

## The use of potent inhibitors of alkaline phosphatase to investigate the role of the enzyme in intestinal transport of inorganic phosphate

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(Received 14 August 1980/Accepted 10 November 1980)

In an investigation of the link between  $P_i$  transport and alkaline phosphatase in mammalian small intestine, the characteristics of  $P_i$  uptake by brush-border membrane vesicles prepared from rat intestine were compared with the properties of the tissue alkaline phosphatase. The NaCl-dependent  $P_i$  uptake had a  $K_m$  of 0.1 mM at pH 7.5 and was inhibited totally by 1 mM-arsenate and by 1 mM-vanadate. These compounds are also potent competitive inhibitors of the alkaline phosphatase activity of the vesicles, with  $K_i$  values less than 5  $\mu$ M at pH 7.5. When the effect on  $P_i$  uptake of several other potent inhibitors of alkaline phosphatase, including phosphonates and phosphate analogues, was tested, however, it was found that there was little, if any, inhibition of transport under conditions in which the inhibition of phosphatase activity was total. Incubation of the vesicles for 20 min with oxidized adenosine 5'-[ $\beta$ -imido]triphosphate followed by rapid gel filtration to remove the inhibitor resulted in an irreversible loss of phosphatase activity, but left  $P_i$  transport unimpaired. Conversely, a similar prolonged incubation with adenosine 5'-[ $\beta$ -thio]diphosphate or adenosine 5'-[ $\gamma$ -thio]triphosphate had no effect on alkaline phosphatase activity but resulted in a permanent partial loss of transport capability. The failure to demonstrate an inhibition of  $P_i$  transport resulting from inhibition of alkaline phosphatase and the different responses of enzymic activity and  $P_i$  transport to irreversible inhibition make it very unlikely that the enzyme is directly involved in the transport system.

The physiological function of alkaline phosphatase (EC 3.1.3.1) has never been adequately explained, in spite of the intense study that the enzyme, prepared from many organisms and tissues, has received in the last 50 years. A recent review (McComb *et al.*, 1979) tabulates the various suggestions made with regard to the role of the enzyme, and it is evident that a connection with a transport process or with phosphate availability is an idea that has been favoured by many who have concerned themselves with this problem. Such views probably prevail because (a) the enzyme is found in high concentrations in tissues, such as intestinal and renal-tubule epithelia, that are known to be asso-

ciated with transport phenomena, (b) many microorganisms respond to growth on low- $P_i$  media by synthesizing large amounts of alkaline phosphatase and (c) the enzyme is known to bind  $P_i$  with affinity.

Moog & Glazier (1972) studied phosphate uptake from free  $P_i$  and from glycerophosphate by intestinal segments of mice and chickens. They found that inhibitors of phosphatase, e.g. L-phenylalanine, inhibited  $P_i$  uptake and concluded that the enzyme is important in  $P_i$  absorption. This view has support in that rat and chick intestinal alkaline phosphatase and also  $P_i$  transport increase in response to cholecalciferol (vitamin D<sub>3</sub>) (Norman *et al.*, 1970; Haussler *et al.*, 1970; Wasserman & Taylor, 1973; Birge & Miller, 1977; Fuchs & Peterlik, 1979). Berner *et al.* (1976) prepared vesicles from rat intestinal brush-border membrane and used the preparation to characterize the Na<sup>+</sup>-dependent  $P_i$ -uptake system of small intestine. Similar preparations were obtained from rat kidney, and some comparisons of the properties of the transport

Abbreviations used: [ $\beta$ -imido]ATP, adenosine 5'-[ $\beta$ -imido]triphosphate; [ $\beta$ -methylene]ATP, adenosine 5'-[ $\beta$ -methylene]triphosphate; [ $\beta$ -imido]GTP, guanosine 5'-[ $\beta$ -imido]triphosphate; [ $\beta$ -thio]ADP, adenosine 5'-[ $\beta$ -thio]diphosphate; [ $\gamma$ -thio]ATP, adenosine 5'-[ $\gamma$ -thio]triphosphate; Hepes, 4-(2-hydroxyethyl)piperazine-ethanesulphonic acid.

system and alkaline phosphatase were made (Stoll *et al.*, 1978). From these studies it appeared unlikely to the authors that the two agents were directly linked. Kempson & Dousa (1979), also working with tubule vesicles, came to the opposite conclusion, however, i.e. that there was a strong correlation between the phosphatase and  $P_i$  transport.

We decided to investigate the link, if any, between transport and phosphatase by testing the effect of a series of potent inhibitors of alkaline phosphatase on  $P_i$  transport. The isolated vesicle preparation of intestinal brush border was chosen as the experimental medium because of the ease of conducting parallel studies of  $P_i$  uptake and enzyme kinetic properties with this system. At the beginning of our study we seemed to find evidence linking the enzyme with  $P_i$  transport (Shirazi *et al.*, 1978), but the further studies with a wider range of phosphatase inhibitors described below have caused us to modify our opinion.

## Experimental

### *Brush-border membrane vesicles*

Vesicles were prepared by a method based on those described by Kessler *et al.* (1978) and Lücke *et al.* (1978). The small intestine was removed from two rats (of Wistar strain, each weighing approx. 100 g) as soon as possible after they were killed, and the serosal face was rinsed with ice-cold saline (0.9% NaCl, pH 7.0). The intestine was everted on a stainless-steel rod. The mucosal face was rinsed with the saline solution, and the excess mucus was removed by blotting with a paper towel. The mucosal surface was scraped gently with a glass slide, and the brush-border cells, mucus and connective tissue released were suspended in an ice-cold solution of 300 mM-mannitol/0.12 M-Tris/HCl buffer, pH 7.1; 30 ml of solution was used for each original rat intestine. The suspension was passed through a Buchner funnel (pore size 1 mm) to remove any large pieces of tissue.

The filtrate was diluted 5-fold with water at 0°C and homogenized for 90 s at maximum speed in an MSE Waring blender. Solid  $CaCl_2$  was added to a final concentration of 10 mM. After standing at 0°C for 20 min, the suspension was centrifuged at 3000 g for 15 min. The pellet, containing nuclei, mitochondria, basolateral membranes, bacteria and cell debris, was discarded. The supernatant was centrifuged at 27000 g for 30 min, and the pellet was suspended in 40 ml of 100 mM-mannitol/10 mM-Hepes/Tris buffer, pH 7.5, in a glass/Teflon homogenizer. The plunger was rotating at 1000 rev./min and ten passes were made. The buffer had been previously sterilized by passage through a Millipore filter of pore size 0.22  $\mu$ m. The suspension was re-centrifuged at 27000 g for 30 min. The resulting

pellet was taken up in 1.5 ml of the mannitol/Hepes/Tris buffer by sucking seven times through a stainless-steel needle (0.9 mm  $\times$  38 mm) into a disposable plastic syringe. The final protein concentration was approx. 5 mg/ml.

### *Assay of sucrase*

Sucrase activity was measured by the method of Dahlqvist (1964). The glucose produced was determined with a commercial reagent kit (Boehringer Corp., London W.5, U.K.). A unit of activity produces 1  $\mu$ mol of product/min.

### *Assay of alkaline phosphatase*

Alkaline phosphatase activity was determined at pH 8.9 or pH 7.5 in 10 mM-Tris/HCl buffer. A 1 ml portion of 1 mM-*p*-nitrophenyl phosphate (diTris salt) dissolved in the same buffer was added to 0.99 ml of the buffer. After temperature equilibration at 30°C, 10  $\mu$ l of a suspension of brush-border vesicles (approx. 50  $\mu$ g of protein) was added. The reaction was terminated at 10 min by the addition of 1 ml of 0.5 M-NaOH. The *p*-nitrophenol liberated was assayed from its absorbance at 400 nm. One unit of activity results in the formation of 1  $\mu$ mol of product/min.

### *Assay of calcium*

The free  $Ca^{2+}$  content of brush-border vesicles was measured spectrophotometrically by complex-formation with Arsenazo II (Harris, 1977).

### *Assay of protein*

The method of Lowry *et al.* (1951) was used, with bovine serum albumin as the standard.

### *Electron microscopy*

The brush-border vesicles were fixed for 2 h at 4°C in 3% (v/v) glutaraldehyde in 0.1 M-phosphate buffer, pH 7.4. The tissue was then washed twice with the phosphate buffer before being treated, for 1–2 h, with 1% (w/v)  $OsO_4$  in 0.1 M-phosphate buffer. The preparation was dehydrated with a series of ethanol solutions before being embedded in resin.

### *Histochemical localization of alkaline phosphatase*

The brush-border vesicles were fixed in 2% (v/v) glutaraldehyde in 0.1 M-cacodylate buffer, pH 7.4, for 15 min at 4°C. After being washed with the buffer solution, the sample was divided into two parts. One was treated with  $\beta$ -glycerophosphate as a substrate for the enzyme, by using the procedure of Essner (1973); the other part was treated similarly, but in the absence of substrate.

### *$P_i$ transport*

The method was based on that of Berner *et al.* (1976). The filters used were Millipore GSW

PO2500 and they were of 25 mm diameter and 0.22  $\mu\text{m}$  pore size. They were soaked in double-distilled water before use. Vesicles (50  $\mu\text{l}$ ; approx. 250  $\mu\text{g}$  of protein) were incubated at room temperature (20°C) with a reaction medium that contained 100 mM univalent-cation chloride, 100 mM-mannitol, 10 mM-Hepes/Tris buffer, pH 7.5, and 0.2 mM- $\text{KH}_2^{32}\text{PO}_4$  (40 Ci/mmol). Other additions are described in the Table and Figure legends. The total incubation volume was 0.1 ml. At appropriate times, 20  $\mu\text{l}$  samples were removed and diluted into 1.5 ml of a solution containing 150 mM-NaCl, 1 mM- $\text{Na}_3\text{AsO}_4$  or 1 mM- $\text{Na}_3\text{VO}_4$  in 5 mM-Hepes/Tris buffer, pH 7.5, at 0°C (stop solution). The diluted sample was filtered immediately under suction. The filter was then washed twice with 5 ml portions of stop solution and then transferred to scintillation vials for determination of the radioactivity retained by the membrane.

Correction for non-specific retention of  $^{32}\text{P}P_i$  by the filters was done by dilution of 10  $\mu\text{l}$  of the brush-border vesicle sample into 1.5 ml of stop solution before addition of 10  $\mu\text{l}$  of the  $^{32}\text{P}P_i$ -containing reaction medium. The mixture was then immediately filtered and washed as described above.

All the solutions for the uptake measurements were freed of bacterial contamination by filtration through Millipore filters (0.22  $\mu\text{m}$  pore size) before use.

#### Glucose transport

A protocol identical with that for  $P_i$  was followed, except that the labelled substrate was 1 mM- $[\text{U-}^{14}\text{C}]$ -glucose, and the stop solution consisted of 150 mM-NaCl plus 0.2 mM-phlorrhizin in 5 mM-Hepes/Tris buffer, pH 7.5. The specific radioactivity was 2 Ci/mmol.

#### Scintillation counting

The vials contained 10 ml of dioxan-based scintillation fluid (Malik & Butterworth, 1976), in which the filters were soluble. Radioactivity was measured in a Beckman LS3100 spectrometer with appropriate internal standards. The efficiency of counting was approx. 50% for  $^{32}\text{P}$  but 80–90% for  $^{14}\text{C}$ .

#### Pretreatment with inhibitors

The vesicles (193  $\mu\text{l}$ , containing approx. 1 mg of protein) were incubated with 7  $\mu\text{l}$  of 52.3 mM oxidized  $[\beta\text{-}\gamma\text{-imidazole}]$ ATP, giving a final concentration of the oxidized compound of 1.83 mM, for 20 min at room temperature. For controls, the vesicles were incubated with 7  $\mu\text{l}$  of 100 mM-mannitol/10 mM-Hepes/Tris buffer, pH 7.5. At the end of the incubation, the vesicles were separated from the bulk solution by rapid gel filtration (Penefsky, 1977; Shirazi *et al.*, 1981). In a similar experiment 198  $\mu\text{l}$  of the vesicle suspension was incubated under the

same conditions as above with 2  $\mu\text{l}$  of 50 mM- $[\beta\text{-thio}]$ ADP or  $[\gamma\text{-thio}]$ ATP before rapid gel filtration. Portions of the vesicles obtained before and after gel filtration were used in  $P_i$ -uptake measurements.

#### Materials

Carrier-free  $^{32}\text{P}P_i$  and the  $[\text{U-}^{14}\text{C}]$ glucose were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). The nucleotides used in inhibitor studies were purchased from Boehringer Corp. Phosphonoacetaldehyde was prepared by the method of La Nauze *et al.* (1977).

#### Results

##### General properties of the brush-border vesicles

The specific activities of alkaline phosphatase at pH 8.9 and sucrase in the vesicle preparation were 0.54 and 0.68 unit/mg respectively. These values represent a 23–25-fold enrichment over that of the original homogenate. These results are very similar to those described by Kessler *et al.* (1978), suggesting that we have a very similar preparation. Kessler *et al.* (1978) showed that the enrichment factor for  $\text{K}^+$ -stimulated phosphatase, a contraluminal plasma-membrane marker enzyme, was only 2.2, suggesting that the vesicles were largely composed of luminal membrane material. Also, their vesicle preparation was essentially free of contamination by cell components and organelles other than the plasma membrane. Electron microscopy of our vesicle preparation fixed in 3% glutaraldehyde showed that the vesicles were 0.2–0.4  $\mu\text{m}$  in diameter and that there was little non-vesicular material present. Histochemical localization of alkaline phosphatase (Essner, 1973) confirmed that the enzyme was confined to the membranes of the vesicles.

The free  $\text{Ca}^{2+}$  content of the brush-border vesicles was found to be approx. 160 nmol/mg of protein. Kessler *et al.* (1978) have reported values of 120–130 nmol/mg of protein for the amount of bound calcium in rabbit brush-border vesicles prepared by a  $\text{Ca}^{2+}$ -precipitation technique.

##### Characteristics of $P_i$ transport

$P_i$  uptake by the brush-border membrane vesicles at pH 7.5 in the presence of 100 mM-NaCl was time-dependent, reaching an equilibration point after about 1 h. A much lower rate of uptake occurred in the absence of NaCl, but the same equilibration point was reached eventually. These results match those obtained by Berner *et al.* (1976). The amount of  $P_i$  taken up at equilibration was about 0.3 nmol/mg of protein (Fig. 1). This value suggests an intravesicular volume of 1.45  $\mu\text{l}$ /mg of protein. From the equilibrium value obtained in glucose-uptake

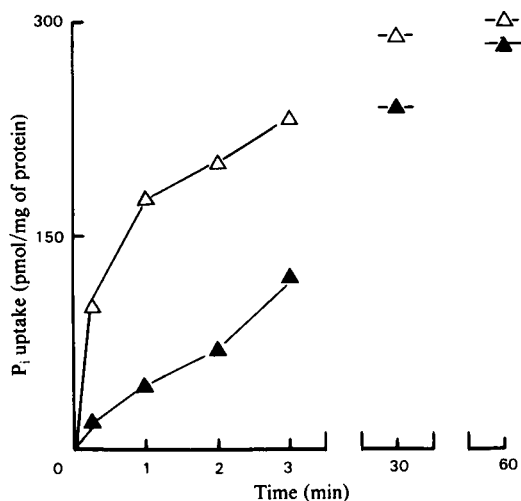


Fig. 1. Time-dependence of  $P_i$  uptake by intestinal brush-border membrane vesicles

The membrane vesicles, suspended in 100 mM-mannitol/10 mM-Hepes/Tris buffer, pH 7.5, were incubated at room temperature for the times shown in a mixture containing 0.2 mM- $^{32}P$  $P_i$  plus 100 mM-NaCl ( $\Delta$ ) or 100 mM-NaCl plus 1 mM-vanadate or 1 mM-arsenate ( $\blacktriangle$ ). For full details see the text.

experiments (results not shown) a volume of 1  $\mu$ l/mg of protein was calculated. These values are close to those given by Berner *et al.* (1976), namely 1.38  $\mu$ l/mg and 1.28  $\mu$ l/mg for  $P_i$  and for glucose respectively. The specific uptake in the presence of NaCl was inhibited by both 1 mM-arsenate and 1 mM-vanadate, competitive inhibitors of alkaline phosphatase. The slower uptake in the NaCl-free system was unaffected by these inhibitors. Replacement of NaCl by either NaCNS or  $Na_2SO_4$  to make the inside of the vesicles more or less electronegative respectively did not change the rate of  $Na^+$ -supported uptake (Table 1). Thus the vesicles exhibit  $Na^+$ -dependent uptake of  $P_i$  with similar characteristics to those of the system already described by Berner *et al.* (1976).

The specificity for the cation component in the co-transport system was investigated by substitution of other univalent-cation chlorides for NaCl. The results given in Table 1 show that  $Na^+$  was the most effective, as judged by the amount of  $P_i$  taken up in the initial period of 15 s, although the  $P_i$  carrier does not appear to be very selective in its choice of cation, at least in these experiments *in vitro*. Choline was found to be as effective as  $Na^+$  in supporting entry. Thus, in subsequent experiments, non-specific, i.e. diffusional, entry of  $P_i$  was estimated from the rate of uptake in the presence of vanadate instead of from the rate in the presence of choline.

Table 1. Effect of anions and of univalent cations on  $P_i$  uptake by intestinal brush-border membrane vesicles. Membrane vesicles were incubated at room temperature with 0.2 mM- $^{32}P$  $P_i$  in 100 mM-mannitol/10 mM-Hepes/Tris buffer, pH 7.5, containing various salts in the presence and in the absence of 1 mM-vanadate to enable correction for the diffusional entry of  $P_i$ . For full details see the text. All the salts were at 100 mM concentration, except for  $Na_2SO_4$ , which was used at 50 mM together with 50 mM-mannitol. Each value given for the initial  $P_i$  uptake in 15 s is the mean of at least two determinations.

Salt	Initial $P_i$ uptake (pmol/mg of protein)
NaCl	90
KCl	46
LiCl	85
RbCl	0
CsCl	0
$NH_4Cl$	38
Choline chloride	94
NaSCN	95
$Na_2SO_4$	92

The rate of  $Na^+$ -supported uptake was concentration-dependent and saturable. Values for  $K_{m(app.)}$  and  $V_{max.}$  at 100 mM-NaCl were found to be 0.1 mM and 0.2 nmol/15 s per mg of protein respectively (Fig. 2).

To investigate the effect of osmolarity on the amount of  $P_i$  taken up at equilibrium, vesicles were prepared and suspended in 100 mM-cellobiose/10 mM-Hepes/Tris buffer, pH 7.5, instead of the mannitol/Hepes/Tris solution used in the other experiments (see the Experimental section). The vesicles were then incubated for 30 min with 0.2 mM- $P_i$  and 25 mM-NaCl plus a range of concentrations of cellobiose, and impermeant solute, before measurement of  $P_i$  uptake.

The equilibrium concentration decreased with increasing osmolarity of the medium (Fig. 3), indicating that measured  $P_i$  uptake represented entry into the intravesicular space rather than from binding to the vesicular membrane. From Fig. 3 it appears that the amount of bound  $P_i$  is approx. 40 pmol/mg of protein. Similar binding is seen in measurements of glucose and lactate uptake by vesicles (Lücke *et al.*, 1978; Hildmann *et al.*, 1980), so this bound  $P_i$  is not necessarily in the form of  $Ca_3(PO_4)_2$ . From the  $Ca^{2+}$  content of the vesicle preparation, it can be calculated that the concentration of  $Ca^{2+}$  in the incubation mixtures was about 0.8 mM. It has been shown that, at this kind of concentration,  $Ca^{2+}$  has no effect on  $P_i$  uptake (Berner *et al.*, 1976). Also, it seems unlikely that accumulated  $P_i$  is precipitated as  $Ca_3(PO_4)_2$  within the vesicle, since the estimate of the intravesicular volume measured by glucose uptake is very similar

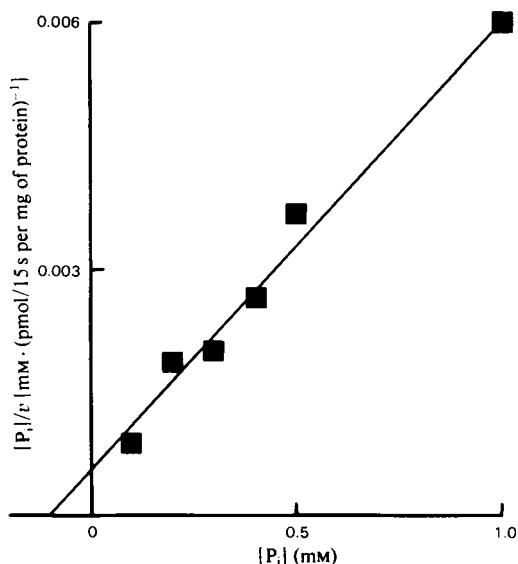


Fig. 2. Concentration-dependence of  $P_i$  uptake by intestinal brush-border membrane vesicles

The vesicles were incubated for 15 s with various  $P_i$  concentrations in the presence of 100 mM-NaCl or 100 mM-NaCl plus 1 mM-vanadate before measurement of  $P_i$  uptake. For full details see the text. The transport rate at each  $P_i$  concentration ( $v$ ) was calculated from the uptake in the presence of NaCl alone minus the uptake in the presence of vanadate. The results are plotted in the  $[P_i]/v$ -versus- $[P_i]$  form of the Michaelis-Menten equation. Each experimental point is the mean of two separate experiments.

to the value obtained from the  $P_i$  experiments. This conclusion assumes that glucose is taken into the intravesicular space, for which there appears to be strong evidence (Murer *et al.*, 1974; Kessler *et al.*, 1978).

#### Effect of inhibitors on $P_i$ transport

Arsenate and vanadate are both potent competitive inhibitors of alkaline phosphatase (Cathala *et al.*, 1975; Lopez *et al.*, 1976). The  $K_i$  values for the vesicle enzyme at pH 7.5 were found to be 2  $\mu$ M and 4  $\mu$ M for arsenate and vanadate respectively. These compounds are also very effective inhibitors of the phosphate carrier (Fig. 1).

Several phosphonate and phosphate-analogue inhibitors of alkaline phosphatase (Shirazi *et al.*, 1981) were included in the  $P_i$ -uptake medium to assess their effects on the initial rate of  $P_i$  uptake. The concentrations of the inhibitors were chosen relative to their  $K_i$  values for alkaline phosphatase at pH 7.5, so that at the concentration of 0.1 mM- $P_i$  in the uptake medium all phosphatase molecules present in the vesicles would be expected to be

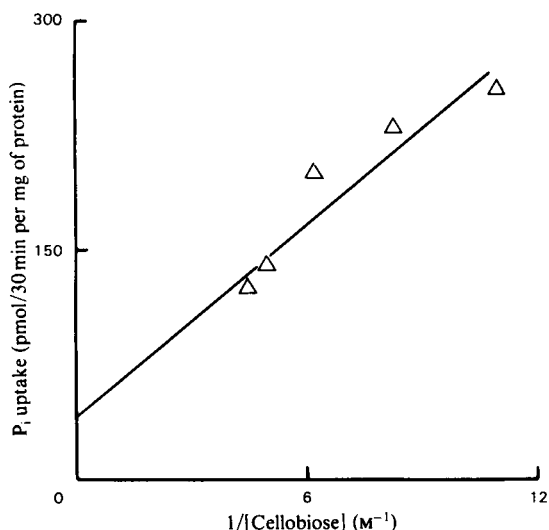


Fig. 3. Influence of osmolarity of the medium on the equilibrium uptake of  $P_i$  by intestinal brush-border membrane vesicles

Vesicles prepared in cellobiose were incubated for 30 min with 0.2 mM- $P_i$ , 25 mM-NaCl and various concentrations of cellobiose before determination of  $P_i$  uptake at equilibrium. For full details see the text. The uptake values are plotted against the reciprocal of the cellobiose concentration. The vertical intercept of 40 pmol of  $P_i$ /mg of protein represents bound  $P_i$ , i.e. that which is attached to the vesicles but not accumulated in the intravesicular space.

complexed with inhibitor. The results given in Table 2 show that the reversible competitive inhibitors of alkaline phosphatase had almost no effect on the initial rate of  $P_i$  uptake. Oxidized [ $\beta\gamma$ -imido]ATP, an irreversible inhibitor of alkaline phosphatase, had no effect on  $P_i$  uptake, but [ $\beta$ -thio]ADP and [ $\gamma$ -thio]ATP inhibited the uptake considerably.

The effects on  $P_i$  transport of prolonged treatment with the phosphatase inhibitors [ $\beta$ -thio]ADP and [ $\gamma$ -thio]ATP and oxidized [ $\beta\gamma$ -imido]ATP (Lowe *et al.*, 1979; Shirazi *et al.*, 1981) were tested by preincubating the vesicles with inhibitor for 20 min followed by rapid gel filtration (Penefsky, 1977; Shirazi *et al.*, 1981) before measurement of alkaline phosphatase activity and of the  $P_i$ -transport capability. The results in Table 3 show that the irreversible phosphatase inhibitor oxidized [ $\beta\gamma$ -imido]ATP is not an irreversible inhibitor of  $P_i$  transport, but the reversible enzyme inhibitors [ $\beta$ -thio]ADP and [ $\gamma$ -thio]ATP caused a permanent partial loss of transport function. The period of preincubation and the separation by rapid gel filtration in these experiments were found to have no deleterious effect in themselves on  $P_i$  transport

Table 2. *Effects of alkaline phosphatase inhibitors on phosphate uptake by intestinal brush-border membrane vesicles*  
Phosphate uptake into the vesicles was studied in the presence of 100 mM-NaCl and of various inhibitors. Other conditions were as described in Table 1. The  $K_i$  values for alkaline phosphatase are taken from Shirazi *et al.* (1981).

Inhibitor and concentration used	Inhibition of alkaline phosphatase		Inhibition of initial $P_i$ uptake (%)
	Type	$K_i$ ( $\mu$ M) at pH 7.5	
Phenylene-1,3-diphosphonate (0.5–5 mM)	Competitive	16	0
2,6-Dinitrophenylphosphonate (0.5–5 mM)	Competitive	78	0
Phosphonoacetaldehyde (0.5–5 mM)	Competitive	47	0
$[\beta\gamma$ -Imido]ATP (0.3–2 mM)	Competitive	68	5–14
$[\beta\gamma$ -Methylene]ATP (0.5–5 mM)	Competitive	22	10
$[\beta\gamma$ -Imido]GTP (0.5–5 mM)	Competitive	80	8
Oxidized $[\beta\gamma$ -imido]ATP (1.83 mM)	Time-dependent irreversible		10 (time-independent)
$[\beta$ -Thio]ADP (0.5 mM)	Mixed	10	48
$[\gamma$ -Thio]ATP (0.5 mM)	Mixed	5	45

Table 3. *Effects of oxidized  $[\beta\gamma$ -imido]ATP,  $[\beta$ -thio]ADP and  $[\gamma$ -thio]ATP on alkaline phosphatase and on phosphate transport by brush-border membrane vesicles*

Membrane vesicles were incubated for 20 min with oxidized  $[\beta\gamma$ -imido]ATP,  $[\beta$ -thio]ADP or  $[\gamma$ -thio]ATP at room temperature in 100 mM-mannitol/100 mM-Hepes/Tris buffer, pH 7.5, or with buffer alone (control) before measurement of alkaline phosphatase activity and  $P_i$  uptake in the presence of 100 mM-NaCl. The vesicles were then separated from the incubation medium by rapid gel filtration (see the Experimental section) and re-tested for phosphatase activity and  $P_i$ -transport capability.

Addition to incubation medium	Alkaline phosphatase activity ( $\mu$ mol/min per mg of protein)		$P_i$ uptake (pmol/15 s per mg of protein)	
	Before gel filtration	After gel filtration	Before gel filtration	After gel filtration
None (control)	0.2	0.2	100	98
$[\beta$ -Thio]ADP (0.5 mM)	0.03	0.2	48	48
$[\gamma$ -Thio]ATP (0.5 mM)	0.03	0.2	45	43
Oxidized $[\beta\gamma$ -imido]ATP (1.83 mM)	0.14	0.14	100	90

(Table 3). Thus impaired  $P_i$  transport was not attributable to non-specific damage of the vesicle tissue, but to modification of the  $P_i$ -transport system in the presence of  $[\beta$ -thio]ADP.

## Discussion

There are few reports in the literature of the use of brush-border membrane vesicles to study intestinal  $P_i$  transport. Thus, before attempting to investigate the role of alkaline phosphatase in such transport, we repeated some of the experiments described by Berner *et al.* (1976) and obtained qualitatively similar results for the properties of the transport system, i.e.  $P_i$  uptake is univalent-cation-dependent, saturable and electroneutral at pH 7.5.

Our studies of the role of alkaline phosphatase seem to rule out the existence of an obvious part for the enzyme in  $Na^+$ -dependent  $P_i$  transport in intestine. It is true that the phosphatase inhibitors arsenate and vanadate are also potent inhibitors of transport. This is not good evidence for the close association of the enzyme with transport, however,

since both of these inhibitors are  $P_i$  analogues and would be expected to compete with  $P_i$  for binding to  $P_i$ -recognition sites on the enzyme and the carrier. From the observations made with the other compounds, it is clear that the transport process is largely indifferent to phosphonates and nucleotide phosphate analogues, yet these compounds are good inhibitors of the hydrolytic activity of alkaline phosphatase. The best evidence for the lack of an enzyme-transport link is provided by the finding that  $P_i$  transport is unimpaired by permanent partial inactivation of alkaline phosphatase, and conversely that compounds can be found, e.g.  $[\beta$ -thio]ADP and  $[\gamma$ -thio]ATP, that irreversibly inhibit transport without affecting alkaline phosphatase similarly. Storelli & Murer (1980) have also concluded that phosphatase is not involved in  $P_i$  transport in kidney tubules from studies with inhibitors of alkaline phosphatase.

If the enzyme has any part in  $P_i$  transport, it can only be as a scavenger, perhaps to increase the localized concentration of  $P_i$  at the transport site. The  $P_i$  could result either from the hydrolytic action

of the enzyme on phosphate esters or from sequestration of free P<sub>i</sub>. The latter could be more significant in kidney tubules and in placenta, where the bulk of phosphate to be transported probably appears as P<sub>i</sub>. The dephosphorylation of metabolites, e.g. sugar phosphates, might have the added advantage that the transport of the non-phosphate moiety of the compound is facilitated.

The authors thank Dr. H. B. F. Dixon (Department of Biochemistry, University of Cambridge, Cambridge, U.K.) and Dr. D. A. Brown (Allbright and Wilson Co., Birmingham, U.K.) for the provision of a number of inhibitors, and Mr. R. Pillinger (Shell Biosciences Laboratory, Sittingbourne, Kent, U.K.) for help with the electron microscopy.

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