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Solubilization and reconstitution of the renal phosphate transporter

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Proteins from brush-border membrane vesicles of rabbit kidney cortex were solubilized with 1% octylglucoside (protein to detergent ratio, 1 : 4 (w/w)). The solubilized proteins (80.2 ± 2.3% of the original brush-border proteins, $n = 10$, mean ± S.E.) were reconstituted into artificial lipid vesicles or liposomes prepared from purified egg yolk phosphatidylcholine (80%) and cholesterol (20%). Transport of P_i into the proteoliposomes was measured by rapid filtration in the presence of a Na^+ or a K^+ gradient (out > in). In the presence of a Na^+ gradient, the uptake of P_i was significantly faster than in the presence of a K^+ gradient. Na^+ dependency of P_i uptake was not observed when the liposomes were reconstituted with proteins extracted from brush-border membrane vesicles which had been previously treated with papain, a procedure that destroys P_i transport activity. Measurement of P_i uptake in media containing increasing amounts of sucrose indicated that P_i was transported into an intravesicular (osmotically sensitive) space, although about 70% of the P_i uptake appeared to be the result of adsorption or binding of P_i. However, this binding of P_i was not dependent upon the presence of Na^+. Both Na^+-dependent transport and the Na^+-independent binding of P_i were inhibited by arsenate. The initial Na^+-dependent P_i transport rate in control liposomes of 0.354 nmol P_i/mg protein per min was reduced to 0.108 and 0 nmol P_i/mg protein per min in the presence of 1 and 10 mM arsenate, respectively. Future studies on reconstitution of P_i transport systems must analyze and correct for the binding of P_i by the lipids used in the formation of the proteoliposomes.

Introduction

Na^+/phosphate (P_i) co-transport in renal brush-border membranes is well documented [1,2]. Although P_i transport has been extensively studied in brush-border membrane vesicles [1,3,4], conclusive identification and characterization of the P_i transporter have not been achieved. Reasons that may be responsible for this include: (1) the sparsity of information about the nature of the chemical groups involved in the transport process prevents the use of highly efficient purification methods; (2) the lack of a suitable assay system for testing the progress in purification. Consequently, reconstitution of the solubilized and (partially) purified renal transporter into artificial membrane vesicles (liposomes) might be a promising approach for addressing this issue, as has been the case for identification of the renal Na^+/D-glucose co-transporter [5–8] and the P_i transporter from mitochondria or bacteria [9–11]. Kinne and Faust [12] first reported an attempt in this direction. They reconstituted a Triton X-100 extract of renal

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Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminoethane; P_i, inorganic phosphate; octylglucoside, *n*-octyl-β-D-glucopyranoside.

brush-border membrane protein into phospholipids, which had been prepared from a chloroform/methanol extract from whole rat kidney. Although the observed uptake of P_i was enhanced by the presence of a Na^+ gradient and was inhibited by arsenate, equilibrium uptake values were not achieved by 90 min (the longest time of incubation reported), despite the finding that uptake of [^{3}H]mannitol (for which there is no facilitated uptake) reached equilibrium values by 40 min in similar proteoliposomes. Moreover, the absolute magnitude of the inhibition of P_i uptake by arsenate was as large at 80 min as at 10 min. This is a finding contrary to that expected if arsenate were only slowing the rate at which P_i entered into the interior of liposomes. The recent demonstration from our laboratory that chloroform/methanol extracts of renal brush-border membranes contain a proteolipid that exhibits arsenate-inhibitable binding of P_i [14], raises the possibility that both the lack of equilibrium uptake of P_i and the quantitatively constant inhibition of uptake of P_i by arsenate found by Kinne and Faust [12] could be attributed, at least in part, to the presence of P_i-binding material in the detergent-solubilized protein or the chloroform/methanol-soluble lipoidic material, or both, that were utilized in their reconstitution system. Therefore, we have re-examined the issue of reconstitution of Na^+-dependent P_i transport while examining rigorously for the extent of adsorption (or binding) of P_i versus the transport of P_i by the proteoliposomes. An important corroborative experiment utilized brush-border membranes in which Na^+-dependent P_i transport (but not Na^+-dependent glucose transport) had been inhibited by treatment with papain [27] prior to solubilization of the membranes with detergent.

Our results demonstrate that detergent-solubilized brush-border membrane proteins reconstituted into liposomes are able to transport P_i into an osmotically sensitive space by a Na^+-dependent and arsenate inhibitable mechanism. Importantly, we show also that destruction of the Na^+/P_i co-transport activity, by treating the brush-border membranes with papain [27] prior to extraction and reconstitution, resulted in the loss of the Na^+-dependent P_i uptake into the subsequently reconstituted liposomes. Nevertheless, future studies utilizing this or similar methods must analyze and correct for an appreciable amount of adsorption or binding of P_i that occurs concurrently with the Na^+-dependent transport of P_i.

Methods

Brush-border membrane solubilization. Brush-border membranes were prepared from rabbit kidney cortex by Mg^{2+} precipitation followed by differential centrifugation according to Booth and Kenny [17]. The purity of the preparation was checked by meausring the relative enrichment in alkaline phosphatase activity (routinely 8- to 10-fold). Absence of mitochondrial contamination was determined by measuring cytochrome *c* oxidase activity. Small aliquots of brush-border membrane vesicles were stored in liquid nitrogen until use without significant loss of activity [16]. Appropriate amounts of membrane vesicles were thawed at 25°C in a water bath, diluted with KHT buffer (150 mM KCl/100 mM mannitol/ 20 mM Hepes-Tris (pH 7.4)/0.1 mM dithiothreitol/0.2 mM EDTA) and mixed with octylglucoside (protein to detergent ratio 1 : 4, w/w, final concentration 1% octylglucoside) [18]. The mixture was stirred on ice for 30 min and the solubilized proteins separated from insoluble material by centrifugation for 45 min at $100\,000 \times g$. $80.2 \pm 2.3\%$ ($n = 10$) of the membrane proteins were solubilized by this treatment. Where indicated, brush-border membrane vesicles were treated with papain according to Rohn et al. [27] prior to extraction with octylglucoside.

Reconstitution procedure. Solubilized membrane proteins were reconstituted into liposomes according to Da Cruz et al. [18], with minor modifications. 4.5 mg of solubilized proteins were transferred into a dialysis membrane and dialyzed against 15% poly(ethylene glycol) 40 000 in KHT buffer to reduce the volume and lower the concentration of detergent. After 2 to 3 hours, 5 to 10 ml of KHT were added to the protein solution and the dialysis continued until the final volume was reduced to approx. 1 ml. 36 mg purified egg yolk phosphatidylcholine (Type V-EA, Sigma, St. Louis, MO) and 9 mg cholesterol (an amount equal to 20% of total lipids) were dissolved under a 100% nitrogen atmosphere in 1 ml of chloroform in a

round bottomed flask. The solvent was evaporated under reduced pressure with a Rotavapor (Büchi, Brinkmann Instruments, Westbury, NY). Complete removal of the solvent was achieved by further evaporation under high vacuum with a liquid nitrogen vapor trap. The thin film of lipids was then resuspended in 1 ml of KHT buffer with a small magnetic stirring bar. The milky suspension was transferred into a test tube and sonicated at room temperature in a bath type sonicator (Bransonic 12, American Scientific Products, McGraw Park, IL) until clarified. All of the above steps were conducted under nitrogen atmosphere. One ml of the sonicated lipids were then frozen in liquid nitrogen and thawed at room temperature prior to the addition to the dialyzed proteins. After addition of 1 ml of KHT buffer, the suspension was dialyzed at 4°C for 36 h against 3×1 liter of KHT buffer. After dialysis, the suspension was diluted to 8 ml with mannitol buffer (300 mM mannitol/20 mM Hepes-Tris (pH 7.4)) and centrifuged for 10 min at $3000 \times g$ to remove any aggregated material. The resulting supernatant was centrifuged for 1 h at $150\,000 \times g$ to sediment the proteoliposomes. The pellet was resuspended in 1 ml of mannitol buffer and gently homogenized by aspirating the suspension into a 1 ml syringe through a 23 gauge needle (10 strokes).

Transport measurement. Uptake of ^{32}P-labelled inorganic phosphate into reconstituted liposomes was measured by a rapid filtration method over Millipore filters (Type GSWP, 0.22 μm) as described elsewhere [16]. Liposomes (10 μl) were mixed with 20 μl of uptake buffer. Final concentrations in the incubation medium were: 100 mM Na^+ or K^+ (nitrate salts), 0.1 mM KH_2PO_4, 100 mM mannitol and 20 mM Hepes-Tris (pH 7.4) and ^{32}P at 25 μCi/ml.

In experiments where the osmotic sensitivity of the liposomes was measured, increasing amounts of sucrose (see legend to figures) were added to the Na^+ or K^+ uptake buffers described above. In some experiments, arsenate was added to the Na^+ or K^+ uptake medium to give a final concentration of 1 or 10 mM.

SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was performed by the method of Shapiro et al. [19]. Samples were made 5% in SDS and 2% in 2-mercaptoethanol and boiled for 5 min. After cooling on ice, the samples were made 10% in sucrose and applied on a polyacrylamide (bisacrylamide/acrylamide 1 : 30) slab gel containing 0.1 M sodium phosphate, 6 M urea and 0.1% SDS (pH 6.8). The running buffer contained 0.1 M sodium phosphate and 0.1% SDS (pH 6.8). After electrophoresis the gels were stained using the silver-reagent method of Sammons et al. [20].

Other methods. Protein was measured using bovine serum albumin as a standard, by the method of Lowry et al. [21], after precipitation of the proteins with trichloroacetic acid. Alkaline phosphatase was measured by the method of Kelly and Hamilton [22] using 16 mM *p*-nitrophenyl phosphate as substrate [23]. Cytochrome *c* oxidase was measured according to Smith [24] by following the absorbance of reduced cytochrome *c* at 550 nm.

Materials. Fresh kidneys were obtained from young adult New Zealand rabbits fed a standard diet. Carrier-free ^{32}P or its potassium salt was obtained from ICN (Irvine, CA). When received from the supplier, occasional preparations of carrier-free ^{32}P contained impurities that produced high background uptake. All results reported were obtained with ^{32}P that produced low ($< 0.04\%$ of total counts) uptake when assayed on the filter without incubation with liposomes.

Results

SDS-polyacrylamide gel electrophoresis

Proteins from total brush-border membrnes, octylglucoside-solubilized proteins of these membranes and reconstituted liposomes were separated by SDS-polyacrylamide gel electrophoresis. The results, shown in Fig. 1, indicate no major differences between the protein composition of the three samples, and suggest therefore (1) that most of the proteins originally present in the intact brush-border memrane were solubilized by the detergent and (2) that most of the solubilized proteins were incorporated into the liposomes by the reconstitution process.

Uptake of P_i into liposomes

Octylglucoside-solubilized brush-border membrane proteins were reconstituted into liposomes and P_i uptake was measured in the presence of

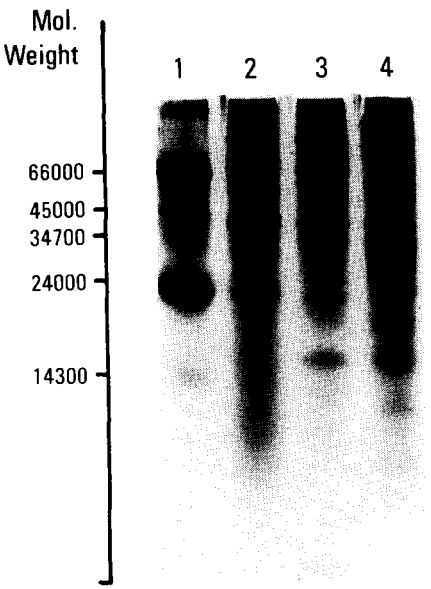

Fig. 1. SDS-polyacrylamide gel electrophoresis. (1) Standards; (2) brush-border membrane vesicles, (3) octylglucoside extract of brush-border membrane vesicles and (4) liposomes reconstituted with octylglucoside extract, were analyzed on a 8.5% polyacrylamide gel. 1 to 2 μg protein were applied in each lane and the gel was silver stained.

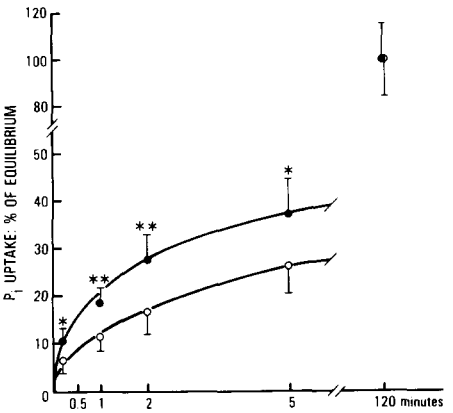

Fig. 2. Uptake of P_i into liposomes reconstituted with octylglucoside extracted proteins from brush-border membrane vesicles. The uptake was measured by a rapid filtration method. At time zero the incubation was started by vortex mixing 10 μl of liposomes with 20 μl of uptake medium. Final composition of the uptake medium was 100 mM mannitol, 100 mM $NaNO_3$ (●) or KNO_3 (○), 0.1 mM K_2PO_4, ^{32}P (approx. 0.5 μCi/sample) and 20 mM Hepes-Tris (pH 7.4). Data are means ± S.E. (vertical bars) of eight different liposome preparations. Equilibrium values, measured after 2 h of incubation were 7.65 ± 1.18 and 7.36 ± 1.15 pmol P_i per assay in the presence of Na^+ and K^+ (out > in) gradients, respectively. Each experiment was run in triplicate. Statistical analysis was done by the paired *t*-test of Student. * $p < 0.01$, ** $p < 0.001$.

either a Na^+ or a K^+ gradient (out > in). Fig. 2 shows the time-course of uptake into the proteoliposomes. As in brush-border membrane vesicles, P_i uptake rates were considerably faster in the presence of a Na^+ gradient than with a K^+ gradient. However, unlike brush-border membrane vesicles, no overshoot in the uptake of P_i above the equilibrium value could be demonstrated in our reconstituted system.

To determine whether the observed Na^+ dependency required the presence of the phosphate transporter in the liposomes, we also reconstituted a protein extract from brush-border membranes that had been treated with papain under conditions which abolish Na^+-dependent P_i transport in brush-border membrane vesicles without abolishing Na^+/D-glucose transport (Fig. 3). In these liposomes the rate of P_i uptake was the same in the presence of either a Na^+ or a K^+ gradient (Fig. 4).

Osmotic sensitivity of the liposomes

P_i uptake was measured in the presence of increasing concentrations of sucrose, a relatively impermeant molecule, and either a Na^+ or a K^+ gradient (out > in) to determine whether the osmotic gradient could induce shrinkage of the liposomes. The results shown in Table I indicate that 25–30% of the total uptake value represented transport into an intravesicular space or that 70–75% of the uptake might represent adsorption or binding. Importantly, the equilibrium accumulation of P_i into the osmotically sensitive space is similar whether Na^+ or K^+ is present in the uptake buffer.

Inhibition of uptake of P_i by arsenate

This suggestion that approx. 75% of the total uptake of P_i at equilibrium (2 h) is due to binding is consistent with the following effects produced by arsenate. Since arsenate is a competitor for binding of P_i by many biological recognition sites for P_i, the equilibrium uptake of P_i in the presence of excess arsenate should represent P_i accumulated into the interior of the vesicles (i.e., unbound P_i).

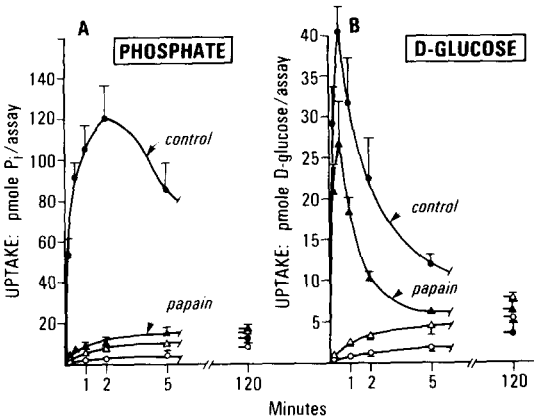

Fig. 3. (A) Uptake of P_i and (B) of D-glucose (0.1 mM) into control (●, ○) and papain-treated (▲, △) brush-border membrne vesicles. Vesicles (4 mg protein/ml were incubated (1 h at 25°C) with or without papain (70 μg/ml). The enzyme was removed by diluting the incubation mixture 10-fold with ice-cold 300 mM mannitol/20 mM Hepes-Tris (pH 7.4) buffer, followed by centrifugation at 48000 × *g* for 30 min. The pellet was resuspended in 35 ml of buffer followed by a second centrifugation. The final pellet was resuspended in buffer at a protein concentration of 6–8 mg/ml. The uptake of P_i and D-glucose was measured simultaneously in the presence of 100 mM Na^+ (●, ▲) or K^+ (○, △) gradient (out > in). Data are mean values ± S.E. (vertical bars) of three different membrane preparations.

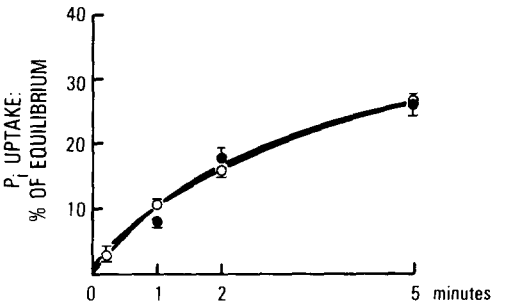

Fig. 4. Uptake of P_i into liposomes reconstituted with octylglucoside extracts of papain-treated brush-border membrane vesicles. Data are means ± S.E. (vertical bars) of three different liposome preparations. Individual equilibrium values (2 h) were, respectively, 8.5, 7.9 and 16.5 (mean 11.0) pmol P_i per assay in the presence of a Na^+ gradient (●) and 6.5, 9.1 and 17.7 (mean 11.1) pmole P_i per assay in the presence of K^+ gradient (○). Other explanations as in Fig. 2.

On the other hand, the equilibrium uptake of P_i in the absence of arsenate should consist of both bound and transported intravesicular (unbound) P_i. Arsenate (10 mM) inhibited equilibrium uptake of P_i, about 85%, as shown in Fig. 5. This suggestion that approx. 80–85% of the total uptake of P_i at equilibrium is P_i that is bound confirms the osmotic equilibrium experiments (Table I). Importantly, the equilibrium values both in the absence and presence of arsenate are not significantly influenced by Na^+ (when compared to K^+). Therefore, there is evidence in these experiments neither for a sodium-dependence of binding of P_i nor for a sodium-dependence of the intravesicular volume.

P_i appeared to bind extensively to the lipoidic component of the liposomes. In control experiments where liposomes were prepared in the absence of any added protein, equilibrium uptake of P_i was inhibited by 71.1 ± 6.2% ($n = 4$ experiments) by 2 mM arsenate as compared to controls (100%).

TABLE I

EFFECT OF EXTERNAL SUCROSE ON UPTAKE OF P_i INTO LIPOSOMES

Liposomes reconstituted from octylglucoside extracts of brush-border membrane vesicles were incubated in uptake media containing increasing concentrations of sucrose in the presence of a 100 mM Na^+ or K^+ gradient (out > in). 10 μl of liposomes were mixed with 20 μl of uptake buffer and the mixture was incubated for 2 h at 25°C. Values are means ± S.E. of three different liposome preparations. Each experiment was run in triplicate. Values are expressed as percentages of equilibrium values determined under isosmotic conditions (zero sucrose).

Gradient	P_i uptake; sucrose concentration (mM)					
	0	50	100	200	400	∞ [a]
Na^+	100	87.7 ± 5.4	82.5 ± 4.2	77.9 ± 7.6	72.4 ± 9.1	72.5 ± 8.5
K^+	100	87.8 ± 7.7	77.6 ± 8.3	75.4 ± 6.9	71.6 ± 7.1	69.8 ± 7.9

[a] Extrapolated values from a graph where uptake was plotted against the reciprocal of the osmolality.

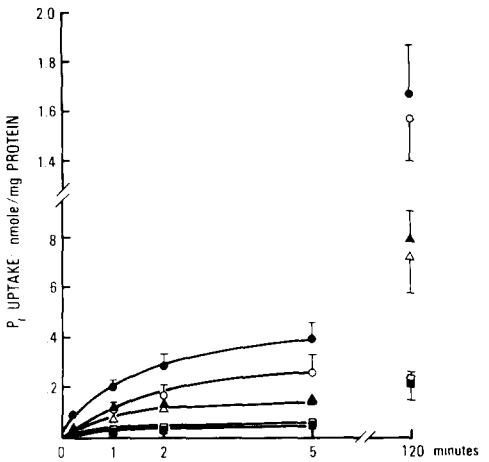

Fig. 5. Effect of 0 mM (●, ○) (control), 1 mM (▲, △) and 10 mM (■, □) arsenate on uptake of P_i into liposomes reconstituted with octylglucoside extracts of brush-border membranes. Uptake was determined in presence of a Na^+ (●, ▲, ■) or a K^+ (○, △, □) gradient (out > in). Data are means ± S.E. of three different liposome preparations. Other explanations as in Fig. 2.

In the reconstituted system, the rates at which the uptake of P_i approaches the equilibrium values in both the absence and presence of arsenate and in the presence of either Na^+ or K^+ are shown in Fig. 4. Notable are the findings that (1) in the absence of arsenate the rate of uptake of P_i in the presence of Na^+ is greater than in the presence of K^+ (shown as Na^+-K^+ in Fig. 6) and (2) in the presence of 10 mM arsenate the rate of uptake of P_i is not influenced by sodium (also shown in Fig. 6).

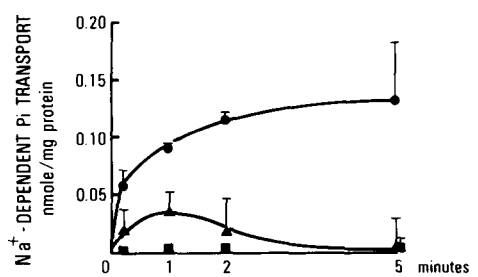

Fig. 6. Na^+-dependent transport of P_i in liposomes reconstituted with octylglucoside extracts. Values are calculated from the data presented in Fig. 4. In controls, the transport was determined by the difference between the uptake in the presence of a Na^+ gradient minus the uptake in the presence of a K^+ gradient (○). In the presence of 1 mM (▲) and 10 mM (■) arsenate the transport was determined by the difference of the P_i uptake in the presence of a Na^+ gradient plus arsenate minus uptake in presence of a K^+ gradient plus arsenate. Initial uptake rates, estimated from 10-s uptake values were 0.354, 0.108 and 0 nmol/min per mg protein in the presence of 0, 1 and 10 mM arsenate, respectively. Values are means ± S.E.

Discussion

Tubular reabsorption of P_i is an important function of the kidney and is essential for the maintenance of phosphate homeostasis. Prior studies on brush-border membrane vesicles from the renal proximal tubule have revealed that P_i is co-transported with Na^+ and that this co-transport is inhibited by arsenate [1,2]. However, little is known about the molecular constituents of this renal phosphate transporter. Isolation of the transporter requires solubilization from its intramembrane location, suitable purification methods and, most importantly, an adequate assay system to monitor the progress in purification. Several publications relate to purification of renal transport systems, mainly to that of the Na^+/D-glucose co-transporter [5–8]. The general strategy applied has been reconstitution of solubilized and partially purified membrane proteins with sonicated lipids to form proteoliposomes. A successful reconstitution should yield vesicles which show the ability to facilitate the transport of the substrate into an intravesicular space. The transport characteristics should be similar to those observed with intact brush-border membrane vesicles. Kinne and Faust [12] reported a proteoliposome system from kidney in which the uptake of P_i was stimulated by a Na^+ gradient, as compared to values observed in the presence of a K^+ gradient, and in which arsenate decreased the uptake of P_i. However, in retrospect and as described in the above Introduction, their findings could be explained in part, or principally, by binding of P_i to their proteoliposomes instead of true transport of P_i into liposomes.

In this study, we critically re-examined the initial observations of Kinne and Faust [12]. We solubilized the brush-border membrane proteins with octylglucoside, a particularly mild non-ionic detergent [25,26]. Qualitative analysis of the protein composition by SDS-polyacrylamide gel elec-

trophoresis indicated that most of the original membrane proteins were extracted by the detergent (Fig. 1). The solubilized proteins were then reconstituted into liposomes. Evidence for physical incorporation of the proteins into the vesicles is shown in Fig. 1. Most of the protein bands present in the detergent extract (lane 3, Fig. 1) were also present in the final liposomes (lane 4, Fig. 1). In contrast, Kinne and Faust [12] found that only a few protein bands were incorporated into their liposomes. This apparent discrepancy could be attributed to the different detergents used for solubilization or to the different methods for staining proteins that were used in the two studies. Our silver staining method [20] is 50–100-times more sensitive than the traditional Coomassie blue staining procedure. Furthermore, Kinne and Faust [12] delipidated their samples with chloroform/methanol prior to electrophoresis. This might have removed some proteins, including the P_i-binding proteolipid [14], that strongly interact with lipids.

Significant contamination of our liposomes with non-incorporated proteins is possible but unlikely, since the procedure we used for harvesting the proteoliposomes involved two successive centrifugations: (1) a low speed spin to sediment aggregated material and proteins precipitated by the removal of detergent by dialysis, and (2) a high speed spin to sediment the proteoliposomes, leaving unincorporated soluble proteins in the supernatant.

Uptake of P_i into the liposomes was measured in the presence of an inwardly directed Na^+ or K^+ gradient. At each measured time point (10 s, 1, 2 and 5 min) uptake of P_i was significantly faster when Na^+ was present in the incubation medium (Fig. 2). This difference disappeared ($p > 0.05$) after 2 h of incubation. Like Kinne and Faust [12], we were unable to observe an overshoot in the uptake of P_i into our liposomes, a feature characteristic for uptake of P_i with intact brush-border membranes. This resembles the findings of several groups involved in the purification of the Na^+/D-glucose co-transport system. A Na^+-dependent overshoot of glucose uptake has been rarely found, except when more or less purified protein fractions were used for the reconstitution [6–8].

An important issue is whether the observed uptake of P_i represented transport into an intravesicular space. Two tests were applied to evaluate this issue. First, we increased the osmolality of the external medium with sucrose and measured uptake of P_i. Our results (Table I) show that the equilibrium uptake of P_i decreased when the medium osmolality was increased, suggesting P_i transport into an intravesicular space. This space was, however, equal to only about 30% of the total equilibrium uptake of P_i, since extrapolated values for infinite osmolality indicated that approx. 70% of the equilibrium uptake was insensitive to osmotic forces in the uptake medium. Second, this suggestion of extensive binding of P_i to the liposomes is in agreement with the experiments demonstrating that 10 mM arsenate also inhibited the equilibrium uptake of P_i by slightly over 80% (Table II).

In the absence of arsenate, therefore, uptake of P_i by our proteoliposomes could consist of three components: (1) accumulation into the intravesicular space via passive permeability (leak pathway); (2) adsorption or binding to lipids and proteins and (3) accumulation into the intravesicular space via mediated pathways. We now discuss the evidence for and characteristics of each of these three components.

First, accumulation of P_i into the intravesicular space via passive permeabiality pathways is the best explanation for the uptake of P_i that occurs in the presence of 10 mM arsenate, since this concentration of arsenate virtually eliminates Na^+-dependent transport of P_i in brush-border membrane vesicles and appears to eliminate binding of P_i in our proteoliposomes (Fig. 5). Concordant values for the apparent intravesicular space were found, whether determined by infinite osmolality (Table I) or arsenate displacement (Table II). Of note is the lack of sodium dependence of the accumulation into the intravesicular space via the passive pathways.

Second, binding of P_i is the best explanation for the arsenate-displaceability of the P_i at equilibrium and for the large uptake of P_i at infinite osmolality. Much of this P_i binding must have been to lipids, since, P_i was also displaced by arsenate (2 mM) when uptake of P_i was measured in liposomes prepared in the absence of added protein. Is binding of P_i a Na^+-dependent process? Several experimental results fail to provide evi-

TABLE II

EFFECT OF ARSENATE ON EQUILIBRIUM P_i UPTAKE INTO LIPOSOMES

Liposomes reconstituted with octylglucoside extracts of brush-border memrbane vesicles were incubated 2 h in uptake media containing 0, 1 or 10 mM arsenate, in the presence of a 100 mM Na^+ or K^+ gradient (out > in). Other explanations as in Table I. *n*, number of experiments. All figures are expressed as percentages of equilibrium values measured in the absence of arsenate.

Arsenate concn. (mM)	*n*	Na^+ gradient		K^+ gradient	
		Uptake	AsO_4-sensitive binding	Uptake	AsO_4-sensitive binding
0		100	0	100	0
1	4	44.0 ± 5.5	56.0	46.5 ± 9.3	53.5
10	6	17.4 ± 4.5	82.6	18.8 ± 4.1	81.2

dence for sodium-dependence of binding: (i) The equilibrium uptakes of P_i with maximal binding (absence of arsenate) (Figs. 2, 4, 5), partial binding (1 mM arsenate) and no binding (10 mM arsenate) are not dependent upon the presence of sodium (Fig. 5 and Table II). (ii) The equilibrium uptake at infinite osmolality was not dependent upon sodium (Table I). (iii) Proteoliposomes containing protein solubilized from papain-treated brush-border membrane vesicles (a treatment that nearly eliminates Na^+-dependent transport of phosphate but produces minimal effect on Na^+-dependent transport of glucose [27]) failed to exhibit Na^+-dependent uptake of P_i even though the rate of uptake in the presence of K^+ during the first 5 min was the same in the papain-treated proteoliposomes (Fig. 4) as in control liposomes (Fig. 2). This suggests that papain eliminated the Na^+-dependent uptake of phosphate, but not the binding to the liposomes. (iv) Binding of P_i to phosphorin (a P_i-binding proteolipid extractable from brush-border membrane vesicles [14]) is not dependent upon the presence of Na^+. (v) Reconstitutions of phosphorin into liposomes by methods analogous to those used in this paper failed to produce Na^+-dependent binding of P_i, despite extensive binding of P_i by the proteoliposomes [13].

Third, the presence of a Na^+-dependent mediated transport for P_i into the intravesicular space can explain the several features of the P_i uptake shown in Figs. 5 and 6, including the dependence upon Na^+ and the inhibition by arsenate. Moreover, the loss of Na^+-dependent uptake into proteoliposomes, when prepared from brush-border membranes vesicles lacking a functional Na^+-dependent P_i transport system (papain treated brush-border membrane vesicles), is also best explained by postulating that the Na^+-dependent uptake of P_i into vesicles reconstituted from normal brush-border membrane vesicles represents reconstitution of the Na^+-dependent phosphate transport system normally found in brush border membrane vesicles. However, future studies utilizing reconstitution of the Na^+-dependent P_i transport system must carefully evaluate the contribution of binding of P_i to the total accumulation of P_i by the reconstituted proteoliposomes.

In summary, this paper presents evidence for reconstitution of the Na^+-dependent P_i transport system into liposomes. The vesicles transport P_i into an intravesicular space, although binding of P_i accounts for a portion of the measured uptake. Na^+-dependent uptake is not observed in the presence of 10 mM arsenate or when, prior to solubilization and reconstitution, the brush-border membranes were treated with papain, a treatment that abolished the Na^+-dependent uptake of P_i in membrane vesicles. However, future studies utilizing reconstitution of the Na^+-dependent P_i transport system must carefully evaluate the contribution of binding of P_i to the total accumulation of P_i by the reconstituted proteoliposomes.

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