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RECONSTITUTION INTO LIPOSOMES OF GLUCOSE ACTIVE TRANSPORT FROM THE RABBIT RENAL PROXIMAL TUBULE

CHARACTERISTICS OF THE SYSTEM

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Summary

This paper describes the characteristics of Na^+-dependent D-glucose transport into liposomes made from soybean phospholipids into which have been reconstituted detergent-solubilized components from the rabbit renal proximal tubular brush border membrane. Conditions for optimal and quantitative reconstitution of glucose carriers are defined. Na^+-dependent D-glucose uptake occurs via a saturable system with a K_m of 0.125—0.135 mM, is responsive to the volume of the internal liposomal space, and shows 'overshoot' as seen in natural membranes. The rate of Na^+-dependent D-glucose uptake and the magnitude of the 'overshoot' are proportional to the concentration of protein used in reconstitution.

Introduction

The molecular basis of transport of D-glucose and other actively transported organic solutes against an electrochemical gradient in kidney and intestinal epithelia is at present unknown. The current concept of the mechanism is that active sugar transport at the luminal membrane of these epithelial cells is mediated by a mobile carrier able to bind both Na^+ and substrate to form a ternary complex, which can simultaneously translocate ion and substrate into the cell in response to the transmembrane electrochemical gradient of Na^+ [1].

We are currently trying to purify the 'carrier' responsible for glucose active transport from the renal brush border membrane in order to understand how

Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

it operates. We have previously reported brief and preliminary data on reconstitution into artificial phospholipid vesicles of Na^+-dependent, stereospecific, phlorizin inhibitable transport of D-glucose by incorporation of Triton X-100 extractable elements of brush borders from rabbit kidney [2] and hamster small intestine [3]. The primary purpose of this effort was to establish an assay to assess our progress toward purification of the 'carrier' and to allow study of the mode of its operation in a defined artificial system. This paper communicates our now more complete understanding of the reconstituted system.

Materials and Methods

I. Chemicals. D-[U-^{14}C]glucose (310 Ci/mol) was obtained from New England Nuclear Corporation. Crude soybean phospholipids (Asolectin; Associated Concentrates, Woodside, NY) were washed with acetone to remove neutral fats as described by Kagawa and Racker [4] dissolved in chloroform and reprecipitated with acetone. The precipitate was collected and stored under acetone at −20°C until required. Triton X-100 was bought from Mallinckrodt and Bio Beads SM-2 from Bio Rad Laboratories. Other chemicals were of the highest available grade.

II. Detergent extraction of brush border membranes. Brush border membranes were prepared from the saline-perfused kidneys of male New Zealand white rabbits by a modification of the method of Schmitz et al. [5] as described [6].

A number of detergents, viz. sodium cholate, sodium deoxycholate, Sarkosyl NL97 (Ciba-Geigy), lubrol, sodium dodecyl sulfate (SDS) and Triton X-100 were tested for their ability to solubilize membrane components. The index of solubilization was taken to be what remained in the supernatant after centrifugation for 1 h at $1 \cdot 10^5 \times g$. Excepting sodium cholate all the detergents tested (at a concentration of 1%) solubilized more than 80% of the membrane proteins. However, only the bile salts and Triton X-100 could be efficiently and quickly removed from the solubilized mixture; bile salts could be removed on an anion exchange resin column (AG 1 X 8, Biorad) and Triton X-100 on a Bio-Bead SM-2 column as described by Holloway [7]. We have chosen to use Triton X-100 in our experiments.

The solubilization procedure is as follows: Triton X-100 (10% in water) was added to the brush border membrane fraction from the kidneys of one rabbit suspended in 9 ml of standard buffer (0.1 M KCl/5 mM HEPES-Tris (pH 7.5) and 1 mM dithiothreitol), to a final concentration of 1% (w/v), and the mixture sonicated at 4°C for one minute using a Bronwill Biosonik sonicator at maximum power. Insoluble components were sedimented by centrifugation at $1 \cdot 10^5 \times g$ for 60 min. The supernatant routinely contained more than 85% of the total membrane protein. Triton X-100 was removed from the supernatant fraction on a 2 X 25 cm column of Bio-Beads SM-2, equilibrated with standard buffer. The column was eluted with standard buffer at 40 ml/h. More than 97% of the Triton X-100 applied to the column was retained by the Bio Beads (estimated using ^{3}H-labelled Triton X-100, a gift to Dr. J. Lenard of this department from Rohm and Haas). Protein recovery was 95 to 100%. The turbid eluate containing protein was centrifuged at $2 \cdot 10^5 \times g$ for 60 min. The

resultant pellets containing the Triton-soluble membrane components were used for reconstitution.

III. Formation of liposomes and reconstitution. Liposomes were prepared essentially as described by Racker [8]. Purified soybean phospholipids were dried under vacuum. Standard buffer was added to the dry lipid to yield a final concentration of 40 mg phospholipid/ml, and the mixture sonicated to clarity in a bath sonicator (Laboratory Supplies Inc., Hicksville, NY) at 30° C.

The pelleted Triton-extractable membrane components were taken up in the liposome suspension with a 23 g needle and a 1 ml syringe, and the mixture sonicated in a glass tube at 25° C in the bath sonicator.

IV. Transport assay. Uptake of labelled compounds was measured using the Millipore filtration technique as previously described, [2,3] with the following modifications: (1) 0.22 μm membrane filters (Millipore GSWP) were used instead of the 0.3 μm filters previously employed, because retention of the liposomes on the 0.22 μm filters was far superior, while still permitting acceptable filtration rates; (2) incubations were at 25° C; and (3) the use of radiolabelled mannitol as marker of the extraliposomal space was found unnecessary and was discontinued.

The final glucose concentration in the incubation media was routinely 0.2 mM.

V. Analytical methods. Protein was assayed by the method of Lowry et al. [9]. Radioactively labelled compounds were measured by liquid scintillation counting. Polyacrylamide gel electrophoresis was performed according to Preiser et al. [10].

VI. Electron microscopy. Liposome samples were fixed by dialysis against 1% paraformaldehyde and 1.5% glutaraldehyde in standard buffer for 2 h at 4° C. After fixation the aldehyde was removed by dialysis against standard buffer for a further 2 h. The liposomes were then suspended in noble agar (to a final concentration of 2% agar) at 42° C, and distributed as a thin layer on a glass slide. After solidification at 4° C, 1 mm^3 blocks were cut from the agar layers and fixed in 1% OsO_4 in standard buffer containing 0.5% tryptone for 15 h in the dark. The blocks were washed repeatedly with standard buffer followed by veronal acetate buffer, pH 6.2, 412 mosM, and then stained in 0.5% uranyl acetate in veronal acetate buffer for 90 min at room temperature. Uranyl acetate was then removed by washing in veronal acetate buffer followed by 30% and 50% ethanol containing 0.1% sodium chloride. The washed material was dehydrated and embedded in a low viscosity resin as previously described [11]. Thin sections (silver-grey intereference color) were prepared in Sorvall MT 2B ultramicrotome stained immediately in Reynold's lead citrate [12] and examined using a JEM 100C electron microscope at 60 kV.

Results

Effects of sonication on reconstitution

The extent of carrier reconstitution was estimated by the ability of liposomes to take up D-glucose under the influence of Na^+. As seen in Fig. 1, only the sonicated mixture of Triton X-100-soluble membrane components and liposomes showed Na^+-dependent D-glucose uptake. In the presence of Na^+ this

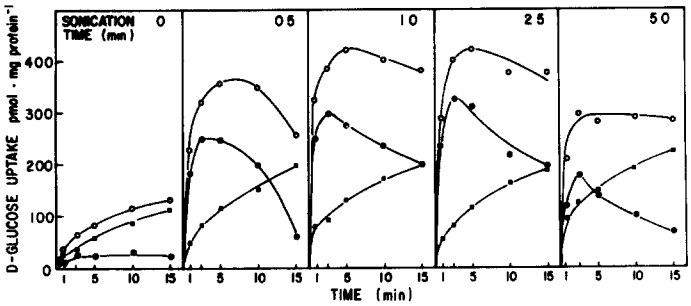


Fig. 1. Influence of sonication upon D-glucose uptake in the reconstituted system. Aliquots of a single batch of solubilized brush border membrane components were sonicated as described (see text) with 0.9 ml-aliquots of a single batch of liposomes for the times indicated. Liposomes contained 0.1 M KCl/5 mM HEPES-Tris (pH 7.5) and 1 mM dithiothreitol. Incubation medium contained 5 mM HEPES-Tris (pH 7.5)/0.2 mM D-[U-^{14}C]glucose and 0.1 M NaSCN (○) or 0.1 M KSCN (□). Na^+-dependent D-glucose uptake ($Na^+ - K^+$). (●).

uptake is stereospecific and inhibited by phlorizin [2,3]. Neither the detergent-soluble membrane components alone, with or without sonication, nor the unsonicated mixture of membrane components and liposomes showed any Na^+-dependent uptake of D-glucose. Maximal rates of Na^+-dependent D-glucose uptake were seen when reparations were sonicated for 1 to 2.5 min. Sonication for longer periods of time resulted in a decrease in Na^+-dependent D-glucose uptake, and loss of the 'overshoot' phenomenon (i.e. a transient uptake of glucose above the final rest point of the system) seen after shorter periods of sonication. Preparations were therefore sonicated routinely for 2 min to achieve optimal reconstitution. Compared to the unsonicated preparation, D-glucose uptake by reconstituted liposomes with KSCN in the incubation medium instead of NaSCN, was increased by sonication for 0.5 min, but longer periods of sonication caused no further increase in Na^+-independent uptake.

Liposomes prepared from pure egg lecithin showed comparatively poor glucose uptake when reconstituted with Triton solubilized membrane components.

Extent of carrier reconstitution

Since Na^+-dependent D-glucose transport can be observed in the reconstituted liposomes it is clear that some material derived from the solubilized brush border membranes becomes associated with the liposomes during the reconstitution procedure and acts as a carrier. However, the proportion of this material which became reconstituted was in question. Rates of Na^+-dependent D-glucose uptake were measured at 0.5 and 2.5 min and were found proportional to the amount of protein in the sonicated liposome preparation (Fig. 2), suggesting that, under the conditions used, carrier reconstitution is complete. An alternative, but less likely explanation is that a constant proportion of the carriers is reconstituted at each protein concentration.

The reconstituted liposomes as osmoreactive vesicles

Na^+-dependent D-glucose uptake was seen only in the sonicated mixture of detergent-solubilized membrane components and liposomes. Because of this, it

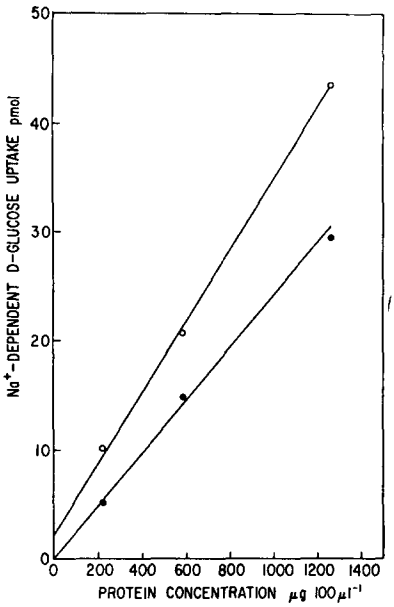


Fig. 2. Relation between protein concentration and Na^+-dependent uptake of D-glucose by the reconstituted preparation at initial rate (0.5 min) (•), and at peak of 'overshoot' (2.5 min) (○). Transport conditions as in Fig. 4.

seemed unlikely that the sugar uptake seen could have been due to binding either to unreconstituted membrane components or to protein-free liposomes. However, binding to a brush-border component reconstituted into liposomal surface, but yet unable to effect sugar translocation into the internal liposomal space remained a possibility. To test this question, uptake of D-glucose was measured in the presence of increasing concentrations of osmotically active solutes in the incubation media. Addition of L-sorbose or sucrose to the incubation media which would be expected to reduce sugar uptake by reducing the internal liposomal space, without any effect on binding-progressively reduced Na^+-dependent uptake of D-glucose (Fig. 3) strongly suggesting that transport into the internal liposomal space rather than surface binding, is the correct explanation for the sugar uptake seen.

Effects of increasing protein concentration on D-glucose uptake

Reconstituted preparations with low protein: lipid ratios (as well as those subjected to prolonged sonication) did not show overshooting D-glucose uptake. However, increasing the ratio of protein to lipid (increasing amounts of a single batch of solubilized membrane compounds reconstituted with aliquots of a single batch of liposomes) caused the appearance of 'overshoot' similar to that seen in natural membrane vesicles (Fig. 4). 'Overshoot' in the reconstituted preparation was, however, delayed compared to that in natural membrane vesicles, peaking at 2.5 to 15 min after the start of the reaction in different preparations. We have no ready explanation for the variations in the timing of 'overshoot' in different preparations, or for the fact that some preparations do not show 'overshoot'. However, the magnitude of the 'over-

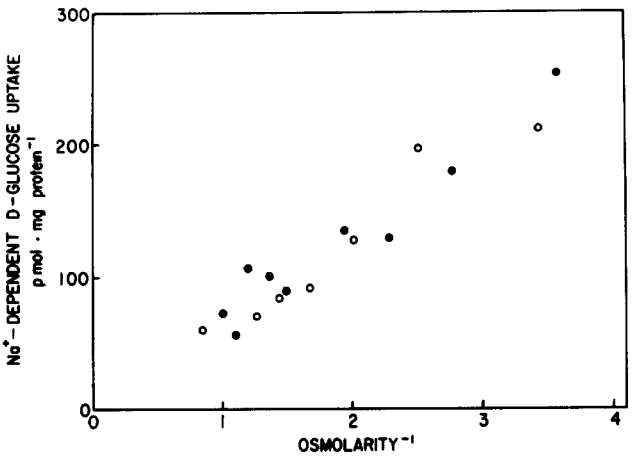


Fig. 3. Effect of increasing incubation medium osmolarity on D-glucose uptake by the reconstituted preparation. Experiment was conducted as in Fig. 1, but with the addition of increasing amounts of sucrose (●) or L-sorbose (○) to the incubation medium. Time of incubation = 2 min. The points with L-sorbose are the same as those previously presented in a published symposium [17].

shoot' phenomenon, like the 0.5 min rate of glucose uptake, is proportional to the protein concentration (see Fig. 2).

As in natural membrane vesicles, an essentially common rest point in D-glucose uptake was reached in the presence of Na^+ and K^+, but equilibration took longer than in natural membranes. Increasing protein: lipid ratios have no effect on the equilibrium concentration of glucose and thus apparently have no gross effect on the liposomal space. The liposomal space, as calculated from the

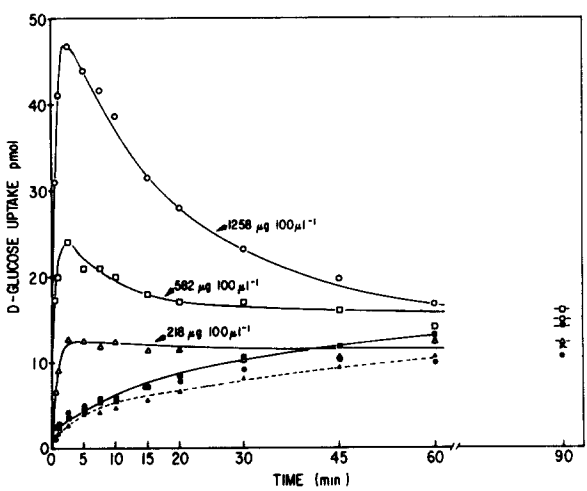


Fig. 4. Influence of protein concentration on D-glucose uptake in the reconstituted system. Aliquots of a single batch of solubilized membrane components were reconstituted into identical volumes (0.9 ml) of a single batch of liposomes. Liposomes were loaded with standard buffer. Incubation media contained 0.2 mM D-[^{14}C]glucose, 5 mM HEPES-Tris (pH 7.5) and either 0.1 M NASCN; △, □, ○, or 0.1 M KSCN, ▲, ■, ●. Protein concentrations are indicated on the graph.

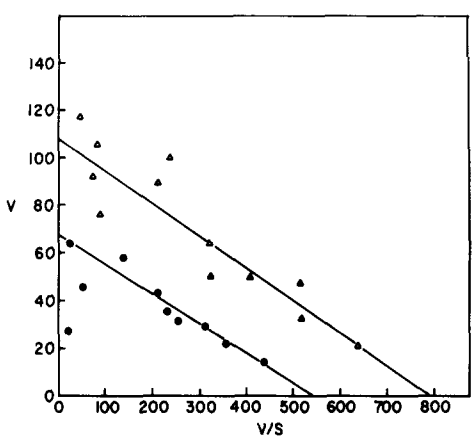
mean equilibrium concentration of D-glucose, was 9 μl/ml of undiluted reconstituted liposome suspension.

Kinetics of D-glucose uptake

D-Glucose uptake by reconstituted liposomes loaded with standard buffer was measured over a range of glucose concentrations from 0.03 to 2.5 mM, using 0.1 M NaSCN in the incubation medium to promote uptake. Eadie-Hofstee plots of the uptake rates during the early part of the upward phase of overshoot show that Na^+-dependent D-glucose uptake occurs via a saturable system with a K_m of 0.125 to 0.135 mM (Fig. 5).

Electrophoresis

The SDS-polyacrylamide gel electrophoresis pattern of the starting brush border membrane, the Triton-solubilized membrane components and the reconstituted preparation in liposomes is very similar (Fig. 6) except that a prominent low molecular weight band in the natural membranes does not appear in the detergent soluble fraction.



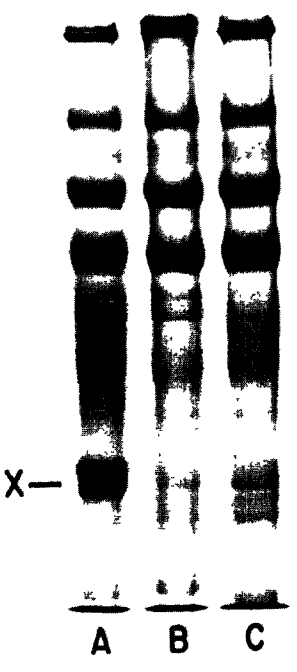


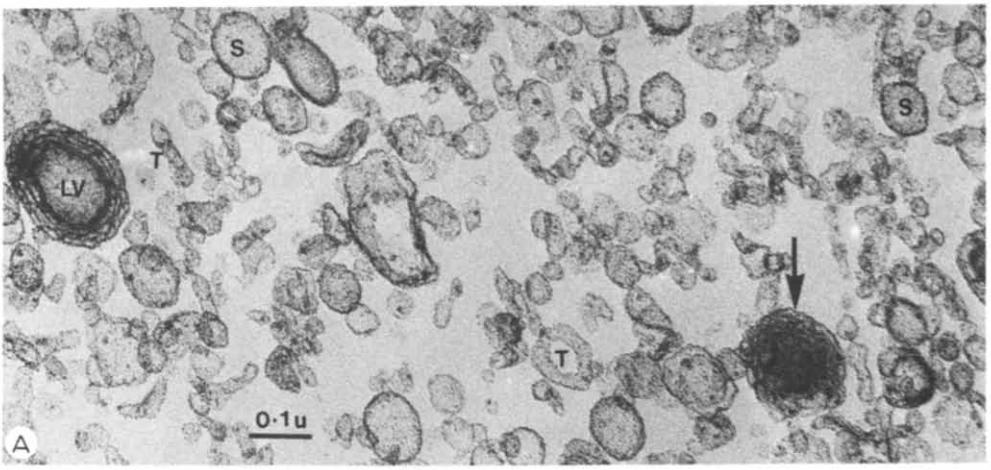
Fig. 5. Kinetics of D-glucose uptake by the reconstituted preparation. Experiment conducted as in Fig. 4. D-glucose concentrations in the incubation medium varied from 0.032 mM to 2.52 mM. Incubations were terminated after 0.5 (•) and 1 min (△). V, Na^+-dependent D-glucose uptake (pmol · mg protein^{-1}); S, D-glucose concentration (mM). Values for K_m were determined from regression lines drawn by the method of least squares.

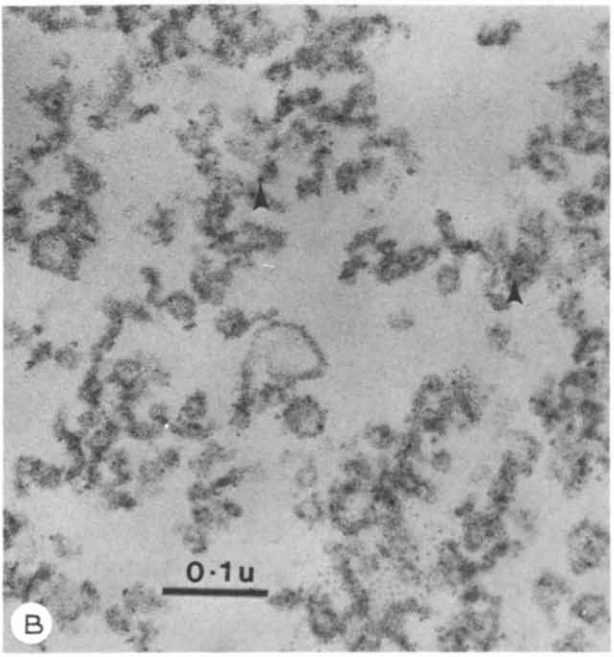
Fig. 6. SDS-polyacrylamide gel electrophoresis patterns of (A) starting brush border membrane. (B) Triton X-100 solubilized membrane components and (C) Triton X-100 sonicated membrane components reconstituted into liposomes.

Electron microscopy

In Fig. 7 are presented electromicrographs of unreconstituted liposomes (A), Triton X-100 extractable membrane elements (B) and the reconstituted liposomes (Fig. 7C is an enlarged view of an average general field shown in Fig. 7D).

Unreconstituted liposomes (Fig. 7A) consisted of vesicles of various sizes, but in the main of 20 to 50 nm diameter. The average diameter of the vesicles determined from the measurement of 100 vesicles at random is 41 nm. The vesicles are delimited bv a membrane 5 nm thick. Only one electron dense layer





of the membrane could be demonstrated. Vesicles varied in shape from spherical to tubular. Multilamellar vesicles and some large multicompartmented or solid bodies were also occastionally seen. These are presumed to be lipid inadequately dispersed by sonication.

Membrane elements extracted by Triton X-100 can be seen as aggregates of amorphous material (Fig. 7B) containing electron dense granules. The

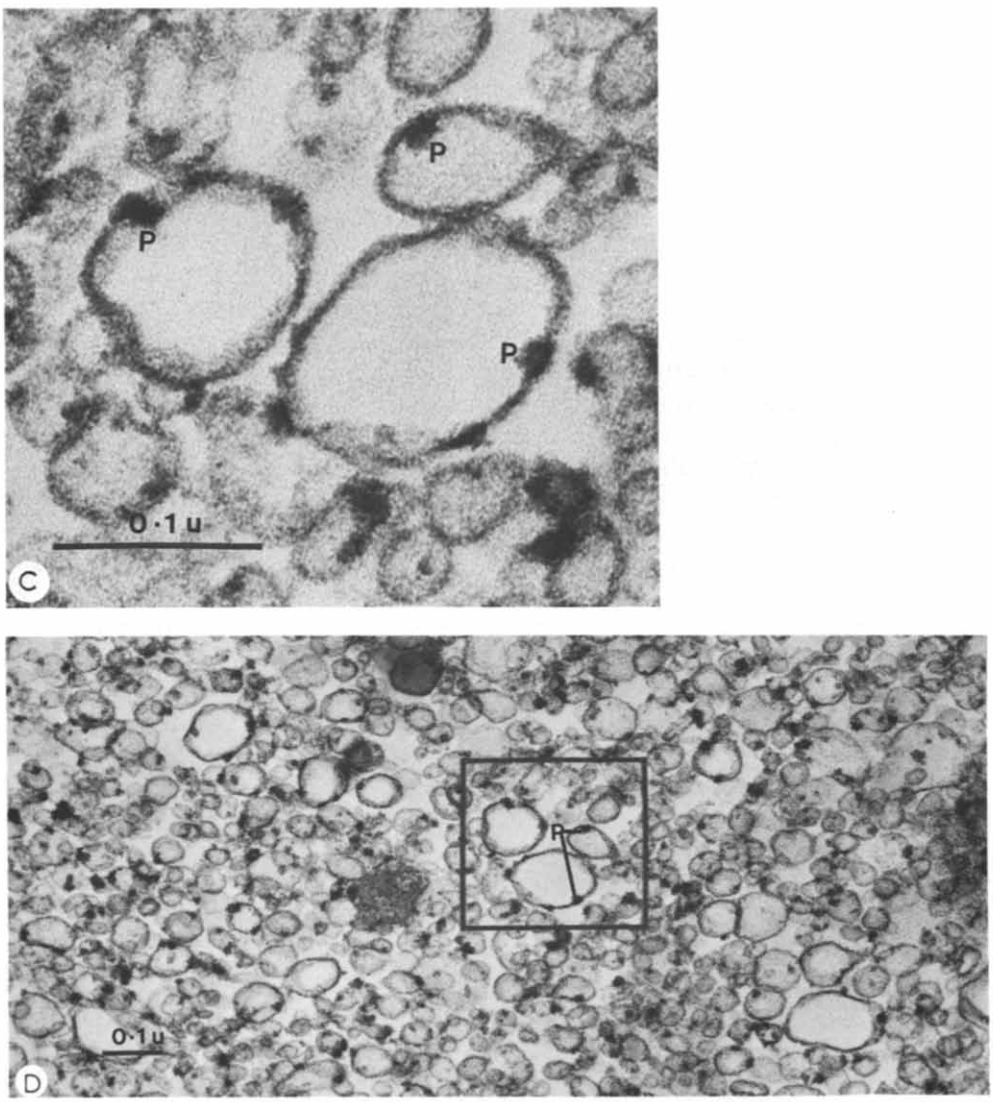


Fig. 7. A. The average view of unreconstituted liposome preparation S, spherical vesicle; LV, multilamellar vesicle; arrow shows inadequately dispersed lipid. B. Triton extracted material of brush border membrane, arrowheads, electron dense particulate material; note that aggregate of similar material is incorporated into the vesicle membrane in C and D. C. and D. Liposome reconstituted with Triton extracted material of brush border membrane; C is an enlarged view of boxed region of D to show incorporated particulate material (P) into the vesicle membrane; note that unlike in A no tubules can be seen in D.

characteristic bilayer appearance of unextracted brush border membrane cannot be demonstrated in this material.

Reconstituted preparations (Fig. 7C and D) differed from the unreconstituted preparations in three ways: (1) tubular vesicles are rarely seen; (2) membrane of the vesicles shows incorporated particulate materials; and (3) frequently the thickness of the vesicle membrane is greater than 10 nm.

Discussion

The previous studies from this laboratory, although preliminary [2,3] convincingly demonstrated reconstitution of stereospecific Na^+-dependent D-glucose transport into artifical phospholipid vesicles. The argument has been made by Kinne and Faust [13] that whole membrane vesicles may have survived extraction with 1% Triton X-100, sonication and centrifugation at 30 000 $\times g$, and could have been responsible for the glucose uptake seen in our preparations. However, unlikely this may seem based on the sum of our earlier communications, our present experimental protocol, namely the use of only those membrane components which do not sediment in 1 h at $1 \cdot 10^5 \times g$ directly answers any criticism in this vein. Although membrane fragments consisting of proteins with their intimately associated lipids may remain in the $1 \cdot 10^5 \times g$ supernatant, it is unlikely that membrane fragments of vesciular size do so. As noted in the Results section above, Na^+-dependent D-glucose uptake is not demonstrable in solubilized, unreconstituted membrane components.

Curiously, in the same paper [13] the use of plant lipids of 'uncharacterized' composition has been questioned in apparent ignorance of the demonstration of Kasahara and Hinkle [14] that mixtures of pure lipids produce liposomes of the same characteristics. Moreover, detailed chemical analyses of the constituents of the soybean lipids used in our studies has already been reported by Kagawa et al. [15] and by Miller and Racker [16]. Kinne and Faust [13] used lipids extracted from whole kidneys to form liposomes by hand shaking. Since the glucose transport activity which these authors seem to have reconstituted into their liposomes is but a feeble fraction of that demonstrated in our present and previous studies, it would appear that the efficiency of the procedures for formation of liposomes and for reconstitution are far more important than the source and 'characterization' of the lipids. We have also used in the course of our studies liposomes made from chloroform-methanol extracted kidney cortex lipids. In our hands, these liposomes perform well and give similar, but no better results than those made from plant phospholipids. We concluded that within certain limits the constitution of the bulk of the membrane lipid is not a major factor. It may be worth once again drawing attention to the fact that our concern has not been to attempt a reconstruction of the natural membrane but rather to establish a reproducible and sensitive assay system to enable the purification and study of the carrier to be pursued.

Na^+-dependent transport of D-glucose in natural brush-border membrane vesicles shows 'overshoot' i.e. a transient accumulation of glucose within the liposomes in concentration exceeding those in the incubation medium. The genesis of this phenomenon is uncertain, but it has been attributed to the electrogenic nature of Na^+-co-flux on the carrier. In contrast to earlier experience

[2,3] we now regularly achieve 'overshoot' in the reconstituted preparations. The reasons for this change in behavior of the preparations are not entirely clear, and do not appear to be due to any single change in our techniques. Two factors, however, do seem to be important; these are (1) protein concentration and (2) controlled sonication. A major factor in the genesis of 'overshoot' in the reconstituted system is protein concentration, and our preliminary purification data indicate that it is specifically the concentration of glucose carriers which is of prime importance. At low ratios of unpurified brush-border membrane protein: lipid (<1 : 20) we do not see 'overshoot' (Fig. 4). At higher protein: lipid ratios 'overshoot' becomes apparent, and increases in proportion to the amount of protein reconstituted (Figs. 2 and 4). Moreover, within single batches of detergent-extracted membrane proteins we have seen the appearance of 'overshoot' in liposomes reconstituted with purified membrane proteins when no 'overshoot' had occurred with the same or greater concentrations of unpurified protein (to be communicated in detail in a later paper). Therefore, it seems that the phenomenon of 'overshoot' is dependent upon a minimal concentration of glucose carriers in the liposomal membrane. At low protein concentrations in the reconstituted system when no 'overshoot' is seen, glucose would enter the liposomes relatively slowly via a limited number of carriers. In this circumstance, the driving force for 'overshoot' may be presumed to have decayed before the internal glucose concentration exceeds that in the incubation medium. On the other hand, if glucose entry proceeds more rapidly via a larger number of carriers, active transport, i.e., 'overshoot' occurs before the driving force is dissipated. This argument would suggest that the driving forces for glucose accumulation are of short duration; e.g., less than 2.5 min in the preparation shown in Fig. 4. Experiments are under way to test this notion.

Our simple treatment of the data assumes that no complications are introduced by a sidedness of the carrier which may be different in the reconstituted system than in the natural membranes. At this stage of development of the project it is not possible to be certain because we know neither the details of structure of the carrier nor the characteristics of carrier function from the inside of the natural membrane. However there are some good reasons to assume that the carrier reconstituted, has the same sidedness as the carrier in the natural membrane. The method of reconstitution used would be expected to select for incorporation into liposome that surface of the reincorporated molecule which is the more hydrophobic as we have found for brush border membrane enzymes [17]. It is reasonable to assume that the carrier is physically different at the two ends, but until isolated it cannot be known.

We also treat the kinetics in a simplified way. We have taken the 0.5 min uptake as a reasonable means for comparing rates under differing conditions. We do not measure true initial rate, nor do we attempt to do so. The reason for this is that the conditions established at zero time with the reconstituted liposomes are not stable but decay rapidly, though, it seems, less rapidly than with natural membrane vesicles. Using D-[^{14}C]glucose as a measure of transport we see no real hope of attaining true initial rate measurements because of inherent limitations of sensitivity of the assay methods. When it may be possible to reconstitute the carrier in planar membranes and measure carrier activity by the electrical consequences of sugar-induced Na^+-flux, true initial

rates may then, hopefully, be closely approximated. The conditions, however, under which we estimate kinetics are certainly equivalent to the conditions used for natural membrane vesicles [18], and probably equivalent to the conditions in intact tissue when one considers the changes in intracellular ion activities and in membrane potential which follow immediately the start of the transport process [19].

The present experiments provide a rational basis for attempts at carrier purification. The SDS-polyacrylamide gel electrophoresis patterns here presented do not indicate which of the protein bands seen contains the glucose carrier, if indeed the carrier is visible using the present techniques. We are currently attempting purification of the glucose carrier on the assumption that it is a protein, using the reconstituted system as our assay procedure.

Acknowledgments

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