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A SIMPLE LIPOSOMAL SYSTEM TO RECONSTITUTE AND ASSAY HIGHLY EFFICIENT Na^+/D-GLUCOSE COTRANSPORT FROM KIDNEY BRUSH-BORDER MEMBRANES

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A simple procedure to reconstitute highly efficient Na^+/D-glucose cotransport from solubilized brush-border membranes of proximal kidney tubules is described. Reconstitution of transport activity was possible with various phospholipid and cholesterol combinations; the presence, however, of cholesterol and at least one phospholipid was essential. When liposomes were synthesized from only one phospholipid and cholesterol, the highest uptake rates were observed with phosphatidylserine; phosphatidylcholine was less effective and phosphatidylethanolamine showed insignificant uptake of D-glucose in the presence of Na^+. The rate at which an inward-directed Na^+ gradient dissipated across the liposomal membranes was reduced if the cholesterol concentration of liposomes was increased. In the optimized system, proteoliposomes were formed from cholesterol and phosphatidylserine by a heat-sonication-freeze-thaw procedure. A Na^+-gradient persisted for hours across these proteoliposomal membranes and a Na^+/D-glucose cotransport with the following characteristics could be demonstrated: (1) dependency on the Na^+ gradient; (2) a transient Na^+-gradient-dependent uptake of D-glucose into liposomes over the equilibrium concentration (overshoot); (3) rheogenicity; (4) stereospecificity; and (5) high-affinity phlorizin inhibition. Since the Na^+-gradient-stimulated D-glucose uptake is linear for minutes, the initial uptake rates can be measured and the Na^+/D-glucose cotransport activity of different protein fractions can be compared.

Introduction

The Na^+/D-glucose cotransporter, localized in the brush-border membranes of intestine and kidney proximal tubules, has been extensively characterized by in vivo measurements [1–3] and by measurements in membrane vesicles [4–8]. To date, attempts to identify and purify the Na^+/D-glucose cotransport system have failed. Labeling experiments in which attempts were made to identify the transport protein by using covalently binding analogs of phlorizin, a competitive inhibitor of D-glucose uptake, have been inconclusive, since mainly unspecific labeling was observed [9,10]. Reconstitution experiments have also not produced any conclusive information as to the identity of either the kidney or intestinal D-glucose transporter [11–17]. In only one report could a Na^+/D-glucose cotransport be unequivocally demonstrated [12]. There it was shown that the liposomal D-glucose concentration increased transiently over the value obtained at equilibrium in the presence of a Na^+ gradient. In other reports, either this overshoot was not demonstrable [11,13,15–17] or the liposomal glucose concentration obtained at equilibrium was not determined [14]. As a starting

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Abbreviations: PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipid.

point for the purification of the Na^+/D-glucose cotransporter, therefore, and, hopefully, also for the purification of other Na^+-cotransport systems, it seemed important to find the optimal conditions for the incorporation of solubilized proteins into liposomes with a low Na^+ permeability so that Na^+/D-glucose cotransport activity could be assayed. Such a system has been found and some of its properties will be described.

Materials and Methods

Preparation of brush-border membranes. Brush-border membranes were isolated from pig kidney cortex using a Ca^{2+}-precipitation method similar to [18]. The brush-border membranes were resuspended in a buffer comprising 10 mM triethanolamine-HCl (pH 7.4)/150 mM NaCl/5 mM EDTA Na_2/10% (v/v) glycerol (buffer 1). 1-ml samples were rapidly frozen and stored in liquid nitrogen.

Solubilization of brush-border membranes. The following were added to a flask on ice while being stirred continuously: buffer 1, 2 M D-glucose solution in buffer 1 and brush-border membrane proteins which had been thawed rapidly at 37°C just prior to use. After cooling, a 1% deoxycholate solution was added drop-wise to the membrane suspension, resulting in the following final concentrations of all the components: 1 M D-glucose, 1.5 $mg \cdot ml^{-1}$ membrane protein and 0.1% deoxycholate *. After stirring for 1 h at 8°C, the suspension was centrifuged for 1 h at 180000 × *g*. The clear supernatant, which showed no intact membrane vesicles after negative staining [16], was concentrated over PM-10 filters (Amicon).

Removal of deoxycholate. The concentrated protein solution was applied to a DEAE A-50 column which had been previously equilibrated with elution buffer (10 mM triethanolamine (pH 7.4)/350 mM NaCl/5 mM EDTA disodium) at 8°C. By this procedure (a) approx. 57% of the total protein applied to the column was bound and (b) deoxycholate was removed from the protein peak which appeared in the void volume, as established in previous experiments with radiolabeled deoxycholate. After the detergent-free eluate was concentrated to a volume of 2–4 ml, it was dialyzed for at least 12 h against 2 l of a buffer comprising 20 mM imidazole cyclamate (pH 7.4)/0.1 mM magnesium cyclamate/100 mM potassium cyclamate (buffer 2).

Optimized reconstitution procedure. To prepare 2 ml of proteoliposomes, 6–13 mg of total lipid, which were stored under nitrogen in a chloroform/methanol (95 : 5, v/v) solution at −40°C, were added to a round-bottomed flask and dried under a stream of nitrogen. As the flask was rotated, a thin lipid film was produced. After the addition of glass beads and 1 ml of buffer 2, the flask was shaken for 3 h under nitrogen at room temperature. 1 ml of the resulting lipid suspension was then mixed with 1 ml of protein solution previously equilibrated with buffer 2. After a 15 min incubation at 41°C, the lipid/protein mixture was sonicated for 30 s under nitrogen at 41°C (Labsonic 1510, Braun, Melsungen AG, probe diameter 4 mm, energy setting 100 W). 500-µl aliquots of the sonicated suspension were placed in vials and stored in liquid nitrogen. Before use, a sample was thawed at 37°C, diluted to approximately 5 ml with buffer 2 (22°C) and centrifuged for 30 min at 150000 × *g*. The centrifugation and subsequent steps were carried out at room temperature. The pellet was resuspended in 400–600 µl of buffer 2. This suspension was then centrifuged for 30 s in an Eppendorf bench-top centrifuge. The resulting supernatant was the liposomal suspension which was used in all uptake and electron microscopic studies.

Uptake assay. Liposomes were preincubated for approx. 30 min at 37°C before uptake measurements were initiated. In all experiments valinomycin was present during the preincubation, except where noted. Uptake was initiated by adding 50 µl of liposomes to 400 µl of incubation medium. The different incubation media contained either 100 mM K^+ or Na^+ and either 0.2 mM D- or L-glucose with tracer amounts of the radiolabeled sugar, or contained tracer amounts of $^{22}Na^+$. At specified time intervals, a 50 µl sample was removed from the incubation medium and diluted in 1 ml of ice-cold 'stop-solution', which has the same composition as the incubation media except that D-glucose was replaced by 0.5 mM phlorizin and, in

* Recently, solubilization has been optimized using 0.2% deoxycholate [19].

the Na^+-uptake experiments, $^{22}Na^+$ was omitted. The diluted sample was immediately mixed and applied to a 0.22 μm cellulose acetate filter (Millipore GSWP) and the liposomes were washed with 6 ml of 'stop solution'. The filters were removed, solubilized in Bray's solution [20] and assayed for radioactivity in a Berthold BF 5000 liquid scintillation spectrometer. The uptake measurements were performed in duplicate or triplicate. The standard deviations are indicated in the figures if they exceed the symbol size.

Electron microscopy. For the freeze-fracture studies, the liposomes were rapidly frozen by a spray-freezing procedure [21]. The digested replicas were examined in a Philips 300 electron microscope operating at 60 kV. Negative staining was performed exactly as previously described [22].

Analytical methods. Protein was determined according to Lowry et al. [23] after precipitating the protein with 10% trichloroacetic acid and resuspending it in 1 M NaOH and 1% sodium dodecyl sulfate (SDS). Bovine serum albumin was used as protein standard.

Polyacrylamide gel electrophoresis was performed as previously described [24] employing a modification of the method described by Weber and Osborn [25].

Lipids from the intact brush-border membrane vesicles were extracted by the method of Folch et al. [26]. In order to separate and identify the individual lipids, thin-layer chromatography (TLC) was performed on glass plates coated with silica gel. The TLC plates were developed in a chloroform/methanol/water (65 : 30 : 3, v/v) solvent system. After the various phospholipids had been recovered from the plates, they were quantitatively analyzed by measuring their phosphate content [27]. Cholesterol was determined according to Clark et al. [28].

Materials. D-[^{3}H]Glucose (30 Ci/mmol), ^{22}NaCl (1.9 mCi/mol) and [^{3}H]phlorizin (6.3 Ci/mmol) were purchased from New England Nuclear (Boston) and [^{14}C]deoxycholate (52 mCi/mmol) was supplied by Amersham Buchler (Frankfurt). Cholesterol, sphingomyelin from bovine brain and valinomycin were obtained from Serva (Heidelberg). Phosphatidylcholine was purified from egg yolk according to Singleton and co-workers [29]. Phosphatidylethanolamine from egg yolk (type III) and phosphatidylserine (PS) from bovine brain were usually supplied by Sigma (Munich). Since different batches of PS showed significantly different results in the D-glucose uptake measurements, PS had been recently purchased from Lipid Products (South Nutfield). DEAE-Sephadex A-50 was purchased from Pharmacia (Uppsala) and phlorizin from Roth (Karlsruhe). All other chemicals used were of highest grade and obtained from Merck (Darmstadt).

Results

Experiments on the reconstitution procedure

Various methods of liposome preparation were tried with lipids of the type and ratio found in the intact brush-border membrane, which were as follows in mol% (mean $\pm$S.E. of four values): phosphatidylserine (PS), 15 ± 0.8; phosphatidylcholine (PC), 15 ± 2; phosphatidylethanolamine (PE), 21 ± 1.6; sphingomyelin, 22 ± 2.9; and cholesterol, 27 ± 2.2. Initially, experiments consisted of using different sonication and/or freeze-thaw procedures. The criteria applied to select appropriate liposomal preparations were their ability to incorporate D-glucose in the presence of an inward-directed Na^+ gradient and their morphological appearance. Attempts were made to select liposomal preparations with diameters between 0.2–0.6 μm by employing negative staining and freeze-fracture techniques. However, quantitative morphological characterizations were not performed during most experiments, since size measurements on freeze-fractures samples were time-consuming and negative staining did not prove to be an appropriate method for quantitation, since shrinkage and aggregation of liposomes were often observed. Consequently, a greater reliance was placed on the results obtained with uptake studies. Good Na^+-dependent D-glucose uptake rates were obtained when the lipids had been sonicated for 30 s in the presence of protein and then frozen only once. Further optimization was obtained by preincubating the protein-lipid mixture at different temperatures and time periods before sonication, by varying the pH and Mg^{2+} concentration during sonication, and by varying the total amount of lipid used for reconstitution.

Two test systems were employed to measure Na^+/D-glucose cotransport into liposomes. Originally a method described by Fairclough and coworkers [12] was used, in which D-glucose uptake was measured in the presence of either 100 mM K^+ or Na^+ thiocyanate into liposomes which contained 50 mM K_2SO_4. However, since there were indications from previous experiments that a sulfate transport system was also reconstituted along with the Na^+/D-glucose cotransporter in our system (unpublished) and, since thiocyanate was found to increase the nonspecific glucose permeability of liposomes (data not shown), we began to assay the uptake of D-glucose in the presence of 100 mM K^+ or Na^+ cyclamate into liposomes preloaded with buffer 2. In this system valinomycin, a K^+ ionophore, was added to prevent the generation of an inside-positive potential with the entrance of the cotransported Na^+ and also to create an inside-negative K^+ diffusion potential to increase the initial driving force for Na^+/D-glucose uptake. The optimal valinomycin effect was found at a concentration of $2 \cdot 10^{-8}$ M (data not shown), some 10^5-times lower than that necessary to obtain optimal D-glucose transport rates in intact brush-border membrane vesicles under the same experimental conditions [19].

Reconstitution experiments with different lipids

Different lipid combinations were employed for several reasons: to see if the reconstitution of transport activity could be further optimized and/or to simplify the system by decreasing the number of lipids utilized. Fig. 1 shows a series of experiments in which the uptake of D-glucose into liposomes synthesized from the five major lipids present in the brush border membrane were compared with liposomes synthesized from only four of the lipids. The D-glucose uptake measured after 28 h, after equilibrium had been reached, was identical in the presence of Na^+ or K^+ when either five lipids were used or when liposomes were synthesized without PC, PE or sphingomyelin. If the liposomes were prepared without PS or cholesterol (data not shown), only a minor (PS) or no (cholesterol), Na^+-specific D-glucose uptake was observed. This indicates that cholesterol, and possibly PS, is essential to reconstitute D-glucose transport activity under these experimental conditions. If the initial uptake of D-glucose is measured at K^+ equilibrium, where the only driving force for the D-glucose transporter is the 0.2 mM D-glucose gradient, the apparent passive permeability * of

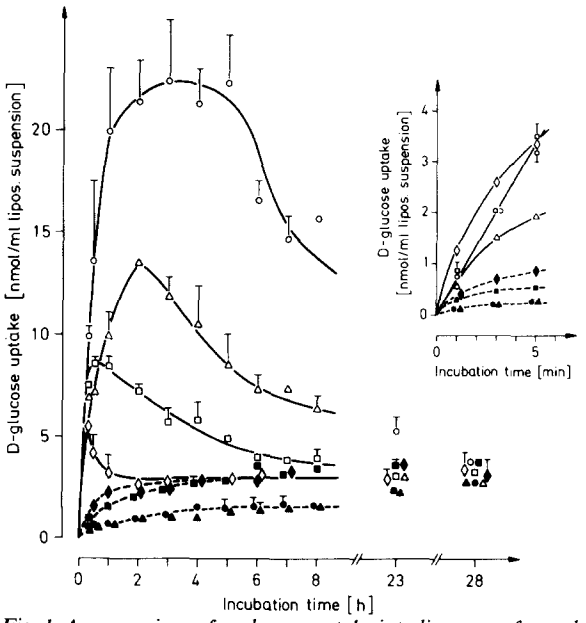

Fig. 1. A comparison of D-glucose uptake into liposomes formed from cholesterol and either four or three different phospholipids. For the liposome formation, the following lipid combinations were used: control, cholesterol, PC, PE, PS, sphingomyelin (■, □); without PC: cholesterol, PE, PS, sphingomyelin (●, ○); without PE: cholesterol, PC, PS, sphingomeyelin (▲, △) or without sphingomyelin: cholesterol, PC, PE, PS (◆, ◇). The protein concentrations of the different liposomal suspensions used for the uptake measurements were 0.40, 0.31, 0.32 and 0.46 $mg \cdot ml^{-1}$, respectively. For the control, 2 ml of a liposomal suspension were formed from approximately 6 mg solubilized, detergent-free brush-border membrane proteins and cholesterol (2.3 mg), PC (2.1 mg), PE (3.2 mg), PS (2.1 mg) and sphingomyelin (3.2 mg) as described in Methods. For the other groups, the amounts of the different lipids used for liposome formation were the same as for the control except that a specific lipid was deleted in each case. The liposomes, preloaded with 20 mM imidazole cyclamate (pH 7.4)/0.1 mM magnesium cyclamate/100 mM potassium cyclamate (buffer 2), were preincubated with $2 \cdot 10^{-8}$ M valinomycin, and D-glucose uptake was measured in buffer 2 (closed symbols) or in a buffer comprising 20 mM imidazole cyclamate (pH 7.4)/0.1 mM magnesium cyclamate/100 mM sodium cyclamate (open symbols). The inset indicates the D-glucose uptake within the first 5 min after initiation of incubation.

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The term 'apparent passive permeability' does not differentiate between D-glucose permeation rates observed through lipid bilayers formed from different lipids or when different fractions of mono- or multilayer liposomes are present, or when

D-glucose can be estimated. The inset of Fig. 1 shows that, for the liposomes exhibiting significant Na^+-dependent D-glucose uptake, the order of apparent passive glucose permeabilities is: without PC = without PE < control < without sphingomyelin. The time at which the peak of the overshoot is reached, however, should, to a greater degree than the initial rate of D-glucose uptake, be dependent on the passive permeabilities of the liposomes to Na^+ and D-glucose. The time required to reach the peak of the overshoot in the different liposomal preparations is: without PC > without PE > control > without sphingomyelin. This is the same order as that observed when the apparent passive permeabilities of D-glucose at K^+ equilibrium are estimated. Since the uptake rate of a cotransport system is dependent on, among other things, the permeability properties of a membrane, not only the intial uptake rate in the presence of a Na^+ gradient but also the maximal amount of the Na^+-gradient-dependent D-glucose content of the liposomes was used as a criterion to optimize the reconstitution.

To further simplify and optimize the reconstitution system, a series of experiments were carried out in which the liposomes synthesized from cholesterol, sphingomyelin, PE and PS, which showed a low apparent passive permeability and a good initial Na^+-gradient-dependent D-glucose uptake rate, were compared with liposomes formed from only three of these lipids. If liposomes were synthesized without PS or PE, only an insignificant Na^+-gradient-dependent D-glucose uptake was observed (Fig. 2). D-Glucose uptake, measured after equilibrium had been reached, was the same for the four lipid control which contained shingomyelin and for liposomes synthesized from cholesterol, PE and PS. Although the apparent passive permeability of liposomes synthesized without sphingomyelin is higher than for the control (inset, Fig. 2), the initial rate of the Na^+-dependent D-glucose uptake showed an even greater increase relative to the control. Since this

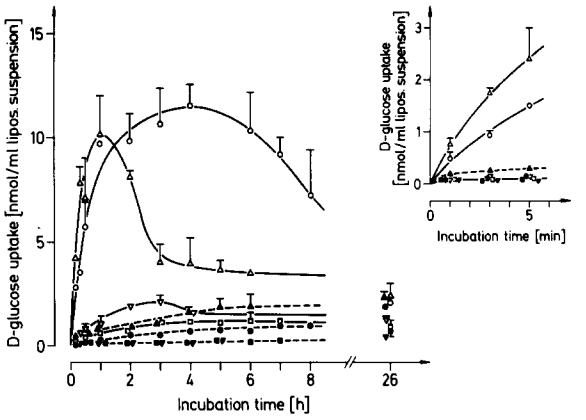


Fig. 2. A comparison of D-glucose uptake into liposomes formed from cholesterol and either three or two different phospholipids. A preparation of brush-border membrane proteins was reconstituted in liposomes as described in Materials and Methods. The total amount of lipid added (11 mg) and the weight ratios of the different lipid components to each other, which were the same as in Fig. 1, were kept constant. The following lipid combinations were employed for the liposome formation: control, cholesterol, PE, PS, sphingomyelin (●, ○); without PE: cholesterol, PS, sphingomyelin (■, □); without PS: cholesterol, PE, sphingomyelin (▼, ▽); without sphingomyelin: cholesterol, PE, PS (▲, △). The protein concentrations in the different liposomal suspensions were 0.22, 0.20, 0.14 and 0.41 $mg \cdot ml^{-1}$, respectively. Liposomes were preincubated with $2 \cdot 10^{-8}$ M valinomycin, and D-glucose uptake was measured at K^+-equilibrium (closed symbols) and in the presence of an inward directed Na^+ gradient (open symbols) as described in Fig. 1. The inset indicates the D-glucose uptake within the first 5 min after initiation of incubation.

increase in the initial Na^+-gradient dependent D-glucose uptake was also observed in the previous series of experiments after removal of sphingomyelin (Fig. 1), this lipid was left out in subsequent experiments.

Finally, reconstitution of the D-glucose transporter was attempted by using only two lipids. In these experiments, liposomes formed from cholesterol, PS and PE served as control. When liposomes were formed from cholesterol and PE, only an insignificant Na^+-gradient-dependent D-glucose uptake was observed (Fig. 3). If cholesterol and PS were used, a low apparent passive permeability to D-glucose (inset, Fig. 3), and approximately the same initial Na^+-gradient-dependent D-glucose uptake was observed as with the vesicles formed from cholesterol, PS and PE. However, the cholesterol/PS liposomes showed a much greater

permeation occurs via incorporated proteins. A correlation between the total amount of protein incorporated into liposomes (without PC, 0.31 $mg \cdot ml^{-1}$ < without PE, 0.32 $mg \cdot ml^{-1}$ < control, 0.40 $mg \cdot ml^{-1}$ < without sphingomyelin, 0.46 $mg \cdot ml^{-1}$) and their apparent passive permeability seems to be present.

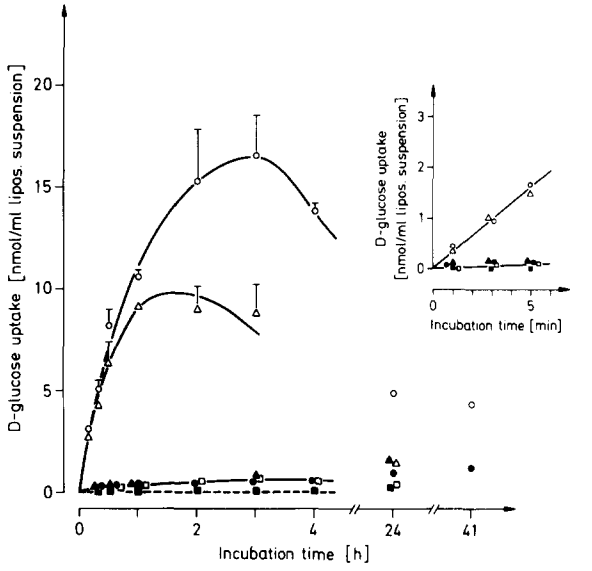


Fig. 3. A comparison of D-glucose uptake into liposomes formed from cholesterol and either two phospholipids or one phospholipid. A preparation of brush-border membrane proteins was reconstituted into liposomes as described in Methods. The total amount of lipid added (10 mg) and the weight ratios of the different lipids, which were the same as in Figs. 1 and 2, were kept constant. For the liposome formation, the following lipid combinations were used: cholesterol, PE, PS (▲, △); cholesterol, PE (■, □); cholesterol, PS (●, ○). The protein concentrations in the different liposomal suspensions were 0.45, 0.13, 0.38 mg·ml^{-1}, respectively. Liposomes were preincubated with $2 \cdot 10^{-8}$ M valinomycin, and D-glucose uptake was measured at K^+ equilibrium (closed symbols) or in the presence of an inward-directed Na^+ gradient (open symbols) as described in Fig. 1. The inset indicates the D-glucose uptake within the first 5 min after initiation of incubation.

transient Na^+-gradient-dependent D-glucose accumulation over the equilibrium value than the control. When attempts were made to form liposomes from only the solubilized brush-border membrane proteins and their associated lipids (0.4 mg phospholipid and 0.2 mg cholesterol per mg protein), or by adding only cholesterol to the solubilized brush-border membrane protein solution, no Na^+-gradient-dependent D-glucose uptake was observed (data not shown). This indicates that addition of extraneous phospholipids is necessary to form liposomes in which the Na^+/D-glucose cotransport system can be successfully reconstituted under these conditions. When liposomes were synthesized from PC and cholesterol, the same apparent passive permeability to D-glucose (proportional to the rate of D-glucose uptake at K^+-equilibrium), but only about half the Na^+-gradient dependent D-glucose uptake was observed as with the cholesterol/PS liposomes.

When identical experiments in different series were compared, a considerable degree of variation in the initial Na^+-dependent D-glucose uptake rate or the height of the overshoot was sometimes observed (compare, for example, the results with liposomes formed from cholesterol, PE, PS, sphingomyelin in Figs. 1 and 2). However, experiments which were performed with the same batch of protein and lipids, were highly reproducible (data not shown). There are several possible reasons for the discrepancies in the results obtained with different liposomal preparations, assuming there are no variations in activity of the transport protein: different amounts of the D-glucose transporter may have been incorporated which could, as described below, be due to differences in the protein concentration during reconstitution or variations in liposome formation may have been introduced by using different sources of lipid. After analysis of our PS by thin-layer chromatography, it was found that some samples contained lysophosphatidylserine (lyso-PS) contamination. To determine if the presence of lyso-PS could effect the transport of D-glucose into liposomes, an experiment was performed using increasing amounts of lyso-PS to replace pure PS in cholesterol/PS liposomes. There was an inverse relationship between the amount of lyso-PS employed for reconstitution and how well the liposomes transported and/or retained D-glucose (data not shown). Liposomes formed from cholesterol and pure PS showed the best transport. When lyso-PS totally replaced PS, almost no Na^+-dependent D-glucose uptake was observed. The uptake of D-glucose in the presence of K^+ equilibrium was not significantly different in any of the groups tested. However, since variations in the results were sometimes still observed when different PS samples without lyso-PS contamination were employed, lyso-PS cannot be the only factor which effects the reproducibility of transport study results when different sources of PS are used for reconstitution. Possibly, differences in fatty acid composition constitute the reason for these observations. This assumption is supported by preliminary experi-

TABLE I

COMPOSITION OF LIPOSOMES SYNTHESIZED WITH INCREASING AMOUNTS OF CHOLESTEROL

Liposomes were formed by adding 1 ml of protein solution which contained 3.9 mg protein, 1.8 μmol cholesterol (Chol) and 1.9 μmol phospholipids (PL) to 1 ml of lipid suspension containing 6 μmol PS and 12, 6, 4, or 1.5 μmol cholesterol. In the table, the amount of lipid and protein which became associated with the liposomes is indicated.

Components present during liposome formation				Composition of the liposomes			
Protein (mg)	Chol (μmol)	PL (μmol)	Chol/PL molar ratio	protein (mg)	Chol (μmol)	PL (μmol)	Chol/PL molar ratio
3.9	13.8	7.9	1.7	1.1	7.2	3.1	2.3
3.9	7.8	7.9	0.99	0.77	4.0	3.4	1.2
3.9	5.8	7.9	0.73	0.63	2.3	3.8	0.6
3.9	3.3	7.9	0.42	0.56	1.2	2.3	0.52

ments in which liposomes were formed from cholesterol and synthetic PC with different types of fatty acids (data not shown).

Reconstitution experiments with liposomes formed from PS and different amounts of cholesterol

After successful reconstitution of brush-border membrane proteins into liposomes formed from only cholesterol and PS, experiments were carried out to optimize the PS/cholesterol ratio for reconstitution. The experimental conditions are given in Table I. The molar ratios of the cholesterol/PS mixtures which were added to the protein solution were 2.0, 1.0, 0.67 and 0.25. However, since the solution already contained cholesterol and phospholipids (PL), the actual molar cholesterol/PL ratios were slightly different (Table I). After reconstitution, the liposomes were analyzed for their protein, cholesterol and PL content. Table I shows that the cholesterol/PL ratios employed for liposome formation were similar to those found in the liposomes. Although the types of protein were identical (data not shown), the amount of protein which was associated with the different liposomes decreased with decreasing cholesterol concentrations. Fig. 4 shows that a decrease in cholesterol content of liposomes leads to a reduced onset, height and duration of the overshoot. At a cholesterol/PS ratio of 0.25, no overshoot and only a minor Na^+-gradient-dependent D-glucose uptake was observed. The observation that the overshoot was higher and lasted longer when the liposomes had a higher cholesterol content suggested that the inward directed Na^+ gradient persisted longer in these liposomes. This was directly demonstrated in experiments where the uptake of radiolabeled Na^+ into liposomes was measured (data not shown). The uptake of Na^+ into liposomes with a cholesterol/PS ratio of 0.25 reached equilibrium after minutes, whereas in liposomes formed from a cholesterol/PS-ratio of 2.0 the equilibrium was reached only after more than 7 h.

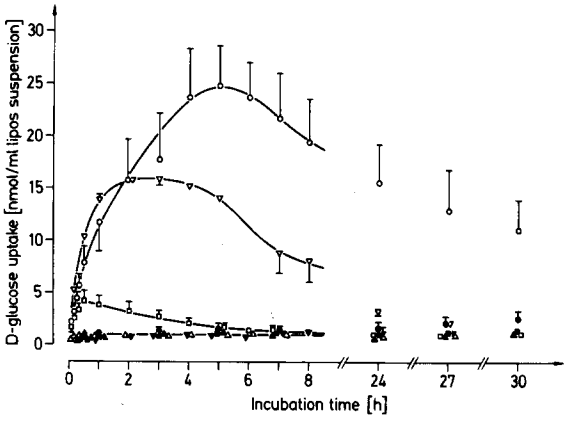


Fig. 4. A comparison of the uptake of D-glucose into PS/cholesterol liposomes containing different amounts of cholesterol. A membrane protein preparation was incorporated into liposomes which were formed from a constant amount of PS and different amounts of cholesterol. The molar cholesterol to PS ratios of the added lipids were 2.0 (●, ○), 1.0 (▼, ▽), 0.67 (■, □) and 0.25 (▲, △). Reconstitution was performed as described in Materials and Methods, and experimental conditions are given in detail in Table I. The liposomes were preincubated with $2 \cdot 10^{-8}$ M valinomycin, and D-glucose uptake was measured at K^+ equilibrium (closed symbols) or in the presence of an inward-directed Na^+ gradient (open symbols) as described in Fig. 1.

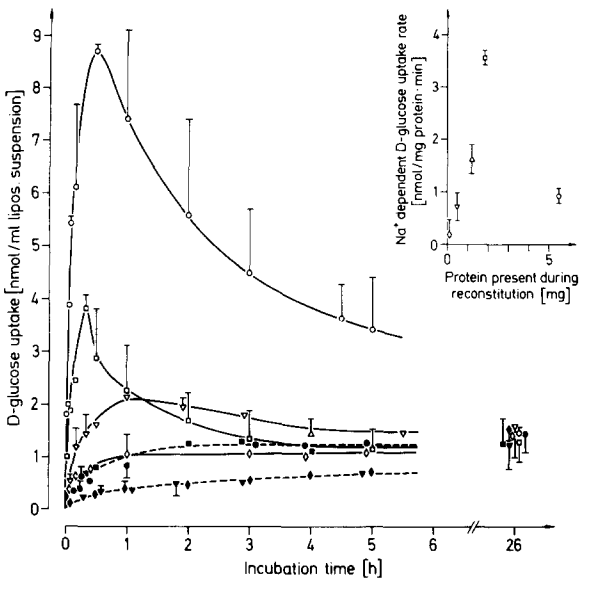


Fig. 5. Effects of changes in the protein concentration during reconstitution on D-glucose uptake. Liposomes were formed from cholesterol and PS (cholesterol/PS molar ratio, 0.67) and reconstitution was carried out in the presence of different concentrations of protein from the same membrane protein preparation. The concentrations of protein present during reconstitution and the protein concentrations in the liposomal suspensions after reconstitution (given in parenthesis) were: (●, ○) 5.50 (1.89); (■, □) 1.85 (0.23); (▼ ▽) 0.50 (0.18) and (◆, ◇) 0.11 (0.16) mg·ml^{-1}. Liposomes were preincubated with 2·10^{-8} M valinomycin, and D-glucose uptake was measured at K^+ equilibrium (closed symbols) or in the presence of an inward-directed Na^+ gradient (open symbols) as described in Fig. 1. In the inset, the initial Na^+-gradient-dependent D-glucose uptake rate per mg liposomal protein is plotted against the protein concentration present during reconstitution. The uptake rate from another experiment in this series is included, which, for sake of clarity, was left out from the main graph.

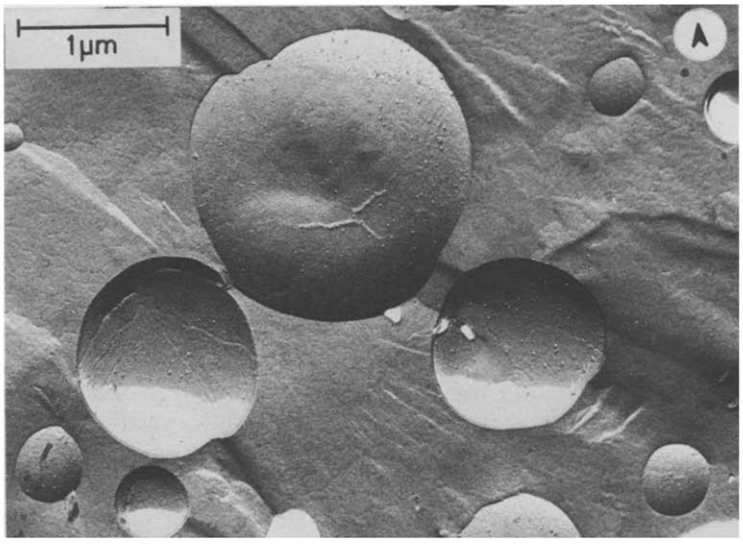
Reconstitution experiments with different amounts of proteins

Fig. 5 shows the effect on D-glucose uptake when reconstitution of proteins into cholesterol/PS liposomes (molar cholesterol/PS ratio 0.67) was carried out at different protein concentrations. Although the protein concentration was varied from 0.11 to 5.5 mg · ml^{-1} during reconstitution, equilibrium values were not significantly different for any of the liposomes. The amount of protein associated with liposomes (0.16–0.23 mg per ml liposomal suspension) remained relatively constant when the protein concentration during reconstitution was increased from 0.11 to 1.85 mg · ml^{-1}. However, initial Na^+-gradient-dependent D-glucose uptake rates per mg protein increased significantly (inset Fig. 5). If the protein concentration during reconstitution was 5.5 mg · ml^{-1}, the amount of protein associated with the liposomes increased substantially and the initial uptake rate per mg protein was therefore decreased. The most probable explanation for these findings is that within a certain range of protein concentration there is a specific amount of protein incorporated into liposomes and that this incorporation becomes selective for the D-glucose transporter as the concentration of protein is increased. This was substantiated in separate experiments using SDS-gel electrophoresis [19].

Properties of the reconstituted proteoliposomes

From the various reconstitution experiments that had been carried out, a reconstitution procedure was selected for subsequent experiments [19] in which reconstitution was performed at a protein concentration of approx. 2 mg · ml^{-1} and liposomes were formed from 6 μmol cholesterol and 6 μmol PS. Some properties of these liposomes and some characteristics of the reconstituted Na^+/D-glucose cotransporter are described. When the liposomes were observed in the electron microscope after spray-freezing and freeze-fracturing it was found that nearly 70% of the liposomes had a diameter of between 0.2 and 0.6 μm. The fraction of monolamellar liposomes was estimated to be greater than 50%. The particles observed on the fracture faces of the membrane, which were only seen when protein was present during reconstitution, appear to represent incorporated proteins (Fig. 6). When the proteins associated with reconstituted liposomes were analyzed (Fig. 7), it was found that there was an enrichment of certain proteins while other proteins were present in lower concentrations or could not be detected when compared to intact brush-border membranes. Since all membrane proteins were solubilized and could be detected after deoxycholate removal (Fig. 7, gel b), some protein selection during incorporation into liposomes had occurred.

In Fig. 8 some of the properties of the reconstituted Na^+/D-glucose cotransporter are shown. As was previously observed, the D-glucose uptake



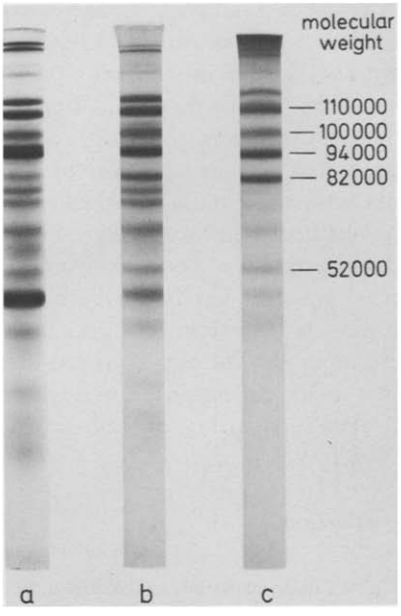


Fig. 6. Electron micrograph of freeze-fractured proteoliposomes containing the reconstituted Na^+/D-glucose cotransporter. Solubilized brush-border membrane proteins were reconstituted into liposomes which were formed from cholesterol and PS (molar ratio 1.0) as described in Materials and Methods. The liposomes were equilibrated at 37°C and then rapidly frozen, freeze-fractured, shadowed and digested (see Methods).

Fig. 7. SDS-polyacrylamide gel electrophoresis of (a) intact brush-border membranes, (b) solubilized brush-border membrane proteins after deoxycholate removal by a DEAE column and (c) reconstituted proteoliposomes which were formed from (b) and from cholesterol and PS as described in Fig. 6. 25 μg of protein were applied to each gel.

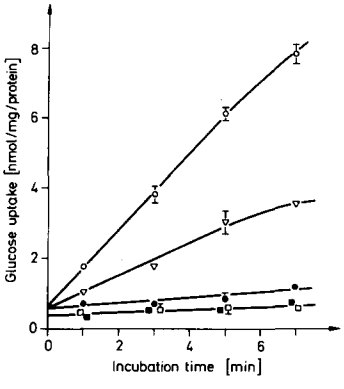


Fig. 8. Phlorizin inhibition and stereospecificity of the reconstituted Na^+/D-glucose cotransporter. Solubilized deoxycholate-free membrane proteins were reconstituted into liposomes formed from cholesterol and PS as described in Fig. 6. After the proteoliposomes, which contained buffer 2, were preincubated with $2 \cdot 10^{-8}$ M valinomycin, they were incubated with either 0.2 mM radiolabeled D-glucose (●, ▽, ○) or with 0.2 mM radiolabeled L-glucose (■, □). The incubation was carried out as described in Fig. 1 at either K^+ equilibrium (●, ■) or in the presence of a 100 mM inward-directed Na^+ gradient in the absence (○, □) or presence (▽) of 20 μM phlorizin. All uptake measurements were corrected for D-[^{3}H]glucose binding to filters. Initial binding of glucose to proteoliposomes was estimated by extrapolating the uptake curve to time zero. The protein concentration in the liposomal suspension was 0.57 $mg \cdot ml^{-1}$.

in the presence of a Na^+ gradient is significantly greater than that observed at K^+ equilibrium. The Na^+-gradient-dependent D-glucose uptake was linear between 1 and approx. 5 min, with an uptake rate of 1 nmol/mg protein per min. In the presence of 20 μM phlorizin, the Na^+-gradient-dependent D-glucose uptake was inhibited by approx. 60%, demonstrating that the sensitivity to phlorizin inhibition is preserved after reconstitution. In these proteoliposomes, Na^+-dependent high-affinity phlorizin binding was also observed (data not shown). The stereospecificity of the Na^+/D-glucose cotransporter was preserved after reconstitu-

tion, since L-glucose uptake was, in contrast to that of D-glucose, not stimulated by a Na^+ gradient. D-Glucose uptake into these liposomes was also shown to be rheogenic, since it was stimulated by an inside-negative diffusion potential of K^+ generated by the addition of valinomycin (data not shown). Finally, it should be stated that the reconstituted liposomal system is appropriate to demonstrate Na^+/D-glucose cotransport. Since the Na^+ gradient persists over hours in these liposomes, a transient Na^+-gradient-dependent increase in the intraliposomal D-glucose concentration over the equilibrium value can be demonstrated even with preparations with low transport activity.

Discussion

Solubilization of membrane proteins and their incorporation into liposomes to assay their activity has become a frequently used tool for purifying active transport proteins. The successful reconstitution of various transport proteins has led, in some cases, to a subsequent protein purification [30,31], indicating the general usefulness of liposomal systems. Although the liposomal systems which have been described up to now have been relatively undefined, reconstitution could still be successfully carried out, in many cases, if the transport protein had a high turnover rate and was present in high enough concentrations [32,33] and/or if appropriate experimental conditions were found to measure the transport activity over a long enough time period.

In the case of the kidney or intestinal Na^+/D-glucose cotransporter, however, since the transport system makes up only a minor fraction of the membrane proteins and the transport activity is coupled to a transmembraneous Na^+ gradient, successful reconstitution has been difficult to demonstrate.

Considering the difficulties in reconstituting Na^+/D-glucose cotransport, a detailed study of the conditions necessary for reconstitution and assaying Na^+/D-glucose cotransport activity in liposomes seemed warranted. Initially, the major five lipid components of the brush-border membranes were used for liposome formation. After appropriate conditions for reconstitution and assaying the Na^+/D-glucose cotransport activity were found with these lipids, further attempts were made to simplify and improve the reconstitution by varying the composition of the liposomes. It was found that an effective reconstitution could be carried out in liposomes made only from cholesterol and phosphatidylserine. This binary lipid system was considered an important step in obtaining reproducible results, since the number of variables had been reduced.

In the reconstituted system described here, Na^+/D-glucose cotransport was routinely demonstrated. Since the liposomes are very impermeable to both Na^+ and D-glucose, a Na^+ gradient can persist for hours across the liposomal membranes and serve as a driving force for D-glucose uptake. A linear rate of D-glucose uptake is observed for minutes, suggesting that over this time period the Na^+ gradient is invariable enough to produce a constant rate of D-glucose uptake. The Na^+-gradient-dependent D-glucose uptake rate measured in the presence of 0.2 mM D-glucose is 1 nmol per mg protein per min. This is about 3.7-times higher than the estimated uptake rate reported by Im and co-workers [14] for proteoliposomes in which a 7-fold purification of the D-glucose transporter was reported (D-glucose concentration 0.1 mM).

A comparison of the absolute Na^+ gradient uptake rates measured in our proteoliposomes with the uptake rates measured by Lin and coworkers [17], who reported a 20- to 30-fold purification of the Na^+/D-glucose cotransporter, is not possible. In this paper the D-glucose uptake in the presence of a 150 mM NaSCN gradient is compared to the D-glucose uptake at KCl equilibrium. Since we have found that SCN^- increases the nonspecific D-glucose permeability of liposomes at K^+ equilibrium, it is possible that the D-glucose uptake data reported by these authors do not represent the specific activity of the Na^+/D-glucose cotransporter, but may be partially due to increased permeability of liposomes in the presence of SCN^-. Furthermore, if the Na^+ to K^+ D-glucose uptake ratios of the proteoliposomes synthesized from both the crude and purified membrane proteins are compared, no significant difference is noted. This suggests that no distinct purification of the Na^+/D-glucose cotransport activity had occurred.

In our proteoliposomes, all properties of the

Na^+/D-glucose cotransporter described in brush-border membrane vesicles were also found. These are: (i) the dependency of D-glucose uptake on the Na^+ gradient; (ii) the stimulation of D-glucose uptake by an inside-negative diffusion potential; (iii) stereospecificity for D-glucose; and (iv) observation of high-affinity phlorizin inhibition and binding. Not only are the proteoliposomes described in this paper well suited to reconstitute Na^+/D-glucose cotransport, they have also been found to be well suited to reconstitute and analyze other Na^+-cotransport systems from brush-border membranes. Properties of the reconstituted Na^+/D-alanine and Na^+/L-glutamic acid cotransport systems are presently being investigated (Korn, Ferguson, Menuhr and Koepsell, unpublished data).

In spite of the nonuniform size distribution of liposomes, the described liposomal system can also be successfully used as an assay system during purification of a specific Na^+-cotransport system provided that the reconstitution and the transport assay are performed under identical experimental conditions. In particular, the protein concentrations during reconstitution should be similar in the different experiments and only the initial, linear rates of the Na^+-gradient-dependent D-glucose uptake should be compared. The establishment of the reported reconstitution system is considered an important step in identifying and purifying Na^+-cotransport systems from brush-border membranes.

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References

1 Kleinzeller, A., Kolinska, J. and Benes, I. (1967) Biochem. J. 104, 852–860
2 Silverman, M., Aganon, M.A. and Chinard, F.P. (1970) Am. J. Physiol. 218, 735–742
3 Ullrich, K.J., Rumrich, G. and Klöss, S. (1974) Pflügers Arch. 351, 35–48
4 Aronson, P.S. and Sacktor, B. (1974) Biochim. Biophys. Acta 356, 231–243
5 Hopfer, U., Nelson, K., Perrotto, J. and Isselbacher, K.J. (1973) J. Biol. Chem. 248, 25–32
6 Kinne, R., Murer, H., Kinne-Saffran, E., Thees, M. and Sachs, G. (1975) J. Membrane Biol. 21, 375–395
7 Murer, H. and Hopfer, U. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 484–488
8 Murer, H., Hopfer, U., Kinne-Saffran, E. and Kinne, R. (1974) Biochim. Biophys. Acta 345, 170–179
9 Hosang, M., Michael Gibbs, E., Diedrich, D.F. and Semenza, G. (1981) FEBS Lett. 130, 244–248
10 Hosang, M., Vasella, A. and Semenza, G. (1981) Biochemistry 20, 5844–5854
11 Crane, R.K., Malathi, P. and Preiser, H. (1976) FEBS Lett. 67, 214–216
12 Fairclough, P., Malathi, P., Preiser, H. and Crane, R.K. (1979) Biochim. Biophys. Acta 553, 295–306
13 Ferguson, D.R. and Burton, K.A. (1977) Nature 265, 639–642
14 Im, W.B., Ling, K.Y. and Faust, R.G. (1982) J. Membrane Biol. 65, 131–137
15 Kinne, R. and Faust, R.G. (1977) Biochem. J. 168, 311–314
16 Koepsell, H., Menuhr, H., Wissmüller, T.F., Ducis, I. and Haase, W. (1980) Ann. N.Y. Acad. Sci. 358, 267–281
17 Lin, J.T., Da Cruz, M.E.M., Riedel, S. and Kinne, R. (1981) Biochim. Biophys. Acta 640, 43–54
18 Booth, A.G. and Kenny, A.J. (1974) Biochem. J. 142, 575–581
19 Koepsell, H., Menuhr, H., Ducis, I. and Wissmüller, T.F. (1983) J. Biol. Chem. 258, 1888–1894
20 Bray, G.A. (1960) Anal. Biochem. 1, 279–285
21 Bachmann, L. and Schmitt, W.W. (1971) Proc. Natl. Acad. Sci. U.S.A. 68, 2149–2152
22 Haase, W. and Koepsell, H. (1979) Pflügers Arch. 381, 127–135
23 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
24 Koepsell, H. (1979) J. Membrane Biol. 48, 69–94
25 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4411
26 Folch, J., Lees, M. and Stanley, G.H.S. (1957) J. Biol. Chem. 226, 497–509
27 Eibl, H. and Lands, W.E.M. (1970) Anal. Biochem. 33, 58–66
28 Clark, B.R., Rubin, R.T. and Arthur, R.J. (1968) Anal. Biochem. 24, 27–33
29 Singleton, W.S., Gray, M.S., Brown, M.L. and White, J.L. (1965) J. Am. Oil Chem. Soc. 42, 53–56
30 Kasahara, M. and Hinkle, P.C. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 396–400
31 Kasahara, M. and Hinkle, P.C. (1977) J. Biol. Chem. 252, 7384–7390
32 Hilden, S., Rhee, H.M. and Hokin, L.E. (1974) J. Biol. Chem. 249, 7432–7440
33 Meissner, G. and Fleischer, S. (1974) J. Biol. Chem. 249, 302–309