an electroneutral process with a stoichiometry of 1 : 1, then the entry of phosphate into the cell from the lumen of the gut would not be favored energetically. Since within the gut the concentrations of Na^+ and phosphate are 140 mM (Wright *et al.*, 1983) and < 1 mM (Davis *et al.*, 1983), and inside the cell, 15 mM Na^+ (Wright *et al.*, 1983) and 5–10 mM phosphate (Kowarski and Schachter, 1969) the tendency would be for the phosphate to leave the cell. The movement of two Na^+ with each phosphate ion in an electroneutral mechanism would be energetically feasible, the movement of each phosphate ion costing 10.1 kJ/mol. The Na^+ gradient will provide 11.58 kJ/mol. However, the results presented here indicate that, at all pH values tested, the mechanism of transport is electrogenic. For the case where the movement of one $H_2PO_4^-$ is linked with two Na^+, the combination of the Na^+ gradient and the membrane potential yields 20.18 kJ/mol. The membrane potential was assumed to be 50 mV, negative inside. The expenditure of these amounts of energy leads to the speculation that the cell will have developed control mechanisms to prevent the leaking of phosphate from the cell either by the reversal of the Na^+ phosphate symporter or via movement through the basolateral membrane. The obvious factor that could be utilized to control the intracellular level of phosphate is the internal phosphate concentration, which might act as an allosteric effector of the transport systems.

A comparison of the properties of the intestinal phosphate carrier with that present in the renal brush-border membrane has not been made in this paper. There are many discrepancies between the data from different laboratories. Indeed, if more effort were made to study the intestinal carriers, a comparable divergence might result. The complexity of the brush-border membrane with respect to the number of proteins that can affect the intravesicular concentration of Na^+ and the difficulties encountered with a

carrier that is sensitive to pH and a substrate that changes its form with pH are complications that cannot be overcome with the techniques that have been used in this and many other studies. There is a great urgency for the recognition, extraction, purification, and reconstitution of this protein in phospholipid vesicles to enable precise experimentation.

The Antigenic Nature of the Phosphate Carrier

Guinea pigs were immunized with detergent-solubilized brush-border membrane vesicles isolated from the duodenum, jejunum, and ileum of the same rabbit (Feracci and Maroux, 1980). The immunoglobulins were purified from the antisera. The constituent proteins of the detergent-solubilized brush-border membrane preparations were resolved by electrophoresis on SDS-polyacrylamide gradient gels. After electrophoresis the proteins were electrotransferred to nitrocellulose paper. The distribution of protein was visualized using Ponceau S, and the antigenic proteins were detected by immunoprinting. The results are shown in Fig. 3. It can be seen that there are few differences in the protein components of the vesicles from the different regions of the gut. Also, the major antigens in the different vesicle preparations are common to all three regions of the gut.

The ability of the anti-duodenum, anti-jejunum, and anti-ileum antibodies to modulate the transport of phosphate by the vesicles from the duodenum was then investigated. The results are presented in Table III. They show that the antibody preparations raised against the ileum and the jejunum both activated the phosphate transport process, while the anti-duodenum antibody preparation inhibited phosphate transport. The maximum inhibition was 43%. Thus in the bulk of the anti-duodenum antibodies there are some that interact with the Na^+-dependent phosphate carrier. This confirms the conclusion that the phosphate carrier is located solely in the membranes of the vesicles isolated from the brush-border of duodenal enterocytes.

Table III. The Inhibition of Phosphate Uptake by Antibodies[a]

Antibody preparation	Phosphate uptake (pmol/mg protein/min)
Nonimmune serum	400 ± 30 (N = 4)
Anti-duodenum	280 ± 19 (N = 4)
Anti-jejunum	500 ± 35 (N = 4)
Anti-ileum	560 ± 59 (N = 4)

[a]Anti-duodenum, anti-jejunum, and anti-ileum antibodies were dialyzed against the standard suspension medium for 24 h. Duodenal vesicles, 0.4 mg of protein, were incubated for 1 hour at 4°C with the dialyzed anti-duodenum, anti-jejunum, and anti-ileum antibodies. The ratio of IGG-protein/mg of vesicle protein was 1 : 1. The final volume was 95 μl. The ability of the vesicles to transport phosphate was then measured. Each assay was done in triplicate. The data presented are the mean of four assays.

It was important to establish that the inhibition of phosphate transport did not arise from agglutination of the vesicle preparations by the antibodies. Control experiments showed that there was no agglutination at IGG/protein ratios up to 1 : 1.7. The ratio used in the experiments described here was 1 : 1. Previous investigations (Louvard *et al.*, 1975; Feracci *et al.*, 1981) have shown that the aminopeptidase activities of either pig or rabbit brush-border vesicles were inhibited only by high antibody–antigen ratios and that the inhibition was never complete. These and the present results can probably be explained in terms of the limited accessibility of the antibody to the antigenic site.

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