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Reconstitution of D-Glucose Transport in Vesicles Composed of Lipids and Intrinsic Protein (Zone 4.5) of the Human Erythrocyte Membrane

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Elucidation of the mechanism of facilitated D-glucose transport in human erythrocytes is dependent on the identification and isolation of the membrane protein(s) mediating this process. Based on the fact that stereospecific D-glucose transport is reconstituted in liposomes prepared by sonication of a lipid suspension with ghosts or fractions derived from ghosts, a quantitative assay for the stereospecific D-glucose transport activity of these fractions was developed (Zala CA, Kahlenberg A: Biochem Biophys Res Commun 72:866, 1976). This assay was used to monitor the purification of ghosts. The solubilized membrane protein fraction was chromatographed on a column of diethylaminoethyl cellulose which was eluted stepwise with NaCl-phosphate buffers of increasing ionic strength. A fraction, eluted at an ionic strength of 0.1, displayed a 13- and 27-fold increase in reconstituted transport activity relative to ghosts and to the unfractionated Triton X-100 extract, respectively. This fraction, when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, consisted predominantly of the ghost proteins with an apparent molecular weight of 55,000, commonly designated as zone 4.5; periodic acid-Schiff-sensitive membrane glycoproteins 1–4 were absent. Transport reconstituted by this preparation of zone 4.5 membrane proteins was almost completely abolished by 1-fluoro-2,4-dinitrobenzene, mercuric chloride, and p-chloromercuribenzene sulfonate, but was unaffected by sodium iodoacetate. Extra- and intraliposomal phloretin and cytochalasin B, respectively, exhibited partial inhibition. The stereospecificity and inhibition characteristics of the reconstituted transport imply that all the components of the erythrocyte D-glucose transport system are contained in the zone 4.5 membrane protein preparation.

Key words: erythrocytes, glucose transport, glucose transport protein, liposomes, reconstitution

Abbreviations: SDS-PAGE – sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DEAE – diethylaminoethyl; PAS – periodic acid-Schiff; FDNB – 1-fluoro-2,4-dinitrobenzene; NEM – N-ethylmaleimide; PMBS – p-chloromercuribenzene sulfonate.

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INTRODUCTION

The human erythrocyte monosaccharide transport system is a typical example of facilitated diffusion (1, 2), whereby a stereospecific mechanism equilibrates the concentration of the permeant across the cell membrane. This transport system is inhibited by protein-alkylating reagents and exhibits a high degree of substrate structural specificity, a property which is not mimicked by erythrocyte membrane phospholipids (for reviews see Refs. 3, 4). Consequently, all models of the erythrocyte monosaccharide transport system invoke a membrane protein(s), hereafter referred to as the D-glucose transport protein, possessing one or more sugar binding sites (3, 4).

Although 3 distinct amino acid residues of the D-glucose transport protein essential for its activity have been identified (5), the results of recent attempts at the identification of this membrane component have not been in agreement. In inhibitor binding studies, band 3 protein (6), bands 3 and 4 (7), membrane proteins of zone 4.5 (8), or protein approximately 180,000 in molecular weight (9) were implicated in the erythrocyte sugar transport system [nomenclature of membrane proteins separated in SDS-PAGE and stained with Coomassie blue is according to Steck (10)]. Other attempts to identify the D-glucose transport protein involved the use of selective extraction procedures coupled to the measurement of stereospecific D-glucose uptake by the residual membrane preparations (11) or to the reconstitution of D-glucose transport catalyzed by Triton X-100 extracts of these preparations when incorporated in sonicated liposomes (12, 13). Based on several considerations (reviewed in Ref. 11), band 3 protein, which represented the major polypeptide component of the membrane preparations or Triton X-100 extracts thereof, was suggested to contain the erythrocyte D-glucose transport protein (11–13).

In this report however, we show that following fractionation of a Triton X-100 extract of erythrocyte membrane protein on DEAE-cellulose, reconstitution of D-glucose transport is associated with a column eluate which is devoid of band 3 protein and PAS-sensitive glycoproteins, PAS 1–4; this fraction consists predominantly of the proteins of the broad, complex region designated zone 4.5 on SDS-PAGE.

MATERIALS AND METHODS

All ^{14}C- and ^{3}H-labeled sugars were obtained from New England Nuclear Corporation (Boston, Massachusetts); SDS from Pierce; cytochalasin B from Aldrich; polyacrylamide gel electrophoresis reagents and Bio-Gel P4 from Bio-Rad; inorganic salts and organic solvents from Fisher or Baker; and all other reagents from Sigma Chemical Company (St. Louis, Missouri). Diaflo PM 10 ultrafiltration membranes and Minicon B15 macrosolute concentrators were obtained from Amicon, and DE 52 DEAE-cellulose from Whatman.

Hypotonic phosphate buffer contained 6.20 mM Na_2HPO_4 and 0.70 mM NaH_2PO_4 and was adjusted to pH 7.5 at 5°C. Solution 1 ($\mu = 0.02$) contained 2 mM NaN_3, 0.56 mM NaH_2PO_4, 4.44 mM $NaHPO_4$, 4.18 mM NaCl, and 0.125% Triton X-100 and was adjusted to pH 8.0 at 5°C. Solution 2 ($\mu = 0.02$) was identical to solution 1 except that the concentration of Triton X-100 was 0.100%. Solutions 3 ($\mu = 0.1$) and 4 ($\mu = 0.5$) were identical to solution 2 except that the concentrations of NaCl were 84 mM and 484 mM, respectively. Liposome-forming buffer contained 20 mM $MgCl_2$, 0.03 mM $CaCl_2$, 5 mM Na_2HPO_4, 3 mM NaN_3, and 115.0 mM NaCl and was adjusted to pH 7.4. A stock solution of 5 mM D-[2-^{3}H]glucose (specific activity 160 μCi/mmole) and 5 mM L-[1-^{14}C]glucose (specific activity 100 μCi/mmole) was prepared in liposome-forming buffer and used for all experiments except the reverse isotope experiments, where a similar solution of 5 mM D-[U-^{14}C]-

glucose (specific activity 100 μCi/mmole) and 5 mM L-[1-^{3}H]glucose (specific activity 100 μCi/mmole) in liposome-forming buffer was used.

Hemoglobin-free erythrocyte ghosts were prepared from recently outdated transfusion blood by hypotonic hemolysis (14) as previously described (15). Extraction of ghosts was performed at 5°C as follows: Twenty-four milliliters of ghosts at a protein concentration of 3–4 mg/ml were added to 96 ml of solution 1; after mixing and incubating for 15 min, the supernatant and pellet fractions were separated by centrifugation at $10^5 \times g$ for 1 h. The pellet was washed once with a 10-fold volume of hypotonic phosphate buffer and made up to 10 ml in this buffer; aliquots of the pellet and supernatant were removed for protein determination (16) and analysis by SDS-PAGE (11, 17, 18). The Triton X-100 extract (105 ml) was adjusted to pH 8.0 at 5°C with 0.5 N NaOH and applied at a flow rate of 50 ml/h to a DE 52 DEAE-cellulose column (2.3 × 7 cm), equilibrated with solution 2 at 5°C. After loading, the column was washed with 10 ml of solution 2, 50 ml of solution 3 ($\mu = 0.1$), followed, in initial experiments, by 50 ml of solution 4 ($\mu = 0.5$). Aliquots of 0.25 ml were removed from each column fraction (4 ml) for protein analysis. The 4 fractions (15 ml) under each peak containing the highest protein values (Fig. 2) were separately pooled, concentrated to 3.5–4.0 ml using an Amicon PM 10 membrane and assayed for protein and reconstitution of D-glucose transport. In the case of the 0.5-μ fraction, polypeptide composition was determined by SDS-PAGE. Prior to similar analysis of the polypeptide composition of the 0.1-μ fraction. SDS was added to a concentration of 1% and the fraction was further concentrated 10-fold in a Minicon B15 macrosolute concentrator. The addition of SDS prevented the appearance on Coomassie blue-stained gels of a second broad band with an apparent molecular weight of 120,000, presumably resulting from an aggregation of approximately one-third of the zone 4.5 proteins during concentration.

Reconstitution of D-glucose transport, based on the stereospecific efflux of D- relative to L-glucose from sonicated liposomes, was measured by a slight modification of the method previously described (12). Briefly, the membrane protein fraction is incorporated into liposome bilayers by sonication with a suspension of erythrocyte lipids containing both D-[2-^{3}H]- and L-[1-^{14}C]glucose. The sonicated suspension is then passed through a Bio-Gel P4 column which retards extraliposomal glucose, resulting in a concentration gradient between the inside and the outside of the liposomes. Thus, liposomes reconstituted in the presence of D-glucose transport protein lose their D-glucose while retaining the L-isomer; this D-glucose is in turn separated from the liposomes during further gel filtration. The liposomes are collected in the void volume and analyzed for ^{3}H and ^{14}C and protein content. The nmoles of D-glucose stereospecifically lost from the liposomes per mg protein is then calculated.

The present procedure differed from that previously described (12) in that a solution containing 25 mg of lipid was evaporated to dryness on the bottom of each Quickfit tube; 1.67 ml of a stock solution of 5 mM D- and L-glucose was then added followed by 0.40 ml of various membrane protein fractions. Appropriate volumes of liposome buffer and inhibitor solution, where indicated, were added to give a final volume of 2.5 ml. Protein-free control samples contained the stock sugar solution, 0.8 ml of liposome buffer, and 0.02 ml of 10% Triton X-100. Tubes were sonicated at 30°C for 12 min, and 1-ml duplicates removed for gel filtration. The concentration of D- and L-glucose before gel filtration was therefore 3.33 mM so that the specific activity of D-glucose transport is equal to

$$\left[\frac{\text{dpm } ^{14}\text{C after}}{\text{dpm } ^{14}\text{C before}} - \frac{\text{dpm } ^{3}\text{H after}}{\text{dpm } ^{3}\text{H before}}\right] \times \frac{3{,}330 \text{ nmoles D-glucose/ml}}{[\text{protein}]\ (\text{mg/ml})}$$

The inhibition experiments were performed as follows. For the covalent inhibitors, solutions of 150 mM FDNB or NEM in ethanol or 150 mM sodium iodoacetate (pH 7.5) in distilled water were prepared; 0.133 ml of these solutions was added to the sonicated liposome suspension. The samples were then incubated for 1 h at 25°C and duplicate aliquots were assayed for reconstituted D-glucose transport activity. For $HgCl_2$ or PMBS, 0.05 ml of 5 mM aqueous solutions of these inhibitors were added to the liposome suspension prior to sonication. This latter procedure was used also for the noncovalent inhibitors, 10 mM phloretin or 0.5 mM cytochalasin B, which were dissolved in ethanol. In all cases, control samples contained the equivalent volume of the corresponding solvent. In experiments where phloretin was present outside the liposomes during gel filtration on Bio-Gel P4, the columns were previously equilibrated with 15 ml of liposome buffer containing 0.2 mM phloretin and 2% ethanol; control samples were chromatographed on columns equilibrated with 2% ethanol in liposome buffer.

RESULTS

Fractionation of Erythrocyte Membrane Proteins and Reconstitution of D-Glucose Transport

Table I summarizes the data on the fractionation of ghost proteins and reconstitution of D-glucose transport catalyzed by each fraction, the polypeptide composition of which is shown in Fig. 1. Extraction of ghosts with 0.1% Triton X-100 ($\mu = 0.02$) resulted in the solubilization of 22% of the membrane protein and 13% of the D-glucose transport activity. The membrane proteins solubilized were portions of bands 3, 4.2, 5, 6, 7, zone 4.5, and all of the PAS-sensitive glycoproteins. The remainder of the reconstituted D-glucose transport activity of ghosts was recovered in the membrane pellet after Triton X-100 extraction, which contained each of the major Coomassie blue-staining polypeptide bands of ghosts but none of the PAS-sensitive glycoproteins, PAS 1–4.

TABLE I. Reconstituted D-Glucose Transport Activity and Protein Content of Various Erythrocyte Membrane Fractions*

Membrane fraction	Protein (mg)	Protein (%)	D-Glucose transport specific activity (nmoles/mg)[a]	Total D-glucose transport activity (nmoles)	Total D-glucose transport activity (%)
Whole ghosts	87.3	100	93.2 ± 8.2 (23)	8,140	100
0.1% Triton X-100 pellet	56.3	65	131.0 ± 32.7 (6)	7,370	90
0.1% Triton X-100 supernatant	19.9	22	52.5 ± 8.4 (6)	1,038	13
0.1-μ fraction	1.1	1	311.6 ± 30.2 (23)	377	4
0.5-μ fraction	6.2	7	13.7 ± 1.7 (6)	85	1

*Membrane fractions were prepared and incorporated into liposomes which were assayed for D-glucose transport activity as described in Methods. The polypeptide composition of each fraction is shown in Fig. 1. Transport specific activity refers to the nmoles of D-glucose stereospecifically lost from the liposomes per mg of membrane protein associated with the liposomes following chromatography on Bio-Gel P4. Total transport activity represents the product of the specific activity of D-glucose transport and the protein content of the membrane fraction.

[a]In this and subsequent tables, values for D-glucose transport activity are the mean ± standard error of the results from the number of experiments shown in parentheses.

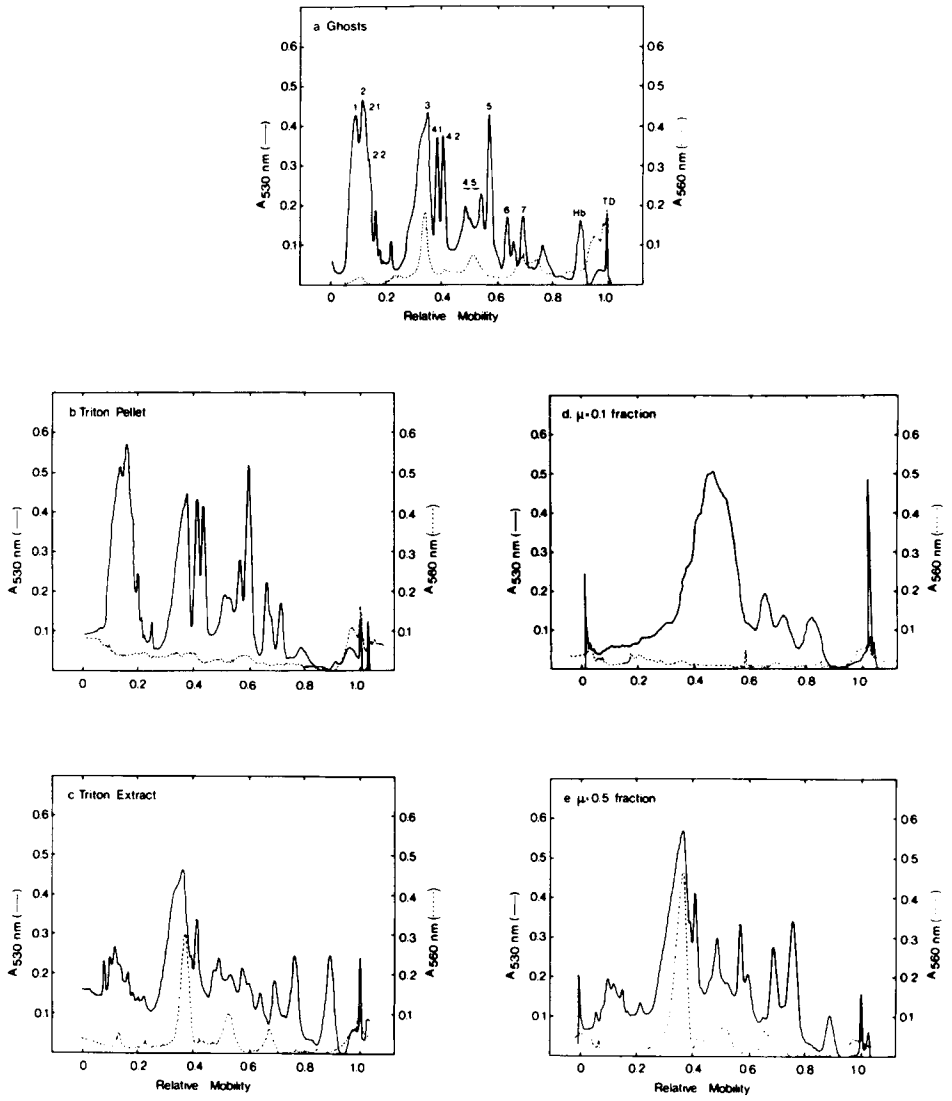

Fig. 1. Protein composition of erythrocyte membrane fractions incorporated into liposomes. Ghosts were extracted with 0.1% Triton X-100 and the resultant solubilized membrane proteins were separated into the 0.1- and 0.5-μ fractions by chromatography on DEAE-cellulose (see Methods for details). Samples from the designated membrane fractions were analyzed electrophoretically on 5% polyacrylamide gels in 0.2% SDS. Solid lines) scans of Coomassie blue-stained gels at 530 nm; dashed lines) scans of periodic acid-Schiff-stained gels at 560 nm. The major Coomassie blue-stained bands were enumerated according to increasing electrophoretic mobility as described by Steck (10). TD) inked needle stab recording the position of the tracking dye; Hb) hemoglobin.

Figure 2 shows the results of chromatography of the Triton X-100 extract of ghosts on a column of DEAE-cellulose. A small amount of protein (12% of that applied) was eluted by solution 3 (μ = 0.1) while 76% was eluted by solution 4 (μ = 0.5). The 0.1-μ fraction consisted primarily of zone 4.5 proteins, with no detectable PAS-sensitive glycoproteins, PAS 1–4. In contrast, the polypeptide and PAS-sensitive glycoprotein composi-

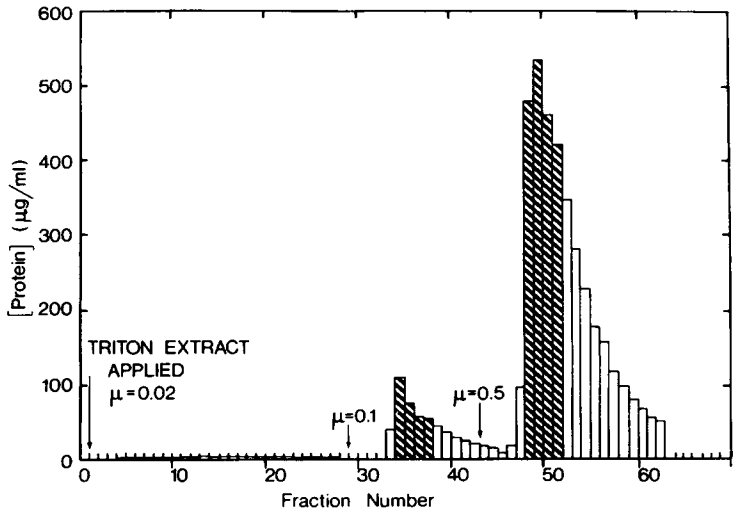

Fig. 2. DEAE-cellulose chromatography of Triton X-100-solubilized erythrocyte membrane proteins. The Triton X-100 extract (105 ml) was applied to a column of DEAE-cellulose and eluted stepwise with NaCl-phosphate buffers of increasing ionic strength (μ = 0.1 and 0.5) as described in Methods. The 4 fractions under each peak containing the highest protein values (hatched lines) were separately pooled and concentrated to yield the 0.1- and 0.5-μ fractions.

tion of the 0.5-μ fraction was similar to that of the original Triton X-100 extract, with some zone 4.5 protein still present (Fig. 1).

In previous reconstitution studies (12), both the membrane residue and Triton X-100 extract of 2,3-dimethylmaleic anhydride-treated ghosts were capable of catalyzing stereospecific D-glucose efflux. The proteins common to these 2 fractions were those of bands 3 and 7 and zone 4.5. The present fractionation of the Triton X-100 solubilized membrane proteins by column chromatography on DEAE-cellulose represents a substantial purification of the D-glucose transport protein. The 0.1-μ fraction displayed a 3.3- and 6-fold increase in D-glucose transport specific activity relative to ghosts and the unfractionated Triton X-100 extract, respectively (Table I). In contrast, the residual Triton X-100 solubilized membrane proteins eluted in the 0.5-μ fraction had a transport specific activity amounting to only 15% of that of the original ghost preparation. Further analysis of the extent of the present purification of the erythrocyte D-glucose transport protein is described below.

Properties of the Reconstituted D-Glucose Transport System

The nmoles of D-glucose lost from the reconstituted liposomes was measured as a function of the amount of membrane protein associated with the liposomes collected in the void volume of the Bio-Gel P4 columns. The lipid suspensions were supplemented with 0.02–0.8 ml of ghosts or the 0.1-μ fraction and sufficient 10% Triton X-100 was added to the latter samples to give a final concentration of 0.16%. With both types of reconstituted liposome preparations, a small amount of membrane protein produced a large efflux of D-glucose (Fig. 3). However, as the protein content of the liposomes was increased, stereospecific D-glucose efflux displayed an asymptotic approach to a limiting value of approximately 30 and 15 nmoles for ghosts and the 0.1-μ fraction, respectively. Thus, the effect on D-glucose transport of incorporation of functional D-glucose transport protein into liposomes formed in the presence of a fixed amount (25 mg) of lipid is a saturable process.

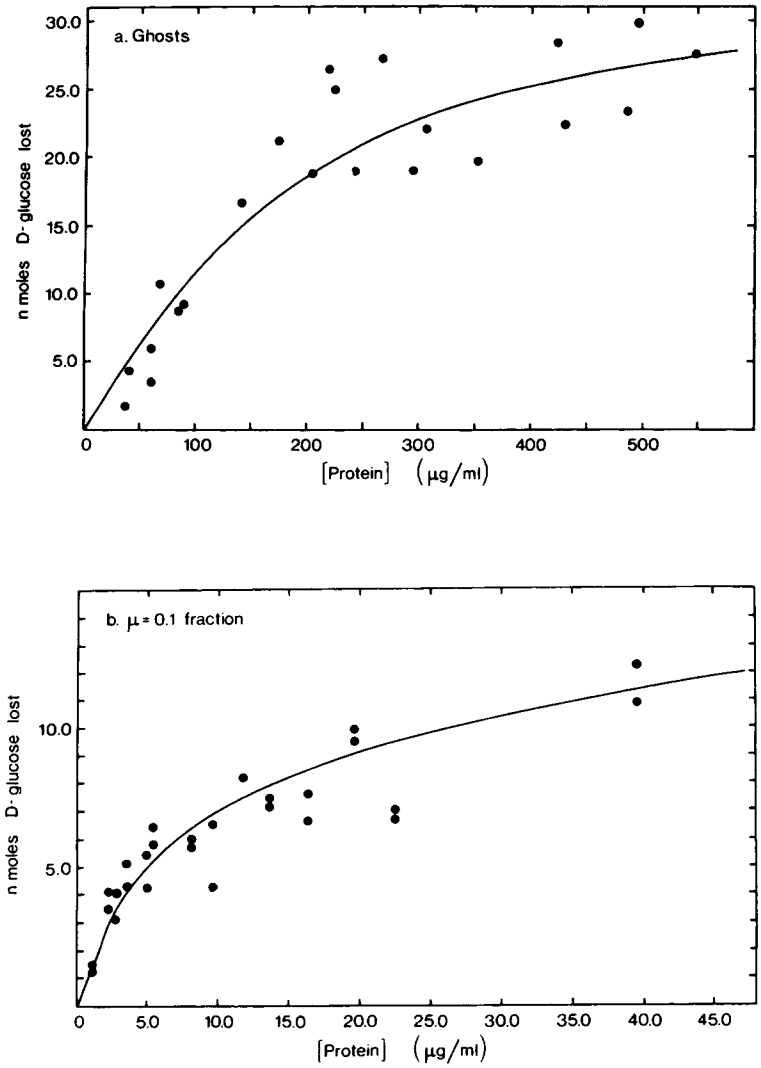

Fig. 3. Reconstituted D-glucose transport activity as a function of membrane protein content of liposomes. Liposomes were reconstituted in the presence of increasing amounts of ghosts (a) or the 0.1-μ fraction (b). The nmoles of D-glucose lost from the reconstituted liposomes was measured as a function of the amount of membrane protein associated with the liposomes collected in the void volume of the Bio-Gel P4 columns (see Methods).

The specific activity values recorded in Table I for the reconstituted transport of D-glucose catalyzed by ghosts or the 0.1-μ fraction were obtained from experiments utilizing a liposomal protein concentration (after gel filtration) of 150–300 and 10–20 μg per ml, respectively. As indicated in Fig. 3, these protein values were in excess of those required for maximum specific activities of reconstituted transport. Since a comparison of the transport specific activity of liposomes reconstituted in the presence of these 2 membrane preparations should be made under conditions in which the liposomal protein concentration is the limiting factor in the amount of stereospecific D-glucose efflux, the values of the initial slopes of the curves in Fig. 3 were used to calculate specific activity.

The slope of the initial linear portion of each curve (8 data points), determined by regression analysis, gave D-glucose transport specific activity values for liposomes reconstituted with ghosts and the 0.1-μ fraction of 111 and 1,420 nmoles per mg membrane protein, respectively. Thus relative to ghosts, Triton X-100 extraction and DEAE-cellulose fractionation results in a 13-fold purification of the erythrocyte D-glucose transport protein, with the recovery of 16% of the initial total transport activity of ghosts in the 0.1-μ fraction based on the protein recoveries listed in Table I.

The concentrated 0.1-μ fraction was unstable and had to be rapidly reconstituted to prevent losses in activity; incubation for 2 h at 20°C decreased activity to 48% of that observed following immediate reconstitution. In addition, incubation for 10 min at 40°C or higher temperatures resulted in complete loss of activity. However, once the fractions had been incorporated into liposomes and stored at 5°C prior to gel filtration, activity was slowly lost, with a half-time of about 1 day (data not shown).

The use of D-[U-^{14}C]glucose and L-[1-^{3}H]glucose as tracers instead of D-[2-^{3}H]-glucose and L-[1-^{14}C]glucose did not affect the D-glucose transport activity values. In 6 experiments, the difference in D-glucose efflux measured by the reverse isotope group (6.76 ± 1.15 nmoles) and the usual mixture of radioactive monosaccharides (8.32 ± 1.89 nmoles) was not significant ($P > 0.1$). This result indicates that the reconstituted D-glucose transport activity measured in the present experiments cannot be due to an isotope effect.

Effect of Inhibitors on the Reconstituted Transport System

Reconstituted transport activity catalyzed by the 0.1-μ fraction was tested for other properties of the erythrocyte D-glucose transport system by exposing the liposomes to various known inhibitors and a noninhibitor of transport. Incubation of the sonicated liposomes with 10 mM FDNB for 1 h at 25°C essentially abolished transport, while 10 mM NEM gave partial inhibition; 10 mM iodoacetate failed to inhibit (Table II). These results are in agreement with those obtained with intact red cells (19).

The entrapment of mercuric chloride and PMBS (0.1 mM) within liposomes during sonication (extraliposomal $HgCl_2$ and PMBS are retarded during gel filtration) strongly inhibited D-glucose transport. Phloretin, however, must be present on the outside face of the liposomes during gel filtration to inhibit D-glucose efflux (Table II). This agrees with the observation of Benes et al (20) that phloretin inhibits transport in ghosts only if present at the face to which diffusion is proceeding. The failure to completely inhibit transport by the high concentration of phloretin (0.2 mM; 80–400 × K_i) (21, 22) may be due to adsorption of the inhibitor to the very high surface area of the liposomes (23), with the concomitant drastic lowering of its effective concentration. Cytochalasin B, a competitive inhibitor of erythrocyte D-glucose transport (24, 25), when present intraliposomally during gel filtration at a concentration of 100 × K_i (24, 25) partially inhibited transport (Table II).

Effect of Increasing Concentrations of Substrate on Reconstituted D-Glucose Transport

The assay for measuring the reconstitution of D-glucose transport activity is based on the fact that the time required for the filtration of liposomes on Bio-Gel P4 is slow enough to allow complete loss of D-glucose from reconstituted liposomes when present at an initial concentration of 3.33 mM. Using liposomes loaded with higher concentrations of D-and L-glucose, one might expect that above a certain substrate concentration the time (2–4 min) of gel filtration might not be sufficient to allow for the complete loss of D-glucose from reconstituted liposomes. Figure 4 shows the results obtained when the

TABLE II. Effect of Known Inhibitors of D-Glucose Transport on the Reconstituted Transport System*

Inhibitor	Transport activity relative to control (%)
1-Fluoro-2,4-dinitrobenzene (10 mM)	3.2 ± 1.7 (4)
N-Ethylmaleimide (10 mM)	56.7 ± 12.6 (4)
Iodoacetate (10 mM)	96.3 ± 6.9 (4)
$HgCl_2$, inside[a] (0.1 mM)	4.2 ± 1.5 (4)
p-Chloromercuribenzene sulfonate, inside (0.1 mM)	7.9 ± 3.5 (4)
Phloretin (0.2 mM)	
inside	110.8 ± 7.6 (6)
inside and outside	44.9 ± 9.6 (4)
outside	61.8 ± 1.4 (4)
Cytochalasin B, inside (0.01 mM)	56.8 ± 8.7 (4)

*The 0.1-μ fraction, recovered from the DEAE-cellulose column (Fig. 2), was used for reconstitution. Mercuric chloride, PMBS, phloretin, and cytochalasin B were added to the lipid suspension before sonication followed immediately by gel filtration; FDNB, NEM, and iodoacetate were added to the liposomes after sonication and the treated samples were incubated for 1 h at 25°C prior to gel filtration. Reconstituted D-glucose transport activity was measured as described in Methods.

[a]Inside and outside refers to the presence during gel filtration of the inhibitor in the intra- and extraliposomal space, respectively.

0.1-μ fraction was sonicated with lipid suspensions containing increasing, but equal, concentrations of D- and L-glucose. Above a concentration of 25 mM, the D-glucose transport activity (solid line) fell below that predicted by extrapolation of the relation defined by the 4 points between 3.33 and 25 mM (dashed line), thus indicating a trend toward substrate saturation of the transport system. At 100 mM, the highest sugar concentration tested, the observed transport activity was 67% of the extrapolated vlaue. It is significant that the substrate concentration used for all other experiments (3.33 mM) is well below that at which nonlinearity in transport activity is apparent.

Substrate Specificity of the Reconstituted Transport System

For the same reasons that an apparent saturation of reconstituted D-glucose transport activity is evident with increasing concentrations of substrate, a varying degree of inhibition of D-glucose transport by sugars with high affinities for the erythrocyte monosaccharide transport system should be observed. The degree of inhibition is a function of the affinity of the sugar analogue, relative to that of D-glucose, for the transport protein. The addition of 100 mM concentrations of sugar analogues to the lipid suspensions ([D- and L-glucose] each equal 3.33 mM) prior to sonication in the presence of the 0.1-μ fraction on the inhibition of D-glucose transport activity is shown in Table III. The most effective inhibition of transport is displayed by the nontransported sugar, maltose (22). Of the transported sugar analogues tested, only 2-deoxy-D-glucose and D-galactose, which have the highest affinities for the D-glucose transport protein (26), inhibited D-glucose efflux.

For the transported sugar analogues, there are 2 effects to consider which contribute to the resultant inhibition of reconstituted D-glucose transport and make quantitative interpretation of the data difficult. 2-Deoxy-D-glucose, by virtue of its high affinity

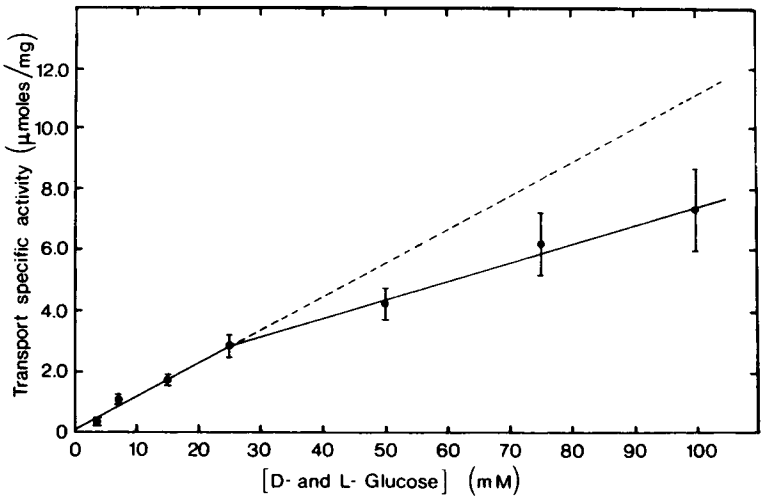

Fig. 4. Reconstituted D-glucose transport activity as a function of increasing concentrations of D-glucose. Liposomes reconstituted in the presence of the 0.1-μ fraction of membrane protein and increasing but equal concentrations of D- and L-glucose were prepared and assayed for D-glucose transport activity (solid line) as described in Methods. The dashed line is a linear extrapolation of the transport activity measured between 3.33 and 25 mM D-glucose. Transport specific activity values are the mean ± standard error of 6 experiments.

together with its high concentration relative to D-glucose, will initially be bound to most of the binding sites on the transport protein and strongly inhibit D-glucose efflux. As efflux progresses, however, the intraliposomal 2-deoxy-D-glucose concentration decreases and with it, the proportion of binding sites occupied by 2-deoxy-D-glucose. With time there will be an increasing efflux of D-glucose. Ultimately, the D-glucose lost would be equal to that lost in the absence of 2-deoxy-D-glucose. However, the time of gel filtration is too short to allow these processes to reach equilibrium, and inhibition of D-glucose efflux by 2-deoxy-D-glucose is observed. A similar argument applies to D-galactose. Sugars of low affinity, despite their high concentration, do not compete with D-glucose for sufficient binding sites to inhibit transport activity under the conditions of the assay. However, since maltose is a nontransported sugar, its intraliposomal concentration remains constant during gel filtration and thus it competes with D-glucose. Therefore, maltose, despite its lower affinity, inhibited D-glucose transport to the same extent as 2-deoxy-D-glucose. As would be expected from its apparent affinity, the degree of inhibition by 2-deoxy-D-glucose is about the same as that observed for D-glucose (Fig. 4) at a concentration of 100 mM.

DISCUSSION

The D-glucose transport system of the human erythrocyte membrane has been the subject of intensive kinetic analysis and many functional properties of the system have been defined (1–4). However, the identity and structure of the D-glucose transport protein(s), its interaction with substrates and membrane lipids, and the molecular processes resulting in the transport event all remain to be elucidated. A first goal in realizing these objectives is the identification of the membrane protein(s) involved in D-glucose transport

TABLE III. Effect of Sugar Analogues on the Activity of the Reconstituted D-Glucose Transport System*

Sugar added	K_m	Transport activity relative to control (%)
Maltose	14	59.4 ± 26.4
2-Deoxy-D-glucose	4	67.1 ± 13.1
D-Galactose	35	73.2 ± 24.8
L-Arabinose	130	112.0 ± 23.4
D-Fucose	240	111.2 ± 22.0
L-Fucose	2,500	112.0 ± 15.4

*Reconstituted D-glucose transport activity, catalyzed by the 0.1-μ fraction, was measured in the presence of 3.33 mM D- and L-glucose as described in Methods. When present, the sugar analogues were added to the lipid suspensions at a concentration of 100 mM prior to sonication. Maltose is a nontransported, competitive inhibitor of the erythrocyte monosaccharide transport system (22). The other transported sugars are listed in a descending order of their apparent affinities (K_m) for the D-glucose transport protein (26).

and the reconstitution of a minimal system capable of catalyzing D-glucose transport. The present study represents a step in this direction.

Upon chromatography of a Triton X-100 extract of erythrocyte ghosts on DEAE-cellulose, a fraction eluted at an ionic strength of 0.1 and consisting predominantly of the polypeptides of zone 4.5, was shown to catalyze D-glucose transport when reconstituted into liposomes. The transport activity of various membrane preparations correlated with their relative amounts of zone 4.5 protein: by SDS-PAGE analysis, zone 4.5 protein accounted for 11, 80, and 12% of the major Coomassie blue-stained membrane polypeptides of ghosts, the 0.1-μ, and the 0.5-μ fractions, respectively. Comparison of the transport specific activities of liposomes reconstituted in the presence of ghosts and the 0.1-μ fraction, under conditions where the amount of membrane protein is limiting, suggests that a 13-fold purification of the D-glucose transport protein had been achieved. However, it should be noted that this degree of purification is an apparent value, dependent on an evaluation of the precise relation between the number of transport proteins in various membrane preparations and their efficiency of reconstitution and consequent ability to support D-glucose transport.

In addition to exhibiting stereospecificity, D-glucose transport reconstituted by the 0.1-μ fraction was shown to have other properties characteristic of the erythrocyte monosaccharide transport system. a) Phloretin, cytochalasin B, and sulfhydryl reagents which inhibit D-glucose transport in erythrocytes (1–4, 26), inhibited the reconstituted transport system. b) There was a tendency to exhibit substrate saturation at high D-glucose concentrations. c) An inhibition of D-glucose transport activity by high-affinity sugars was observed. These data taken together constitute strong evidence that a partially purified, functionally intact D-glucose transport system has been reconstituted in liposomes.

At a Triton X-100 concentration of 0.1%, only 13% of the total D-glucose transport activity of ghosts was extracted. Although we attempted to improve the extraction of this activity by using higher concentrations of Triton X-100, activity was found to decrease, possibly because of detergent-induced denaturation; the optimal concentration for extraction was found to be 0.1% (unpublished data). Furthermore, use of higher concentrations

of Triton X-100 results in a decrease in the selectivity of extraction of intrinsic membrane proteins (27), so that subsequent purification of the transport protein might prove more difficult. Since extraction and recovery of D-glucose transport activity were incomplete, the possibility that a distinct subpopulation of D-glucose transport proteins may have been selectively purified cannot be excluded.

The present assay for reconstituted D-glucose transport does not provide a measure of the rate of efflux but rather monitors the amount of D-glucose completely lost from functionally reconstituted liposomes during gel filtration. The assay is therefore not suitable for measurement of kinetic parameters and other rate-dependent properties such as competitive inhibition. However, the method is well suited for estimation of the relative amounts of functional transport protein and is especially useful in the measurement of irreversible inhibition.

Sonicated lipid suspensions are known to contain 2 general classes of liposomes: a population of small, fairly uniform, single-walled spherules 25–30 nm in diameter and a more heterogeneous population of larger size (28). The predominant presence of the former in our sonicated ghost-lipid suspensions was confirmed by electron microscopy (unpublished data). The aqueous volume contained by a small liposome of 11 nm internal radius is 5.6×10^{-18} ml. At a D-glucose concentration of 3.33 mM, the average small liposome would therefore contain about 11 molecules of sugar. This type of calculation helps explain several of our observations if it is assumed that the bulk of transport observed is due to loss of D-glucose from these small liposomes. First, a single transport protein need only catalyze the net efflux of 11 molecules of D-glucose during the time of filtration on Bio-Gel P4 (2–4 min). Even if the reconstituted transport protein had an unusually low turnover number, such a small number of molecules could be lost extremely rapidly. Second, the presence of 2 or more functional transport proteins per liposome would have no further effect on the loss of D-glucose, which would approach a maximum value as an increasing number of transport proteins are incorporated into a constant number of liposomes (Fig. 3). Third, at a concentration of 100 mM, approximately 340 molecules of a high-affinity sugar are present inside the small liposomes; most, if not all, would be lost during gel filtration, and inhibition of D-glucose transport, if seen at all, would be weak (Table III).

Similar considerations apply to the inhibition by chemical reagents (Table II). If $HgCl_2$ and PMBS, at an overall concentration of 0.1 mM, were uniformly distributed throughout the suspension, mercuric and PMBS ions would be present in one out of every 3.4 small liposomes; similarly, at 0.01 mM, cytochalasin B molecules would be included in one out of every 34 liposomes. The observed complete inhibition by mercurials may be explained on the basis of their extremely high reactivity with sulfhydryl groups, so that they would remain complexed with these groups on the D-glucose transport protein during gel filtration. On the other hand, binding of cytochalasin B is rapidly reversible (24), so that essentially all cytochalasin B would be removed from the extraliposomal space during gel filtration. Only intraliposomal cytochalasin B would therefore cause inhibition. If cytochalasin B had bound to a substantial number of sites on the intraliposomal exposed portion of the D-glucose transport protein, this intraliposomal cytochalasin B would cause substantial inhibition of D-glucose efflux (Table II). In this situation, the value of inhibition would be between the small value predicted by liposomal entrapment of cytochalasin B on the basis of its overall concentration and the value of about 100% expected if the binding equilibrium were very slowly reversible.

Collectively, the polypeptides of zone 4.5 account for approximately 11% of the Coomassie blue-staining material of ghosts; with an average apparent molecular weight of 55,000 (10), they would be present in a total of 7.3×10^5 copies per cell. This is in two- to fourfold excess of previous estimates of the number of D-glucose transport proteins per erythrocyte (6, 11). As evident from the SDS-PAGE of ghosts, the broad zone 4.5 region has a complex structure, comprising at least 3 polypeptides, each of which is present in sufficient quantity to account for $2–3 \times 10^5$ D-glucose transport proteins per cell (6, 11). The resolution of these components on SDS-PAGE, however, is impaired by the purification procedure so that a single broad band results. Consistent with the exposure of zone 4.5 proteins on the cytoplasmic surface of the erythrocyte membrane (29), preliminary studies indicate that these proteins contain 4–8% carbohydrate by weight (data not shown).

Although zone 4.5 protein accounts for 80% of the Coomassie blue-stained protein of the 0.1-μ fraction, band 7 and lower-molecular-weight proteins are present as minor contaminants. As indicated previously (6, 11), since a protein exposed on the extracellular surface of the erythrocyte membrane is part of the transport mechanism (8, 30), it is unlikely that band 7 protein which is exposed only on the cytoplasmic membrane surface (10) contains the D-glucose transport protein. However, the possibility that a minor protein contaminant or some non-Coomassie blue-staining component of the 0.1-μ fraction is involved in D-glucose transport has not been definitely eliminated in the present study. In this connection, Batt et al. (8), employing a two-step differential labeling procedure, have recently identified an exofacial component of the erythrocyte hexose transport system which migrated with the polypeptides of zone 4.5 on SDS-PAGE. Both D-glucose and cytochalasin B protected this exofacial transport site from alkylation by impermeant maleimides which irreversibly inhibit sugar transport. In addition, Hinkle and Kasahara (31) have recently demonstrated that the incorporation of zone 4.5 protein in sonicated liposomes results in the reconstitution of D-glucose transport. The present results support and extend these findings. The stereospecificity and inhibition characteristics of the reconstituted D-glucose transport catalyzed by the 0.1-μ fraction imply that all the components of the transport system are contained in this membrane preparation consisting predominantly of zone 4.5 polypeptides. Thus, this soluble membrane fraction should prove invaluable for further pruification of the human erythrocyte monosaccharide transport system in a functional form, suitable for study at the molecular level.

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