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Partial Purification of the Na^+-Dependent D-Glucose Transport System from Renal Brush Border Membranes

Wha Bin Im*, Kit Yin Ling and Robert G. Faust

Department of Physiology, University of North Carolina School of Medicine, Chapel Hill, Noth Carolina 27514

Summary. A membrane extract enriched with the Na^+-dependent D-glucose transport system was obtained by differential cholate solubilization of rat renal brush border membranes in the presence of 120 mM Na^+ ions. Sodium ions were essential in stabilizing the transport system during cholate treatment. This membrane extract was further purified with respect to its Na^+-coupled D-glucose transport activity and protein content by the use of asolectin-equilibrated hydroxylapatite. The reconstituted proteoliposomes prepared from this purified fraction showed a transient accumulation of D-glucose in response to a Na^+ gradient. The observed rate of Na^+-coupled D-glucose uptake by the proteoliposomes represented about a sevenfold increase as compared to that of the reconstituted system derived from an initial 1.2% cholate extract of the membranes. Other Na^+-coupled transport systems such as L-alanine, α-ketoglutarate and phosphate were not detected in these reconstituted proteoliposomes.

Key words cholate solubilization · D-glucose · Na^+-dependent transport · renal brush border membranes

Introduction

The transport characteristics of the Na^+-coupled D-glucose uptake system located in brush border membranes of renal proximal tubules have been examined with intact kidneys (Silverman, Aganon & Chinard, 1970), with intact epithelial preparations (Schultz & Curran, 1970; Frömter & Luer, 1973; Frömter & Gessner, 1974; Ullrich, Rumrich & Klöss, 1974) and with isolated brush border membrane vesicles (Kinne, 1976; Sacktor, 1977; Hilden & Sacktor, 1979; Ullrich, 1979; Hopfer & Groseclose, 1980). All of these studies indicated that: 1) stereospecific and phloridzin-sensitive D-glucose uptake is primarily energized by a sodium gradient across the brush border membrane, and 2) transport is a rheogenic process cotransporting Na^+ and D-glucose. Several recent studies have reported the reconstitution of the renal Na^+-dependent D-glucose transport system into proteoliposomes (Crane, Malathi & Preiser, 1976; Kinne & Faust, 1977; Fairclough, Malathi, Preiser & Crane, 1979), the transport properties of which were comparable to those of the intact membrane.

Further understanding of the molecular basis of the renal Na^+-D-glucose cotransport system, however, largely depends on progress in the purfication of the membrane transport protein(s) responsible for it. One major problem in purifying membrane transport proteins is the selection of a suitable solubilizing agent; ideally such an agent should not inactivate membrane functions of interest and should be removed with ease during reconstitution in order to assay for transport function. In the reported reconstitution studies with the renal Na^+-coupled D-glucose transport system, Triton X-100 was used in solubilizing brush border membranes (Crane et al., 1976; Kinne & Faust, 1977; Fairclough et al., 1979). The suitability of other detergents has yet to be examined.

The present study was initiated to examine cholate as a solubilizing agent of renal brush border membrane components with particular emphasis on the purification of the Na^+-coupled D-glucose transport system. Cholate has been shown to be removed efficiently by dialysis (Racker, 1972) or by Sephadex column (Brunner, Skrabal & Hauser, 1976; Allen, Romans, Kercret & Segrest, 1980). Also, it takes much higher concentrations of cholate (1 mM) as compared to Triton X-100 (0.025 mM) to render membrane vesicles leaky to glucose and Na^+ ions (Goldin & Tong, 1974; Schlieper & DeRobertis, 1977; Bangham & Lea, 1978; Beesley & Faust, 1980). Therefore, the use of cholate may produce reconstituted proteoliposomes with a tighter diffusional permeability barrier. In our study we have found that cholate, in the presence of Na^+ ions selectively solubilizes renal brush border membrane proteins. Thus a membrane extract enriched with

* *Present Address:* Department of Experimental Biology, Upjohn Company, Kalamazoo, Michigan 49001.

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the Na^+-dependent D-glucose transport system was obtained by differential cholate extraction. Further purification of the transport system in this fraction was accomplished with hydroxylapatite. Purification of this transport system was monitored by Na^+-coupled ^{14}C-D-glucose uptake by reconstituted proteoliposomes and SDS-polyacrylamide gel electrophoresis.

Materials and Methods

Preparation of Renal Cortical Brush Border Membranes

The brush border membrane vesicles from rat renal cortex were prepared by a modification of the method previously reported (Im, Misch, Powell & Faust, 1980). Briefly, renal cortical slices from 12 kidneys of male Wistar rats were suspended in 60 ml of a solution containing 150 mM mannitol, 10 mM Tris-HCl, 5 mM $MgCl_2$, 30 mM succinate, 0.1 mM $MnCl_2$ and 5 mM potassium phosphate, pH 7.4. The suspension was homogenized in a Sorvall Omni-Mixer for 3 min. After incubation for 30 min on ice, the homogenate was centrifuged at $8,000 \times g$ for 12 min. The supernatant was decanted and centrifuged at $110,000 \times g$ for 20 min. The pellet was resuspended in 6 ml of a buffer containing 100 mM mannitol, 20 mM Hepes-Tris, 2 mM $MgCl_2$, pH 7.4 (mannitol transport buffer) and was layered on a density gradient consisting of 27, 31, 34, 37 and 40% (wt/wt) of sucrose containing 2 mM Hepes-Tris and 2 mM $MgCl_2$, pH 7.4. The centrifugation was at $107,000 \times g$ for 90 min with a SW 50.1 rotor. The interfacial band between 34 and 37% of sucrose was most enriched in the brush border membrane markers, maltase and alkaline phosphatase. This fraction represented about 30% of the total homogenate activities of the marker enzymes. It was purified about 10-fold as compared to the homogenate.

Solubilization of Renal Brush Border Membranes

The brush border membranes were washed with the mannitol buffer and suspended in a solution containing 70 mM NaCl and 40 mM sodium phosphate, pH 7.4 (phosphate-buffered saline solution) to a final protein concentration of about 5 mg/ml. In order to study solubilization of renal brush border membrane proteins by cholate, a 2.4% cholate-phosphate-buffered saline solution was added proportionately to the membrane suspensions making final cholate concentrations 0.1 to 1.2%. The mixtures were incubated on ice for 10 min and then were centrifuged at $170,000 \times g$ for 1 hr. This $170,000 \times g$ supernatant will be referred to as the membrane extract. To assess the degree of solubilization of the membranes as a function of cholate concentrations, the $170,000 \times g$ pellet was assayed with respect to its protein content and alkaline phosphatase and maltase activities. In some experiments, the residual membrane pellet was reextracted with cholate at a higher concentration or with another detergent, octyl-β-D-glucopyranoside. This will be called the second membrane extract. In order to study the role of Na^+ ions in the extraction media, all the sodium salts were replaced with their corresponding potassium salts but the extraction procedures remained unchanged.

Reconstitution of Various Membrane Extracts

The Sephadex G-50 column procedures (Brunner et al., 1976) were used with the following changes. A column (2.5 × 40 cm) of Sephadex G-50 was equilibrated with a buffer containing 70 mM KCl, 50 mM potassium phosphate, pH 7.4, and 0.5 mM $MgCl_2$. Typically, 5 to 10 ml of membrane extracts were applied to the column. The column was eluted with the equilibrating buffer at a rapid flow rate, 2.5 ml/min. Turbid effluents in the void volume were collected and centrifuged at $170,000 \times g$ for 60 min. The pellet, which contained most of the reconstituted vesicles, was usually resuspended in the mannitol transport buffer to a final protein concentration of about 5 mg/ml. In some experiments, membrane extracts were supplemented with acetone-washed asolectin (20 mg/ml) (Kagawa, Kandrach & Racker, 1973; Miller & Racker, 1976) which were dispersed in phosphate-buffered saline containing 1.2% cholate before reconstitution.

Chromatographic Separation of Membrane Proteins with Hydroxylapatite

About 2 ml of packed hydroxylapatite, previously equilibrated with the phosphate-buffered saline containing 1.2% cholate and asolectin (1 mg/ml), were mixed with 2 to 4 ml of membrane extracts. The mixture was centrifuged at $220 \times g$ for 5 min and the supernatant was saved. About 5 ml of fresh equilibrating buffer were mixed with the pellet. The centrifugation step was repeated and the supernatants were combined. Then the resin was extracted with 5 ml of a buffer containing 200 mM sodium phosphate, pH 7.4, 1.2% cholate and asolectin (1 mg/ml). This extraction procedure was repeated once more and the supernatants were combined. These fractions were reconstituted according to the procedures described previously.

Measurement of Transport Activities of Proteoliposomes

The technique of rapid filtration (Murer, Hopfer, Kinne-Saffran & Kinne, 1974; Kimmich, 1975) over a nitrocellulose membrane (0.2 μm pore size) was used to estimate the amount of solute trapped within proteoliposomes. For measurements of Na^+-coupled D-glucose uptake, the incubation medium contained 100 mM NaSCN, 100 mM mannitol, 2 mM $MgCl_2$ and 10 mM Hepes-Tris, pH 7.4. Uniformly labeled [^{14}D]-D-glucose was added at a concentration of 100 μM (100 to 120 cpm/pmole). For the Na^+-independent component of solute uptake, NaSCN was replaced with KSCN. Usually the incubation media and the proteoliposome suspensions were preincubated separately at 37 °C for 5 min. The transport activity was then initiated by mixing 100 μl of the proteoliposome suspension and 500 μl of the incubation media. An aliquot of 60 μl (50 μg protein) was withdrawn as a function of time and mixed rapidly with 2 ml of an ice-cold solution containing 150 mM KCl and 1 mM phloridzin. Then the mixture was filtered. The filter was washed three times with 1.5 ml of the stopping solution, dried and placed in 10 ml of tritosol scintillation fluid (Pande, 1976). Radioactivity was counted with a Nuclear Chicago Mark I Liquid Scintillation System. The rate of Na^+-coupled D-glucose uptake was obtained by computing the difference in uptake after 1 min when measured in a Na^+ and K^+ medium. To study Na^+-dependent ^{14}C-L-alanine, ^{14}C-α-ketoglutarate or ^{32}P-inorganic phosphate transport activity, the identical conditions outlined above were employed. In the last case, sulfate replaced phosphate in the buffered solutions. All the transport data presented in this paper represent the average of three experiments.

Other Assays

Protein was estimated by the method of Lowry, Rosebrough, Farr and Randall (1951) modified by Wang and Smith (1975). The alkaline phosphatase activity of membrane fractions was

determined according to the method of Hübscher and West (1965) by measuring hydrolysis of *p*-nitrophenolphosphate in the presence of potassium fluoride. Maltase activity was measured using the method of Dahlqvist (1968). Polyacrylamide gel electrophoresis (5.6% gel, 1% sodium dodecyl sulfate) of various membrane samples were carried out as described by Fairbanks, Steck and Wallach (1971). Approximately 20 µg of membrane proteins were applied to each gel. Standard proteins used for estimation of molecular weights were γ-globulin (127,000 daltons), bovine serum albumin (67,000 daltons) and cytochrome *c* (12,000 daltons).

Materials

Nitrocellulose filters (0.2 µm, 25 mm diameter) were purchased from Schleicher and Schuell. Sephadex G-50 (medium) was purchased from Pharmacia Fine Chemicals. Hydroxylapatite was obtained from Bio-Rad. [32P]-inorganic phosphate was purchased from New England Nuclear. Cholic acid, obtained from Sigma, was recrystallized from 50% ethanolic solution after an activated charcoal treatment. All other chemicals used were of reagent grade from standard sources.

Results

Figure 1 shows the extent of solubilization of renal brush border maltase, alkaline phosphatase and overall proteins as a function of cholate concentrations. About 55% of the brush border membrane proteins became soluble at a 1.2% cholate concentration. More interestingly, cholate solubilization appears to be selective. For instance, no alkaline phosphatase was solubilized up to a 1.2% cholate concentration, but most of the maltase was solubilized at this concentration. It should be noted that the cholate treatment had no apparent effect on the alkaline phosphatase activity that remained in the insoluble membrane fractions since more than 90% of the initial enzyme activity was recovered after cholate extraction.

Figure 2 shows the time course profiles of D-glucose uptake by reconstituted proteoliposomes derived from two types of brush border membrane extracts: one (panel *A*) was prepared in the usual sodium phosphate-buffered saline containing 1.2% cholate, the other (panel *B*) in the same medium except potassium replaced sodium. The extract prepared in the presence of sodium salts showed, upon reconstitution, a pronounced Na^+-dependent D-glucose uptake (panel *A*), about 3 to 5 times greater than that of the extract prepared in the presence of potassium salts. There was no noticeable difference observed, however, in the amount of protein in the extracts or in the SDS polyacrylamide gel electrophoresis patterns of the proteoliposomes used in panels *A* and *B* (*data not shown*). This Na^+-coupled D-glucose transport activity, $38 \pm 4(3)$ pmoles/mg protein × min, was inhibited by phloridzin (Fig. 2, panel *A*) and was also sensitive to osmotic pressures

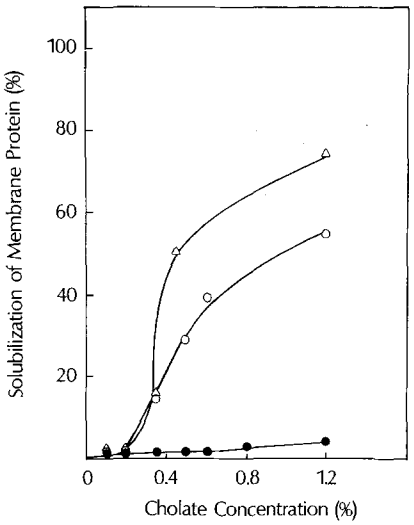

Fig. 1. Plot showing the extent of solubilization of overall proteins (○), maltase (△) and alkaline phosphatase (●) in renal brush border membranes as a function of cholate concentrations. The assays for membrane protein and the enzyme activities were carried out with the 170,000 × *g* pellets obtained after cholate treatments (*see* Materials and Methods). The differences in their quantities from untreated renal membrane were used to compute the percentage of solubilization of various membrane proteins

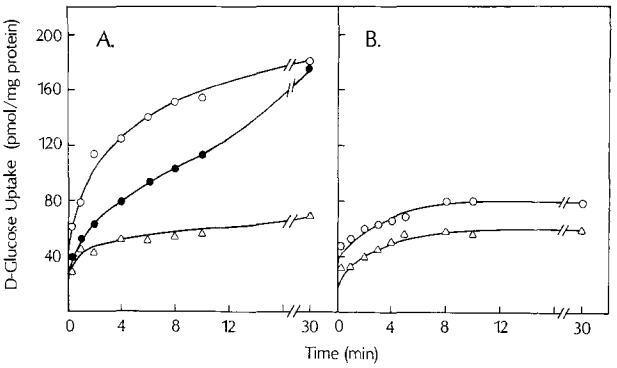

Fig. 2. Time course profiles of D-glucose uptake by reconstituted proteoliposomes derived from 1.2% cholate extracts of the brush border membranes. Panel *A* represents the extract prepared in the presence of 120 mM Na^+ ions, panel *B* in the equivalent amount of K^+ ions replacing sodium ions. D-glucose uptake was measured in a medium containing 80 mM NaSCN (○), 80 mM NaSCN plus 1 mM phloridzin (●) or 80 mM KSCN (△). The concentration of D-glucose was 100 µM, and the incubation was performed at 37 °C

created by adding sucrose to the medium (*data not shown*). These results indicate that the presence of Na^+ ions in the detergent solution stabilizes the solubilized renal Na^+-dependent D-glucose transport system. Supplementation of the detergent medium with D-glucose (10 mM), however, had no additional protective effect on the transport system.

This finding of Na^+-ion protection enabled us to assay quantitatively the Na^+-dependent D-glucose

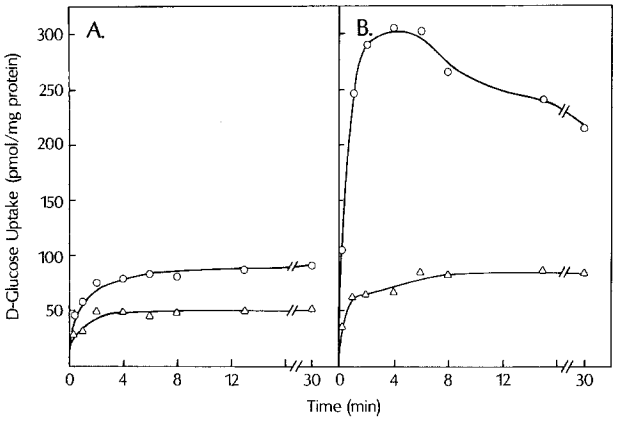

Fig. 3. Time course profiles of D-glucose uptake by reconstituted proteoliposomes obtained from a 0.7% cholate extract of brush border membranes (panel *A*) and from a 1.2% cholate extract of the residual membranes (panel *B*). D-glucose uptake was measured in a medium containing 80 mM NaSCN (○) or 80 mM KSCN (△). The other conditions were the same as those described in Fig. 2

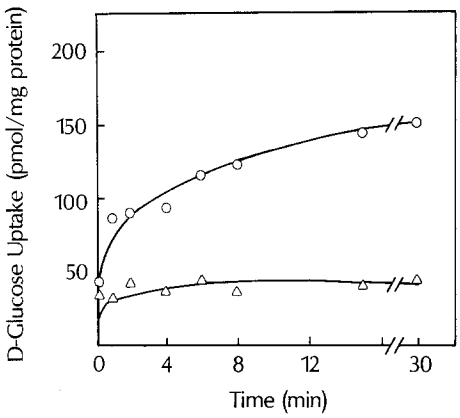

Fig. 4. Time course profiles of D-glucose uptake by the reconstituted proteoliposomes prepared from a 1% octylglucoside extract of the residual brush border membranes initially treated with 0.7% cholate buffer. D-glucose uptake was measured in a medium containing 80 mM NaSCN (○) or 80 mM KSCN (△). The other conditions were the same as those described in Fig. 2

transport activity of various renal brush border membrane extracts obtained at different cholate concentrations. As the cholate concentration was raised from 0.3 to 0.7%, almost 40% of the brush border membrane proteins were extracted (*see* Fig. 1). Nevertheless, the membrane extract at 0.7% cholate, when reconstituted into proteoliposomes, showed only limited Na^+-dependent D-glucose uptake (panel *A*, Fig. 3). The residual membrane pellet (170,000 × g) obtained after the 0.7% cholate extraction was re-extracted with 1.2% cholate buffer. This second extract contained only 15% of the brush border membrane proteins. But its reconstituted proteoliposomes showed a very rapid rate of D-glucose uptake in the sodium medium (Fig. 3, panel *B*). The observed rate of Na^+-coupled D-glucose uptake, 188 ± 12(3) pmoles/mg protein × min, was about four- to fivefold higher than that of the control system consisting of proteoliposomes initially prepared from the 1.2% cholate extract of the brush border membranes (*see* Fig. 2, panel *A*). In addition, the proteoliposomes derived from the second cholate extract displayed an overshoot of Na^+-dependent D-glucose accumulation. The appearance of such an overshoot is an apparent indication of the active transport of D-glucose into the proteoliposomes energized by an artificial sodium gradient.

To compare the effectiveness of a nonionic detergent with cholate on the reconstitution of the Na^+-dependent D-glucose transport system, the membrane initially treated with 0.7% cholate buffer was reextracted with the phosphate-buffered saline containing 1% octylglucoside. This nonionic detergent solubilized almost 40% of the brush border membrane proteins. As is shown in Fig. 4, the reconstituted proteoliposomes obtained from this octylglucoside extract showed a rate of a Na^+-dependent D-glucose uptake less than 50 pmoles/mg protein × min and no transient accumulation of D-glucose. It is possible that this observed rate reduction could be attributed to the increased protein content of the membrane extract.

Up to this point, the membrane extracts were reconstituted without supplementation of exogenous phospholipids. Conceivably some membrane extracts, however, may not contain a sufficient amount of phospholipids to allow complete formation of proteoliposomes. To check this point, asolectin dispersed in 1.2% cholate buffer was added in an amount equal to the protein content of each membrane extract. This mixture was applied to a Sephadex G-50 column (*see* Materials and Methods) in order to prepare the proteoliposomes containing asolectin. The reconstituted vesicles thus supplied with this mixture of exogenous phospholipids, however, showed no noticeable change in their transport activity as compared to those shown in Figs. 2 and 3. However, further increases in the amount of supplemented asolectin, up to 5 times, led to a significant decrease in the rate of Na^+-coupled D-glucose uptake by the reconstituted proteoliposomes. For instance, the reconstituted vesicles obtained from a mixture of the second cholate extract and the fivefold excess of asolectin showed a rate of Na^+-coupled D-glucose uptake equal to only one-third of that observed without the supplementation. Furthermore, no sign of an overshoot of D-glucose accumulation was observed during the 30-min incubation period. These data clearly indicate that a simple addition of exogenous phospholipids does not im-

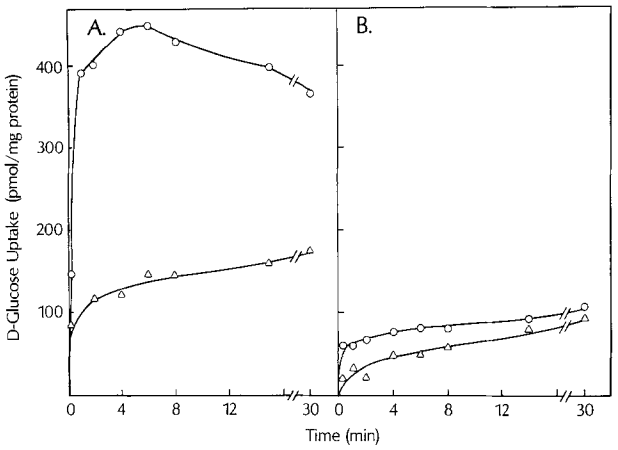

Fig. 5. Time course profiles on D-glucose uptake by the reconstituted proteoliposomes obtained from hydroxylapatite fractions. Panel *A* represents the fraction unadsorbed at a 50-mM phosphate concentration and panel *B* the adsorbed fraction which was recovered by raising the phosphate concentration to 200 mM. D-glucose uptake was measured in the medium containing 80 mM NaSCN (○) or 80 mM KSCN (△). The other conditions were the same as those described in Fig. 2

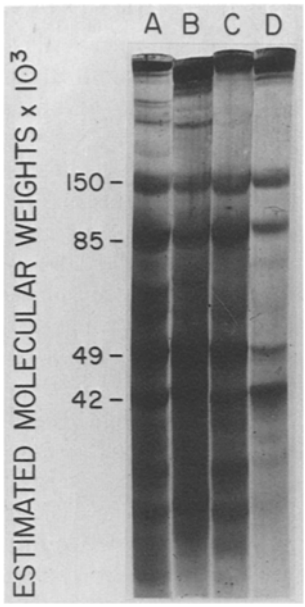

Fig. 6. SDS-polyacrylamide gel electrophoresis patterns. (*A*) Renal brush border membranes; (*B*) reconstituted proteoliposomes obtained from the 0.7% cholate extract of brush border membranes; (*C*) reconstituted proteoliposomes prepared from the 1.2% cholate extract of the residual brush border membrane treated initially with 0.7% cholate buffer; (*D*) reconstituted proteoliposomes obtained from the unadsorbed hydroxylapatite fraction at a 50-mM phosphate level of the eluting buffer. The amount of protein applied was 30 μg in *A* and 20 μg in *B*, *C* and *D*, respectively

prove the efficiency of reconstitution of the Na^+-coupled D-glucose transport system.

Although hydroxylapatite has been often used to resolve cell membrane proteins (Carter-Su, Pillon & Czech, 1980; Wohlrab, 1980), our preliminary experiments showed that the resin inactivates the renal Na^+-dependent D-glucose transport system. It was further learned that such an inactivation can be avoided by equilibrating the resin with asolectin, although such a treatment has no effect on the protein composition of the eluted fractions as determined by SDS polyacrylamide gel electrophoresis (*data not shown*). Therefore, we attempted to resolve the membrane components in the second cholate extract of the brush border membranes by using asolectin-treated hydroxylapatite. About 60% of the membrane proteins in the second extract were not adsorbed to the resin equilibrated with 50 mM sodium phosphate buffer. The proteins adsorbed to the resin were recovered by raising the sodium phosphate level of the buffer to 200 mM. Figure 5 shows the time course profiles of D-glucose uptake by the reconstituted proteoliposomes from these hydroxylapatite fractions. Most of Na^+-coupled D-glucose transport activity was associated with proteoliposomes derived from the unadsorbed fraction recovered in 50 mM phosphate; the observed rate, 275 ±20(3) pmoles/mg protein × min, was about seven- to eightfold higher than that of the control system (Fig. 2, panel *A*). It should be noted that other Na^+-dependent transport activities, i.e. L-analine, α-ketoglutarate and phosphate, were not detected with these proteoliposomes or proteoliposomes derived from the initial 1.2% cholate extract although these transport systems did exist in the native renal brush border membrane vesicles (*unpublished observation*).

Figure 6 shows the SDS polyacrylamide gel electrophoresis patterns of intact renal brush border membranes, the reconstituted proteoliposomes from the 0.7% cholate extract, the second extract with 1.2% cholate and the unadsorbed hydroxylapatite fraction in 50 mM phosphate buffer. Considerable variations in the intensities of the major bands can be observed among these fractions. Nevertheless, the hydroxylapatite fraction was most purified. It has only four prominent bands in the 150,000, 85,000, 49,000, and the 42,000 dalton range.

Discussion

In this study we have demonstrated that the integral membrane protein(s) responsible for Na^+-dependent D-glucose transport can be selectively solubilized by differential cholate treatments of renal brush border membranes. It appeared that at a concentration of 0.7% or below, membrane solubilization by cholate was limited to perhaps peripheral proteins or a few integral proteins easily accessible from the surface, such as maltase. Malpartida and Serrano (1980) also

reported the beneficial effect of 0.5% cholate treatment on enriching the integral ATPase in yeast plasma membranes prior to its selective solubilization with Zwittergen TM 314. Thus the limited solubility effects of cholate at low concentrations may serve a useful purpose in the purification of many integral membrane proteins. Even at concentrations higher than 0.7% we found that cholate solubilized only selective regions of renal brush border membrane as evidenced by its inability to solubilize alkaline phosphatase. This confined capacity of cholate may reflect the presence of various microenvironments in the membrane distinctively heterogeneous in their composition and function (Sackman, Albrecht, Hartman & Galla, 1977). In fact, in our study these properties of the detergent and the membranes were maximally utilized in obtaining a membrane extract enriched with the Na^+-coupled D-glucose transport system. Further purification of the transport system was aided by the use of hydroxylapatite equilibrated with asolectin. As described earlier (*see* Results), such an equilibration with soybean phospholipids prevents inactivation of the transport system by hydroxylapatite. It is very likely that the untreated hydroxylapatite may disturb lipid environments of membrane proteins by interacting strongly with the phospholipids in the vicinity of the membrane proteins.

Prerequisite for all of these experimental manipulations, however, was the stability of the Na^+-dependent D-glucose transport system in the presence of cholate. During the course of this study, several agents were tested for their ability to protect the transport system during cholate treatment. Those tested without success included sucrose, dithiothreitol and D-glucose. High levels of Na^+ ions, on the other hand, sufficiently stabilized the transport system so that we could estimate the distribution of the transport system among various cholate membrane extracts. It should be noted that even in the presence of Na^+ ions a prolonged exposure to cholate or an increase in the cholate concentration relative to the protein content of the membrane extracts led to a considerable decrease in Na^+-coupled D-glucose transport activity. The high concentration of Na^+ ions in the membrane extracts raised the possibility that the reconstituted proteoliposomes might trap enough Na^+ ions to nullify an imposed Na^+ ion gradient. It became apparent, however, that the Na^+ ions in the cholate extracts were diluted and retarded adequately during the chromatography over a large column (2.5×40 cm) of Sephadex G-50. Consequently, we have been able to observe a Na^+-dependent D-glucose overshoot with some reconstituted proteoliposomes.

Most reconstituted proteoliposomes examined in this study showed a very slow rate of D-glucose uptake in the absence of Na^+ ions. Such a tight diffusional permeability may be attributed partly to the efficiency of the Sephadex G-50 column procedures that were used to remove cholate and partly to the high threshold concentration of the detergent which renders the proteoliposomes leaky (Goldin & Tong, 1974; Bangham & Lea, 1978; Beesley & Faust, 1980).

We have failed to detect Na^+-dependent L-alanine, α-ketoglutarate or phosphate transport activities with the various cholate extracts of renal brush border membranes. It is possible that these transport systems were not solubilized by cholate. Alternatively, under our experimental conditions, cholate may have inactivated these transport systems.

Recently, Lin, DaCruz, Riedel and Kinne (1981) reported a 20- to 30-fold purification of the hog kidney sodium-D-glucose transport system using phloridzin affinity chromatography. Since the SDS gel electrophoresis pattern of their preparation was also complex but somewhat different from that of ours (*D*, Fig. 6), it is conceivable that the cholate-selective solubilization and the hydroxylapatite purification of the transport system may be complementary to the purification procedures using phloridzin affinity chromatography. If these two purification procedures are combined, then it is possible that the membrane protein responsible for Na^+-dependent D-glucose transport could be isolated.

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