Reconstitution and "Transport Specificity Fractionation" of the Human Erythrocyte Glucose Transport System

A NEW APPROACH FOR IDENTIFICATION AND ISOLATION OF MEMBRANE TRANSPORT PROTEINS

STANLEY M. GOLDIN and VICTORIA RHODEN

From the Department of Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

A stereospecific \( \alpha \)-glucose transport system was reconstituted from human erythrocyte ghosts by hollow fiber dialysis of a cholate-solubilized supernatant protein fraction (20 to 25% of the ghost protein) in the presence of added phospholipid and cholesterol. \( \alpha \)-Glucose transport was inhibited by cytochalasin B (\( K_a \) = 3 to 5 \( \times 10^{-7} \) M), Hg\(^{2+} \), and phloretin. \( \alpha \)-Glucose uptake exhibited saturation kinetics, with a \( K_m \) of 20 to 25 mM. A more rapid, saturable \( \alpha \)-glucose exchange process had a \( K_m \) of 40 to 45 mM. This reconstituted glucose transport system was associated with single bilayer vesicles, primarily 450 to 650 Å in diameter, and was reconstituted under conditions such that 15 to 25% of the vesicles contained the transport system. At least 63,000 transport sites/red cell have been reconstituted. When the entire vesicle population was preloaded with 0.8 mM \( \alpha \)-glucose and subsequently incubated in glucose-free medium, most of the glucose leaked out of specifically those vesicles containing the sugar transport system; the concomitant reduction in intravesicular density of the sugar-transporting vesicle fraction permitted the separation of this fraction from the rest of the vesicle population on density gradients ("transport specificity fractionation"). This vesicle fraction was predominantly enriched only in Coomassie-staining protein(s) of sodium dodecyl sulfate gel electrophoretic mobility corresponding to "Band 4.5" (nomenclature of Steck, T. L. (1974) J. Cell Biol. 62, 1-19). These results, together with cytochalasin B binding data from other laboratories which indicate that the sugar transport system is a major protein component of the erythrocyte membrane, provide strong evidence that a component of Band 4.5 is part of the sugar transport system.

The kinetics and specificity of the human erythrocyte sugar transport system have been extensively studied in erythrocytes and resealed erythrocyte ghosts (1-3). Inhibitor binding (4-6) and differential labeling (7, 8) evidence has implicated several different polypeptides as possible components of the sugar transport system; the conflict among these findings may be attributable to the lack of specificity for the sugar carrier of the ligands employed in these studies.

The molecular identity of the membrane protein(s) responsible for this facilitated diffusion process has yet to be unambiguously established. The objectives of the study reported here were to identify and purify the polypeptide(s) comprising the erythrocyte sugar transport system. Toward these ends, a novel approach, termed "transport specificity fractionation," was developed. This approach, which may prove to be of general utility for identification and purification of certain membrane transport proteins, involves the processes discussed in the following sections:

Reconstitution of Transport System of Interest into Vesicles before Purification - A crude membrane fraction containing the transport system is solubilized with cholate in the presence of a large excess of added lipid; undissolved material is pelleted. Under appropriate conditions, the supernatant fraction contains the transport activity (along with impurities). Vesicles containing the transport activity are formed by a dialysis technique (9) that is a modification of that of Racker (10).

Identification of that Fraction of Vesicles Containing Transport System - Recent studies on the process of reconstitution of purified (NaK)-ATPase by this technique (9) indicate that one obtains the apparently random distribution of functional monomer units of the transport system among dimensionally homogeneous (400 to 600 Å) unilamellar vesicles. Based on these findings, one might expect that, when the starting material employed for reconstitution is a heterogeneous membrane fraction (rather than a purified membrane protein), the cholate-solubilizable components of this membrane fraction might be randomly distributed among the vesicle population upon reconstitution; thus, at a sufficiently high lipid/protein ratio, each vesicle will contain only a few protein molecules; just a fraction of the vesicles will contain the membrane transport protein of interest.

Isolation by Use of Specific Permeability Properties of Transport System - Vesicles containing the transport system are fractionated from the rest of the vesicle population. This is done by exploiting some physical property of the vesicles that can be specifically modulated according to a given vesicle's
transport capabilities. In the instance reported here, we find that the presence of the sugar transport system in a fraction of the vesicles permits us to create changes in intravesicular density of this fraction that lead to its isolation on density gradients and to concomitant (partial) purification of the sugar transport system.

Like affinity labeling and affinity chromatography, this method depends for its viability on a specific biological property (transport activity rather than ligand binding) of the protein one wishes to identify and isolate. We report here the reconstitution, in vitro characterization, and partial purification of the human erythrocyte sugar transport system.

**EXPERIMENTAL PROCEDURES**

**Materials**

Cholesterol was from Fisher Chemical Co. Cholic acid and standards for gel electrophoresis were from Sigma Chemical Co. All radioisotopes were from New England Nuclear. Scintillation fluid consisted of Triton X-100 (Rohm and Haas) and Scintiverse (Fisher Chemical) in a 1:9 (v/v) ratio. L-Glucose was from Calbiochem. Bio-Gel A-150m, biacrylamide, bisacrylamide, TEMED (N,N,N',N'-tetramethylethylenediamine), 2-mercaptoethanol, and Bio-Fibet 50 Minutues were from Bio-Rad Laboratories. Freshly drawn blood was obtained from the Red Cross Center, Boston, Mass. Sephadex and Ficoll were from Pharmacia; bromphenol blue, Allied Chemical; phloretin, ICN; cytochalasin B, Aldrich; phosphatidylcholine, a gift of Avanti Biochemical Co. (Catalogue No. 830051, shipped in dry ice under acetone/N₂). Standards for calibration of Bio-Gel A-150m column included 850 and 380 Å diameter monodisperse latex spheres, Dow Chemical; Sindbis virus was a gift of Steve Harrison, Biological Laboratories, Harvard University.

**Methods**

**Preparation of Lipid** — Egg yolk was subjected to the chloroform/methanol extraction and acetone/ether precipitation procedures of Litman (11). The product was stored under liquid N₂ as a 50 to 80 mg/ml solution in 20:1 (v/v) decane/ether. 2-Mercaptoethanol (1 mM) was used as an antioxidant in all solvents employed in lipid preparation and storage; care was taken to minimize lipoxidation (9). Thin layer chromatography (9) revealed that the product of five successive acetone/ether precipitations was ~95% phosphatidylcholine. Egg phosphatidylcholine (>99%) prepared by Avanti Biochemical Co. could be effectively substituted for the above lipid preparation.

Cholesterol was recrystallized twice from acetone/water and stored under liquid N₂ as a 100 mg/ml solution in benzene.

**Reconstitution of Glucose Transport** — Human erythrocyte ghosts were prepared from freshly drawn blood by the method of Dodge et al. (13), with the following modifications. The acidic phosphate buffer contained 5 mM 2-mercaptoethanol. Ghosts were lysed and subsequently washed in 10 mM Tris, 5 mM 2-mercaptoethanol, pH 7.8. All washing and handling of ghosts were performed in a 4° cold room. The partially void ghosts were finally suspended, sedimented, and stored at ~85° in 10 mM imidazole, pH 7.4, 5 mM 2-mercaptoethanol, 0.25 M sucrose as suspensions of 6 to 10 mg/ml of protein.

Solutions of phosphatidylcholine and cholesterol were mixed under N₂ to give 1.9 mol of phospholipid/mol of cholesterol. The more volatile components of this mixture were evaporated under an N₂ stream; the remainder was frozen and lyophilized for 2½ to 3 h to remove remaining solvent. The lipid was reconstituted in a final phospholipid concentration of 11.6 mg/ml by blending on a Vortex mixer under N₂ in 0.25 M sucrose, 0.55 mM EDTA, 5 mM Tris, 10 mM imidazole, 0.235 M KCl, 35 mg/ml of cholic acid, pH 7.2, 5 mM 2-mercaptoethanol.

A 1.6 ml sample of this turbid lipid suspension was placed under N₂ in a tube (10 x 75 mm); the suspension was clarified by immersion for 15 to 30 s in a boiling water bath, and immediately chilled on ice. Two milligrams of ghost protein as a 0.2- to 33-ml aliquot was added to the clarified lipid and mixed thoroughly. The mixture was incubated on ice for 30 min. It was then centrifuged at 20,000 rpm for 4 min (4°) in a Beckman Ti-50 rotor. One to several simultaneously prepared batches of this supernatant were used immediately for hollow fiber dialysis.

**Two types of reconstituted D-glucose transporting vesicle preparations were formed:** "9:1 phospholipid/protein" vesicles and "18:1 phospholipid/protein" vesicles. (This nomenclature was based on the ratio of the total exogenous phospholipid to total ghost protein contained in the reconstituted vesicles.) The 9:1 vesicle membranes were dialyzed against the supernatant for 2 hr in Bio-Gel 50 Minutues, as previously described for reconstitution of (Na⁺K)·ATPase (9); each Minube was connected to a Tygon tubing in series with the interior of the hollow fibers to form a unit of 0.7 ml total internal volume. As required, mixtures of 0.5- to 2.8 ml of vesicle preparations were produced by dialysis in 1 to 4 of these hollow fiber units connected in series.

The dialytes contained 200 mM imidazole, 5 mM 2-mercaptoethanol, 0.1 mM EDTA, 125 mM NaCl, pH 6.9 (these ingredients comprised Buffer A). To create vesicles of relatively high internal density, 800 mM D-glucose was included in this dialyse. Portions of the studies of transport kinetics and for control vesicle preparations of lower intravesicular density, vesicles were formed using dialyse consisting of Buffer A and 800 mM glycerol instead of Buffer A and 800 mM D-glucose. For the determination of intravesicular transport, the dialyse was loaded into the vesicles by including the radioactively labeled species in the dialyse, 0.1 to 0.3 μCi/μl. To prevent the binding of radioactive glucose or sucrose (which would occur if the radioisotopes were added in carrier-free form) from interfering in the solution, compartment, the dialyse was added to the dialyse together with cold carrier sucrose or glucose, 0.1 to 1 mM; dialyse containing 800 mM D-glucose did not require additional cold D-glucose when D-[3H]glucose was employed for aqueous compartment determinations. [3H]Glucose and [3H]sucrose each gave similar values for intravesicular aqueous compartment. A 750-Minube was used for the reconstitution of (Na⁺K)·ATPase (9); each Minube was then subjected to the dialysis procedure.

The resulting dialyzed material was the reconstituted D-glucose transporting vesicle preparation. Vesicles as controls were made on occasion by a procedure identical with the above, but without added glucose.

**Protein Assays** — Vesicles and ghosts were assayed for protein by the procedure of Lowry et al. (15), as modified to avoid interference by lipid (9).

**Total Phosphate** — Total lipid phosphate was determined by the method of Ames (14).

**Transport Assays** — Solutions of L-[3H]glucose in Buffer A + 800 mM glycerol + 2 mM MgSO₄ containing 6 to 12 × 10⁶ cpm/ml were prepared; the [glucose] was adjusted to the desired level (0.2 to 50 mM) by addition of 2 M cold D or L-glucose from stock solutions in Buffer A. For determination of the radioinqueous compartment, the radioisotope was added to the dialyse across the vesicle membrane to avoid vesicle shrinking or swelling due to osmotic forces; such dimensional change would decomplicate measurement of the kinetics of glucose transport. Assaying glucose transport at [glucose] greater than 50 mM thus necessitated preparation of 9:1 vesicles and 18:1 vesicles with [glucose] > 50 mM. One ml portion of dialyse was used for each hollow fiber unit employed.

The 18:1 vesicles were produced by diluting the supernatant used for the formation of 9:1 vesicles with an equal volume of clarified, solubilized phospholipid/cholesterol suspension prepared as above; the resulting mixture was then subjected to the dialysis procedure.

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Data reported as "stereospecific" n-glucose transport is the excess of n-glucose transport over l-glucose transport determined under the same conditions.

Gradient Centrifugation of Vesicles—The 18:1 vesicles were formed in the presence of 800 mM n-glucose as described above. Samples containing 2.5 ml of these vesicles were placed on a 25-ml column of Sephadex G-50M and eluted at 4° at a flow rate of 0.6 ml/min with 800 mM glycercol, 2 mM MgSO₄ in Buffer A to replace external glucose with glycercol of equal osmolarity. Five milliliters of fractions containing the vesicles were pooled and incubated at 23.5 ± 0.5° for 2 h. The incubated vesicles were chilled on ice, and concentrated on an Amicon PM-30 membrane in an Amicon 8MC ultrafiltration cell (6 p.s.i. of N₂, 4°) to a final volume of 1.0 to 1.1 ml.

Linear gradients were formed in 5 ml Beckman ultracentrifuge tubes on top of a 0.3-ml cushion of 9.5% Ficoll, 1 M n-glucose in Buffer A. The gradients were formed from equal volumes of a low density solution of 800 mM glycercol, 2 mM MgSO₄ in Buffer A; and a higher density solution of 500 mM n-glucose, 375 mM glycercol, 62.5 mM NaCl, 20 mM imidazole, 0.1 mM EDTA, 2 mM MgSO₄, 5 mM 2-mercaptoethanol, pH 6.9.

On each gradient, 0.5 to 0.7 ml of the above concentrated vesicles were placed and centrifuged at 50,000 rpm for 6 to 8 h in an SW 50.1 rotor. Gradients fractions of ~150 µl volume were collected, and assayed for lipid phosphate.

Pooled fractions of the gradient were assayed for stereospecific n-glucose transport activity as follows. A 125-µl aliquot of each set of pooled fractions was eluted on Sephadex G-50M in a Pasteur pipette; the column was equilibrated and eluted with Buffer A + 800 mM glycercol + 2 mM glucose as described under "Transport Assays." The void fractions, containing the vesicles, were pooled. This manipulation resulted in removal of all extravesicular glucose and its replacement by iso-osmotic amounts of glycercol. These pooled void fractions were then assayed for uptake of 1 mM n-glucose versus 1 mM l-glucose as described under "Transport Assays." These pooled void fractions were also assayed for lipid phosphate. Recovery of transport activity was 60 to 110%.

Sodium Dodecyl Sulfate Gel Electrophoresis—Sodium dodecyl sulfate-acrylamide gels (7.5% acrylamide, 0.1% bisacrylamide) were prepared in 6-mm (inner diameter) tubes, electrophoresed at pH 8.5, and stained with Coomassie blue according to Davies and Stark (16). The high ratio of lipid to protein in the vesicles imposed severe limitations on the amount of protein that could be loaded on a gel without clogging and fouling of the gel by the excess lipid. To circumvent this problem, the following procedure was developed for delipidation of the samples before they were subjected to SDS gel electrophoresis.

Each sample, containing 2.5 mg of phospholipid, was concentrated by ultrafiltration as described above to a volume of 300 µl. The sample was exchanged at 4° on columns of Sephadex G-25F formed in Pasteur pipettes, into 5 mM phosphate buffer, pH 8.3. The column void fractions (300 µl), containing all the protein and phospholipid, were concentrated and warmed to room temperature. SDS (3%) was added to the sample to a final concentration of 1.5%; the sample was immediately heated to 100° for 3 min. The clarified, boiled sample was extracted after cooling with 2.5 ml of 2:1 chloroform/methanol by blending on a Vortex mixer for 2 min; the two phases were separated by centrifugation in a tabletop centrifuge. The lower (organic) phase was discarded, leaving the interfacial pellicle and aqueous phase; this material (containing virtually all the protein and most of the SDS) was frozen, lyophilized, and resuspended in 10% glycercol to a volume of 200 µl. The sample was made 30 mM in 2-mercaptoethanol. Bromphenol blue was added as tracking dye; the sample was heated to 100° for 3 min and incubated for 1 h at 45°. This sample was applied to the gel.

Erythrocyte ghosts, when processed by the above method, exhibited an SDS gel pattern of Coomassie-staining bands that significantly differed from ghosts that were not extracted with chloroform/methanol. Samples of vesicles that were not chloroform/methanol-extracted could be successfully subjected to gel electrophoresis if they contained less than 0.5 mg of lipid; the sample exhibited banding patterns that were qualitatively similar to, but much fainter than, the delipidated sample of the same material. The high concentration of SDS required to treat the 2.5-mg vesicle sample before delipidation and lyophilization (final concentration, ~9% in resuspended samples) caused some broadening of the protein bands. A trade-off was observed between the processing of a large amount of vesicles to produce darker but broader bands, and processing smaller vesicle samples which produced fainter (albeit narrower) bands that were more difficult to quantitate by densitometry. Coomassie-stained gels were scanned at 550 nm in a linear transport attachment for the Gilford 240 spectrophotometer.

RESULTS

The conditions employed for cholate solubilization of red cell ghosts in the presence of added phospholipid and cholesterol produce a supernatant fraction containing 20 to 25% of the ghost protein. Removal of the cholate by dialyzing this supernatant results in formation of vesicles, a fraction of which exhibits stereospecific n-glucose uptake (Fig. 1).

Identification and Characterization of n-Glucose-Transporting Vesicle Fraction—The total volume of aqueous medium enclosed in the vesicles is determined by forming the vesicles in the presence of a soluble radioactive marker, such as sucrose or glucose, and assaying for the fraction of this marker enclosed in the vesicles; these vesicles enclose 1.8 to 2.2 ml of aqueous medium/g of phospholipid. As shown in Fig. 1, we consistently see that about one-fourth of the aqueous compartment equilibrates rapidly with n-glucose. Vesicles incubated at 23° in the presence of 1 mM cold n-glucose for 1 h subsequently exhibited the same uptake curve for n-[3H]glucose, demonstrating that there was insignificant decay of the transport activity during the incubation. Vesicles

![Fig. 1. Uptake of 1 mM n-glucose (O-O) and of 1 mM l-glucose (-----) by vesicles. Vesicles contained ghost supernatant protein fraction and were formed in Buffer A + 800 mM glycercol by the 9:1 procedure (see "Methods"). Uptake measurements were made at 23°. The intravesicular aqueous compartment was determined by including 1 mM n-[3H]glucose in the dialysate, and is depicted in the figure as the level that would be attained (---) if the total intravesicular aqueous compartment was equilibrated with externally added 1 mM n-[3H]glucose. Aqueous compartment determinations were made both before (O-O) and after (-----) an incubation for 5 min at 23° in the absence of externally added n-[3H]glucose.](http://www.jbc.org/)
formed in the absence of membrane protein exhibit identical D- and L-glucose permeability, similar to the L-glucose permeability shown in Fig. 1.

The elution profile of the vesicles on a Bio-Gel A-150m column (Fig. 2) indicates that they are primarily 450 to 650 Å in diameter. Fig. 2 also shows that the stereospecific n-glucose transport activity parallels the elution profile of the phospholipid, demonstrating that those vesicles containing the transport activity are similar in size to the bulk population of vesicles. The A-150m profiles, together with the demonstration that one-fourth of the aqueous compartment rapidly equilibrates with n-glucose, show that about one-fourth of these vesicles contain the transport activity. Reconstitution performed at double the initial lipid-protein ratio (18:1 versus 9:1, see “Methods”) results, by these criteria, in rapid D-glucose transport in 13 to 17% of the vesicles (data not illustrated).

The experimentally determined trapping of aqueous medium (1.8 to 2.2 ml/g of phospholipid) correlates well with what one would expect for vesicles of mean diameter 550 Å surrounded by a single bilayer of (1.0 mol/mol) lecithin/cholesterol. One can calculate (9) this theoretical quantity (1.82 ml/g) from the x-ray diffraction data for hydrated lecithin/cholesterol bilayers (18, 19). This agreement indicates that the vesicles must be primarily of single bilayer structure rather than multimolecular. Vesicles formed by an identical procedure but without added ghost protein have the same size distribution and unilamellar structure.

Vesicles were formed in the absence of added ghost protein, in the presence of n-[^3H]glucose; Fig. 3 shows that most of this internally trapped n-glucose remains inside the vesicles over several hours at either 24° or 0° after dilution into either iso-osmotic or hypo-osmotic glucose-free medium.

**Kinetics and Inhibition of Glucose Transport**—The concentration dependency of the initial rate of stereospecific n-glucose uptake shows saturability, with a $K_m$ of 20 to 25 mM (Fig. 4); the low level of uptake of L-glucose is not saturable over the L-glucose concentration range studied. Our most active preparations have a $V_{max}$ for n-glucose uptake about 2-fold greater than that derived from Fig. 4. The concentration dependency for stereospecific D-glucose uptake under conditions which allow exchange diffusion was determined by forming these vesicles in the presence of 800 mM n-glucose, removing the external glucose on Sephadex, and measuring uptake of externally added n-[^3H]glucose (Fig. 5). This latter process is also saturable, with a $K_m$ of 40 to 45 mM; the maximum velocity is 4- to 5-fold higher than under the former conditions, which allow only unidirectional movement of D-glucose.

Fig. 6 depicts the inhibition of D-glucose uptake by externally added cytochalasin B ($K_i = 4 \times 10^{-9}$ M); cytochalasin B, in the concentration range studied, had no significant effect on L-glucose uptake. Table I demonstrates asymmetric, thiol reagent-reversible inhibition of D-glucose uptake by internally incorporated Hg²⁺ and internal phloretin. Externally added Hg²⁺ or phloretin are much less effective in inhibiting D-glucose uptake.

**Purification of D-Glucose-transporting Vesicle Fraction**—The transport data presented so far serve to compare the kinetics of D-glucose uptake of the vesicle fraction containing the sugar transport system with the passive D-glucose permeability inherent to the lipid portion of vesicles formed by this technique. The data indicate that, even at saturating D-glucose concentrations as high as 800 mM, the vesicle fraction containing the sugar transport system should be severalfold more permeable to D-glucose than the rest of the vesicles. By
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Transport Specificity Fractionation

...glucose-free iso-osmotic glycerol medium for 2 h at 24°C only slightly alters the peak position; this is expected because most of the glucose should remain in such vesicles.

Vesicles were formed with added ghost protein in the presence of 800 mM glucose at the lipid/protein ratio (18:1) demonstrated to insert the d-glucose transport system into vesicles.

Exploiting this d-glucose permeability difference, conditions were achieved to selectively reduce the intravesicular fluid density of the d-glucose-transporting vesicle fraction. This enables that fraction to be purified on a density gradient as follows.

Vesicles are preloaded with 800 mM glucose by substitution of 800 mM glucose for the 800 mM glycerol normally employed in the dialysis medium (see "Methods"). Bio-Gel A-150m chromatography and intravesicular aqueous compartment determinations showed that the substitution of 800 mM d-glucose for 800 mM glycerol in the dialysate does not alter the vesicle size distribution or unilamellar structure.

The particle density of single bilayer vesicles of 550 Å diameter containing 800 mM glucose would be expected to differ by 0.024 g/ml from that of vesicles containing, instead, 800 mM glycerol. As shown in Fig. 7, vesicles formed in the presence of 800 mM glycerol can be separated from vesicles containing 800 mM glucose on a glucose/glycerol density gradient kept iso-osmotic with the intravesicular medium.

The peak positions of the two classes of vesicles on this isopycnic gradient correspond well to their respective particle densities calculated for single bilayer vesicles of this size. Incubation of the glucose-containing protein-free vesicles in

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**FIG. 4.** Concentration dependency of the initial rate of glucose uptake. Stereospecific (d- minus L-) glucose uptake (O—O) and L-glucose uptake (O—O) measured at 23.5°C for 40-s incubations. Vesicles were formed by same procedure as in Fig. 1.

**FIG. 5.** Concentration dependency of the initial rate of stereospecific d-glucose isotope exchange. Conditions permitting exchange diffusion were obtained by forming vesicles under the same conditions as reported in Fig. 1, but with 800 mM d-glucose substituted for 800 mM glycerol in the dialysate. External d-glucose was removed by Sephadex elution in Buffer A + 800 mM glycerol. Vesicles were assayed for incorporation of d- and L-[3H]glucose after dilution with an equal volume of [3H]glucose solution, 2 to 500 mM, as described under "Methods."

**TABLE 1**

| Compound | Concentration | Internally trapped | Externally added |
|----------|---------------|--------------------|------------------|
| Phloretin | 100 μM        | 7 (3)              | 87 (4)           |
| HgCl₂    | 10 μM         | 88 (2)             |                  |
|          | 25 μM         | 73 (2)             |                  |
|          | 50 μM         | 56 (2)             |                  |
|          | 100 μM        | 53 (2)             |                  |
|          | 500 μM        | 42 (2)             |                  |
| HgCl₂    | 100 μM        | 2.5 (3)            |                  |
| HgCl₂    | 100 μM + 5 mM 2-mercaptoethanol | 78 (3)            |
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**FIG. 7.** Profile of protein-free vesicle phospholipid on an isosmotic density gradient. Vesicles formed in Buffer A + 800 mM glycerol • • • •, vesicles formed in Buffer A + 800 mM glucose, then exchanged into Buffer A + 800 mM glycerol on Sephadex, then subjected to incubation at 23.5°C for 0 • 0 or 2 h, • • • •. All vesicles were prepared in absence of added ghosts. Aliquots (15 µl) of each gradient fraction were assayed for phospholipid. Fraction 1 is top of gradient. Preparation of gradients described under "Methods."

about 15% of the vesicles. Vesicles were exchanged on Sephadex into isosmotic glucose-free medium, and incubated for 2 h at 24°C. After concentration by ultrafiltration, the preparation was subjected to the density gradient procedure described above (Fig. 8). Assay of the gradient for stereospecific D-glucose transport activity indicated that the low density portion of the gradient denoted Region A (about 25% of the vesicles) reproducibly contained most of the transport activity. This clearly demonstrates separation of the vesicles containing the sugar transport system from the rest of the population on the basis of transport specificity.

**Polypeptide Composition of Reconstituted Sugar Transport System** —The inclusion of 2-mercaptoethanol as an antioxidant in the buffers used to prepare ghosts results in ghosts with polypeptide patterns on SDS gels (Fig. 9) that are qualitatively similar to those of ghosts prepared by the conventional procedure of Dodge et al. (12). Relative to the "standard" pattern (20), these 2-mercaptoethanol-washed ghosts are somewhat depleted in Bands 5 and 6 (nomenclature of Steck et al. (20, 21)). Fig. 9 also shows the polypeptide pattern of the vesicles before their fractionation on a density gradient.

Fig. 10 shows densitometer tracings of Coomassie blue-stained SDS gels prepared from vesicles after density gradient fractionation. These gels compare the polypeptide pattern of the three gradient regions corresponding to A, B, and C in Fig. 8. Region A, the D-glucose-transporting vesicle fraction, is reproducibly and specifically enriched in protein only in the Band 4.5 area of SDS gels. The tracings indicate that 35% of the Coomassie staining in Region A is in Band 4.5. The Coomassie-stained Band 4.5 of Region A is relatively broad and diffuse. Some of this broadening may be attributable to the method of processing samples to avoid interference by the 140 12 10 12 10 Phospholipid phospholipid, moles

**FIG. 8.** Density gradient fractionation of D-glucose-transporting vesicles. Vesicles containing added ghost protein (18:1 vesicles) formed in the presence of 800 mM intravesicular glucose were processed for and subjected to density gradient centrifugation as described under "Methods." Aliquots (15 µl) of each fraction were assayed for phospholipid (• • • •). Pooled fractions from regions of gradient denoted A, B, and C, were assayed for stereospecific D-glucose transport after the removal of external glucose (see "Methods"); the transport activity is depicted by crosshatched bars.

**FIG. 9.** Coomassie-staining polypeptide patterns of SDS gels of 1, erythrocyte ghosts (25 µg of protein) prepared by conventional procedure of Dodge et al. (12); 2, ghosts (25 µg of protein) prepared in presence of 5 mM 2-mercaptoethanol, as described under "Methods;" 3, unfractionated D-glucose-transporting (18:1) vesicles. Numbers above protein peaks are according to the nomenclature of Steck et al. (20, 21).
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The results clearly demonstrate reconstitution of a stereo-specific $\alpha$-glucose transport system from human red blood cell membranes, and are qualitatively consistent with findings recently reported (15), employing a sonication rather than a dialysis technique to reconstitute $\alpha$-glucose transport. The data reported here, however, generate a more detailed comparison of the reconstituted $\alpha$-glucose transport system with carrier-mediated glucose transport in the red cell.

**Number of Transport Sites Reconstituted per Red Cell Ghost**—Based on the mean vesicle diameter of 550 Å, their single bilayer structure, and x-ray diffraction data on lecithin/cholesterol bilayers (18, 19), each vesicle should contain an average of $\sim 1.5 \times 10^5$ daltons of phospholipid. When the reconstitution was performed by processing 1 mg of red cell ghost protein for every 18 mg of phospholipid employed to form vesicles, what resulted was the insertion of at least one copy of the sugar transport system into 15% of the vesicles; this means that we have reconstituted at least one sugar carrier for every $10.2 \times 10^5$ daltons of phospholipid, hence one carrier/5.74 $\times 10^6$ daltons of ghost protein processed. Dodge et al. (12) have determined the mass of protein per red cell ghost to be $8 \times 10^{12}$ g (3.6 $\times 10^{13}$ daltons/ghost). Thus, we have unambiguously demonstrated the reconstitution of $(3.6 \times 10^{13}$ daltons/ghost) $\div$ (one carrier/5.74 $\times 10^6$ daltons of protein) $= 0.63 \times 10^3$ (±20%) carriers per red cell ghosts. This is a minimum estimate of the number of sugar carriers per red cell for two reasons: some of the vesicles may contain more than one carrier, and not all the carriers in the red cell may have been reconstituted. Our findings unambiguously demonstrate that the sugar carrier comprises at least $\sim 2\%$ of the estimated $3.5 \times 10^6$ polypeptides per red cell ghost (derived from the data of Steck et al. (20, 22)).

**Glucose Transport and Cytochalasin B**—The $K_i$ ($3 \sim 5 \times 10^{-7}$ M) for inhibition of reconstituted $\alpha$-glucose transport by cytochalasin B agrees quantitatively with its $K_i$ for inhibition of glucose transport in the red cell (23–25) and is similar to its $K_i$ for $\alpha$-glucose-competitive cytochalasin binding to red cell membranes (1 to $5 \times 10^{-7}$ M) (25, 26). Recent results indicate that $1.5 \times 10^4$ (25) to $3 \times 10^4$ (26) high affinity cytochalasin B binding sites appear to be intimately associated with the glucose transport system. The number of reconstituted sugar carriers we obtain per ghost (63,000) is substantially lower than the above estimate of cytochalasin B sites. Because our estimate of sugar carrier number by the reconstitution approach is a minimum one, and because more than 1 cytochalasin B molecule may bind for each functional monomer of carrier, our findings do not inherently conflict with the cytochalasin B binding data.

**Inhibition of $\alpha$-Glucose Uptake by Phloretin and Hg$^{2+}$**—Phloretin inhibits carrier-mediated glucose flux in erythrocytes (27). Benes et al. (28) have shown asymmetry in the action of phloretin; externally added phloretin selectively inhibits glucose efflux, but internal phloretin is required to inhibit uptake of glucose in the red cell ghost. Our finding that internal phloretin is required to inhibit influx of $\alpha$-glucose agrees with this study.

Externally added Hg$^{2+}$ inhibits $\alpha$-glucose transport in the red cell (29); this inhibition is reversed by thiol reagents. We find marked asymmetry in our sulfhydryl reagent-reversible inhibition of $\alpha$-glucose uptake by Hg$^{2+}$; only about one-half of the transport is inhibitable by externally added Hg$^{2+}$, complete inhibition requiring internal Hg$^{2+}$ as well. A likely enormous excess of phospholipid present in the vesicle fractions (see "Methods"). A large amount of Band 6 which was present in the vesicle preparation before density gradient centrifugation (Fig. 9) does not co-sediment with the vesicles on the gradient.

![Coomassie-staining polypeptide patterns](image-url)
"Transport Specificity Fractionation" of Sugar Carrier

reason for this is that, as has been found for reconstituted
(NaK)-ATPase (9), the vesicles contain populations of reconsti-
tuted transport protein that are oriented both "inside out" as well as "right side out" with respect of its normal in vivo orientation; thus, internal Hg²⁺ is required to inhibit glucose uptake by this inside out fraction of reconstituted sugar carrier. The fact that virtually all of the D-glucose uptake is inhibited by external cytochalasin B may be because the relatively hydrophobic cytochalasin B molecule can readily enter the vesicle.

Maximum Velocity of Reconstituted Glucose Transport —
The Kₐ values of the reconstituted transport system for D-
glucose uptake (20 to 25 mM) and for exchange diffusion (40 to 45 mM) are similar to those values (25 and 38 mM, respectively) for these processes as observed in the erythrocyte (30, 31).

Under saturating conditions, efflux of glucose at 20° from resealed ghosts depletes the internal concentration of glucose at the rate of about 100 mm/min (32). Assuming that the maximum estimate of 3 x 10⁹ transport-associated cytocha-
lasin B binding sites (26) represents the number of sugar carriers per red cell and given a volume per cell of 110 μm³ (12), the rate of glucose efflux at saturation is 0.35 x 10⁻⁹ mol/site/min. Based on the maximal rate for exchange diffusion of glucose at 20° of 260 mm/min (30), a calculation along the above lines yields 0.87 x 10⁻⁹ mol/site/min as maximal D-
glucose exchange rate. Assuming one carrier/vesicle shown to contain the transport system, our measurements of Vₘₐₓ for unidirectional influx and diffusion exchange suggest that the sugar carrier contains a protein that does not bind Coomassie stain. It is expected that further utilization of the transport specificity fractionation approach will facilitate the attainment of this objective. This novel approach may be of utility in the identification and isolation of other membrane transport components.

Acknowledgments — We wish to thank Guido Guidotti, Robert Farley, Kurt Drickamer, Kathleen Sweadner, and members of the Pharmacology Department of Harvard Medical School for useful discussions.

Note Added in Proof — Since this manuscript was submitted, a full paper describing one of the reconstitution studies cited above in abstract form (34) appeared in this journal (Kasahara, M., and Hinkle, P. C. (1977) J. Biol. Chem. 252, 7384-7390).

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