RECONSTITUTION OF SPECIFIC Na^+-DEPENDENT D-GLUCOSE TRANSPORT IN LIPOSOMES BY TRITON X-100 EXTRACTED PROTEINS FROM PURIFIED BRUSH BORDER MEMBRANES OF HAMSTER SMALL INTESTINE

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Summary Liposomes were prepared from soybean phospholipids and were sonicated together with Triton X-100 extracted proteins from purified brush border membranes isolated from hamster small intestine. The treated liposomes showed a specific Na^+-dependent uptake of D-glucose as compared to L-glucose, which was inhibited by phlorizin and D-galactose.

Introduction In 1960, Crane et al. (1) proposed that active sugar transport at the brush border membrane of the animal small intestine is mediated by a carrier which binds both Na^+ and substrate to form a ternary complex which can respond to the transmembrane electrochemical potential gradient. Over the years, numerous studies with intact tissue (2) and vesiculated membrane preparations (3,4) have provided substantial support for the proposal which has come to be called, generally, the Gradient Hypothesis. However, such studies have been largely phenomenological and studies at a molecular level have been tentative at best. In order to proceed with studies of gradient-coupled carrier function at a molecular level it has been necessary to devise a means to study the isolated molecule in a defined system. We wish to report our first successful experiments in this direction. We have

reconstituted Na^+-dependent D-glucose transport in liposome vesicles by the addition of Triton X-100 extracted proteins from purified brush border membranes of hamster small intestine.

Materials and Methods D-[U-^{14}C] glucose (200 m Ci/m mole), L-[1-^{3}H(N)] glucose (17.46 Ci/m mole), D-[1-^{14}C] mannitol (47.6 m Ci/m mole) and D-[1-^{3}H(N)] mannitol (2.65 Ci/m mole) were purchased from New England Nuclear Corp. Crude soybean phospholipids (Asolectin) was obtained from Associated Concentrates, Woodside, N.Y., and freed of neutral lipids by acetone extraction as described by Kagawa and Racker (5). Triton X-100 was from J. T. Baker & Co. and Bio-Beads SM2 from Bio-Rad Laboratories. All other chemicals and materials were from commercial sources and of known purity. Male hamsters (9-10 weeks) were obtained from Charles River Breeding Labs. Inc.

Purified brush border membranes were prepared according to Schmitz et al. (6) and suspended in a buffer containing 0.1 M KCl, 5 mM HEPES-Tris (pH 7.5), 1 mM dithiothreitol, 0.1 mM $MgSO_4$. Triton X-100 (1% final concentration) was added to the suspension which was allowed to stand for 60 min at 4° and centrifuged for 30 min at 30,000 xg. Under these conditions, 70-75% of the membrane proteins solubilized. To remove Triton X-100, the supernatant was passed through a column of Bio-Beads (1 g/ml) equilibrated with the above mentioned buffer.

The method described by Racker (7) was used for reconstitution with some modifications. The soybean phospholipids, dried in vacuo and kept under dry N_2, were suspended in buffer, kept flushed with N_2, and sonicated for 5 min at 15-30° (Biosonik, Generator model, 120 W, 20 KHz) to give a clarified suspension of 50 mg/ml phospholipid. The solubilized membrane fraction was added to the liposomes (25 mg/ml phospholipid and 1-2 mg/ml protein) and the mixture further sonicated for 5 min under a stream of N_2, at 15-30°. The resultant mixture was centrifuged at 100,000 x g for 90 min. The pellet thus obtained was resuspended in a small volume of buffer and tested for transport properties.

Sugar transport was measured by adding an aliquot (50 µl) of the suspended liposomes into appropriately constituted buffers containing the radioactive (^{14}C or ^{3}H) sugars in a final volume of 350 µl. Aliquots (50 µl) were removed at intervals and diluted into 1 ml of cold stopping solution (0.154 M NaCl + 0.5 mM phlorizin at 4°) containing tracer amounts of radioactive (^{3}H or ^{14}C) mannitol which served as the extravesicular marker. The mixture was rapidly filtered through 0.3 µm millipore filters and washed with 5 ml of the ice-cold stopping solution. The filters were removed to counting vials and the radioactivity measured in a Beckman LS counter.

Protein was assayed according to Lowry et al. (8).

Results and Discussion Liposomes reconstituted with extracted proteins in 0.1 M KCl buffer, showed time-dependent non-specific uptake of both D- and L-glucose from a 0.1 M KCl medium (Fig. 1). Protein-free liposomes showed a similar non-specific but greatly reduced (25-30%) uptake characteristic. Extracted proteins subjected to the procedures involved in reconstitution but without phospholipids showed no uptake.

In order to better understand the experiments done, it may be recalled that Na^+-dependent glucose uptake by intact cells (2) and by membrane vesicles (4) is electrogenic. That is, Na^+ and sugar bind to the carrier and move into the cell without a counterion. With intact cells, entering sodium may be removed by the sodium pump or may exchange with intracellular potassium. With membrane vesicles which lack the pump and do not exchange cations readily (4) Na^+-dependent substrate entry can best be observed under experimental conditions where Na^+ movement is induced by movement of an anion in the same direction or a cation in the opposite direction (4). The same is true with liposomes vesicles. Thus, when inward Na^+ movement is induced by addition of the lipophilic anion, SCN^-, it can be seen from Fig. 1 that substantial specific entry of D-glucose also occurs. Under the same condition the entry of L-glucose is not appreciably affected.

Phlorizin, a powerful, competitive inhibitor of glucose transport by intestinal epithelial cells (9,10) substantially inhibited sugar transport by reconstituted liposomes in the presence of NaSCN. Phlorizin had little effect in the absence of Na^+. Galactose, which shares the glucose active

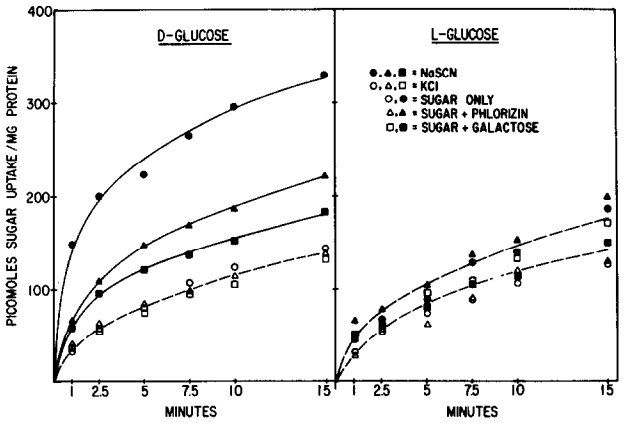

Figure 1. Triton X-100 extracted membrane proteins were reconstituted into soybean phospholipid liposomes in 0.1 M KCl - 5 mM Hepes-Tris buffer containing 1 mM dithiothreital and 0.1 mM $MgSO_4$, as described in the text. Incubation was at room temperature ($\approx$ 25°) in a total volume of 350 µl containing 0.1 mM D-[U-^{14}C] glucose or 0.1 mM L-[1-^{3}H(N)]-glucose, 0.1 M KCl or NaSCN, 5 mM Hepes-Tris (pH 7.5) and 0.1 mM $MgSO_4$. The reaction was started by the addition of 50 µl of reconstituted liposomes. When present, phlorizin was 5 mM and D-galactose, 10 mM.

transport system in the intestine (9), also inhibited glucose transport in the presence of Na^+ (Fig. 1).

We believe that these data indicate that reconstitution of the Na^+-dependent glucose transport system of hamster small intestine has been achieved. Similar results have been obtained from rabbit kidney cortex brush border membranes (11).

The use of total Triton X-100 extractable proteins for these experiments was dictated by the fact that separation of the membrane entity responsible for Na^+-dependent entry from other membrane elements has had to wait upon the development of a suitable assay system. The demonstration of

reconstitution described above is equally a demonstration that an assay system has been achieved.

In the experiments in Fig. 1, the excess entry of D-glucose in the presence of Na^+ is maintained over the time tested. We have not tested whether the same equilibrium is reached with D-glucose in the presence and absence of sodium. Movement of sugar against a gradient has not yet been achieved and this may suggest that there may be volume changes in the liposomes induced by D-glucose entry.

Since sugar and Na^+ bound to the carrier are transferred simultaneously across the membrane, attempts were made to test Na^+ transport in the presence of sugar. A very large Na^+ uptake was observed with the reconstituted liposomes which was not significantly altered in the presence of D-glucose. We suspect that the use of a heterogeneous protein extract has probably reconstituted a number of other transport systems, not yet tested for, which may provide substantial "leaks" of Na^+. In fact, our preliminary data indicate a Na^+-dependent uptake of L-alanine by these reconstituted liposomes.

Some of the brush border hydrolases are incorporated into the liposomes along with the D-glucose transport activity. Consequently, the question may be raised that D-glucose enters by binding to a hydrolase and undergoing translocation in a form of Hydrolase Related Transport (HRT) (12,13). This is highly improbable for the following reasons. First, experiments on HRT indicate that it is not a significant route for the entry of free glucose. Second, HRT is not dependent on the presence or the coflux of Na^+. Third, phlorizin has no appreciable effect on

hydrolase activity or on HRT. Fourth, $Tris^+$ is a potent, competitive inhibitor of several disaccharidases (14-16) and the concentration used for buffering in these experiments is high enough (3.3 mM) to cause substantial inhibition of glucose (0.1 mM) binding to the active sites of these enzymes.

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