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STEREOSPECIFIC D-GLUCOSE TRANSPORT IN MIXED MEMBRANE AND PLASMA MEMBRANE VESICLES DERIVED FROM CULTURED CHICK EMBRYO FIBROBLASTS

CEDRIC A. ZALA and JAMES F. PERDUE *

Lady Davis Institute for Medical Research, Sir Mortimer B. Davis Jewish General Hospital, 3755 Cote Sainte Catherine Road, Montreal, Quebec H3T 1E2 (Canada)

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Summary

Mixed membrane vesicles prepared from cultured chick embryo fibroblasts possess a stereospecific D-glucose transport system, the properties of which are identical to those of the system in intact cells. Uptake of D-glucose proceeds without chemical alteration. The rate of stereospecific uptake of D-glucose into the mixed vesicles is 70% greater than that of the homogenate and uptake is directly proportional to membrane protein concentration. Stereospecific D-glucose uptake appears linear for 0.3 min, reaches a maximum at 2—5 min, and declines to zero by 5 h as L-glucose enters the vesicles. Uptake is osmotically sensitive and inhibited by cytochalasin B (K_i = 0.13 μM) and the structural analogues of D-glucose : D-mannose, 2-deoxy-D-glucose, 3-*O*-methyl-D-glucose, D-galactose and maltose, but not by sucrose or L-glucose. Uphill counterflow can be demonstrated and the apparent activation energy displays a transition from 47.7 kcal/mol below 11°C to 18.1 kcal/mol above 11°C. Stereospecific uptake rates of mixed vesicles prepared from Rous sarcoma virus-transformed cells are increased 30% over control values, and are increased 66% in vesicles derived from cells incubated for 24 h in glucose-free medium.

Plasma membrane vesicles prepared from these cells by a dextran cushion centrifugation procedure display a 9-fold increase in the specific activity of stereospecific D-glucose uptake relative to the homogenate. Extraction of these membranes with dimethylmaleic anhydride (5 mg/mg protein) results in substantial or complete removal of major polypeptides of molecular weight 40 000, 55 000, 75 000, 78 000 and 200 000 with no loss in total uptake activity. Following extraction, major polypeptides of molecular weight 28 000, 33 000 and 68 000 remain in the membrane residue.

* To whom reprint requests should be addressed.

Introduction

The use of bacterial membrane vesicles in studies of the mechanism and energetics of nutrient uptake is well established [1,2]. In recent years, similar approaches have been employed for the study of transport in eucaryotic tissues [3—20] and cultured cells [3,21—35]. Because these membrane preparations are essentially free from cytosol kinases and metabolites, e.g., ATP, transport becomes the rate-limiting step of uptake. The use of membrane vesicles therefore permits an analysis of nutrient transport of cells uncomplicated by subsequent metabolism of the accumulated substrates; another advantage of vesicular systems is the ease with which the composition of intra- and extravesicular solutions may be controlled. This approach has been applied to Na^+-dependent D-glucose transport by renal [4—6] and intestinal [7—13] plasma membranes and to facilitated diffusional D-glucose uptake into adipocyte membrane vesicles [14—20]. In addition, workers in several laboratories have used membrane vesicles from cultured cells in studies of amino acid [21—30] and nucleoside [31—34] transport of quiescent, actively growing, and transformed cells. However, few studies have appeared characterizing sugar transport into vesicles derived from cultured cells [23,35].

The kinetics of facilitated diffusional uptake of 2-deoxy-D-glucose into chick embryo fibroblasts have been investigated in normal and virus-transformed cells [36—40]. These studies have shown that increases in transport activity following transformation, serum addition, or glucose starvation are due to an increase in V with no change in K_m. Furthermore, the activity was found to be subject to transcriptional, translational, and post-translational control. For these reasons, a vesicular preparation from chick embryo fibroblasts would be expected to be well suited to analysis of both mechanism and regulation of D-glucose transport.

In this report, we characterize stereospecific D-glucose uptake by a mixed membrane vesicle preparation from chick embryo fibroblasts and show its properties to be identical to those of the intact cell. In addition, we describe a simple procedure for the preparation of transport-competent plasma membrane vesicles from these cells and the selective extraction of certain polypeptides from these plasma membranes, using dimethylmaleic anhydride, under conditions where stereospecific D-glucose uptake activity is quantitatively retained by the membrane residue.

Materials and methods

Materials. D-[U-^{14}C]-Glucose and L-[1-^{3}H(n)]-glucose were purchased from New England Nuclear, Boston, MA; sodium dodecyl sulfate (SDS) from Pierce Chemical Co., Rockford, IL; cytochalasin B from Aldrich Chemical Co., Inc., Milwaukee, WI, and L-glucose from Pfanstiehl Laboratories, Inc., Waukegan, IL. Other sugars and inorganic salts were obtained from local sources. Dextran of mean molecular weight 80 700 and dimethylmaleic anhydride were supplied by Sigma Chemical Co., St. Louis, MO. Metricel GA-6 membrane filters (0.45 μm) were obtained from Gelman Instruments, Ltd., Montreal, Quebec. All materials for polyacrylamide gel electrophoresis were obtained from Bio-Rad Laboratories, Richmond, CA.

Solutions. All buffers were adjusted to pH 7.4. Phosphate-buffered saline contained 140 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$. Phosphate/saline/azide buffer contained 114 mM NaCl, 2 mM NaN_3, 8.33 mM Na_2HPO_4 and 1.67 mM NaH_2PO_4. Hypertonic buffer was prepared from phosphate/saline/azide buffer by increasing the concentration of NaCl to 164 mM. A stock solution of 5 mM D-[U-^{14}C]-glucose (1 mCi/mmol) and 5 mM L-[1-^{3}H(n)]-glucose (2.5 mCi/mmol) was prepared in phosphate/saline/azide buffer and used for all uptake experiments.

Cell culture. Chick embryo fibroblasts were prepared from 12-day-old embryos by using the method of Temin [41]. The cells were incubated at 37°C in a humidified CO_2 incubator in Temin-modified Eagle's medium containing 4% fetal calf serum. Primary cells were trypsinized, transferred at $2 \cdot 10^6$ cells per plate into 100-mm plastic dishes and grown for 4 to 5 days until they became confluent; the medium was changed 24 h prior to harvest. Glucose starvation was started 20 h prior to harvest by the addition of medium containing 4% dialyzed fetal calf serum and all components of Temin-modified Eagle's medium except glucose. Fibroblasts were transformed by addition of a concentrated suspension of the Bratislava 77 strain of Rous sarcoma virus at the time of plating, according to previously published procedures [38].

Preparation of mixed membrane vesicles. Mixed vesicles were prepared from chick embryo fibroblasts (30—50 100-mm plates) as follows, all steps being carried out at 0—4°C. Cells were washed three times with phosphate-buffered saline, scraped from the dishes with a rubber policeman, and pooled. After sedimentation for $7500 \times g \cdot$ min, the fibroblasts were resuspended in 20 ml phosphate-buffered saline and homogenized during three up-down strokes in a Potter-Elvehjem homogenizer driven by a TRI-R stirrer motor (Rockville Centre, NY) at a setting of 3. Where appropriate, aliquots were removed from the homogenate and stored on ice until assayed. The remaining homogenate was sedimented for $7500 \times g \cdot$ min, the supernatant decanted, and the pellet suspended in 20 ml phosphate/saline/azide buffer, resedimented for $24\,000 \times g \cdot$ min, and resuspended in phosphate/saline/azide buffer to a protein concentration of 1—2 mg/ml. This material was designated the low-speed pellet. The supernatant from the first low-speed spin was centrifuged for $6 \cdot 10^6 \times g \cdot$ min, the pellet suspended in 20 ml phosphate/saline/azide buffer, resedimented as above, and resuspended in phosphate/saline/azide buffer. This material, designated the mixed membrane vesicle preparation, was stored on ice until assay.

Preparation of plasma membrane vesicles. All steps were carried out at 0—4°C; where appropriate, aliquots were removed and stored on ice until assayed. The cells were washed, scraped, and sedimented as above. They were then suspended in 40 ml 10 mM Tris-HCl (pH 7.5 at 4°C) containing 0.25 M sucrose and homogenized as described above. The homogenate was centrifuged for $45\,000 \times g \cdot$ min and the resulting supernatant stored on ice. The pellet was rehomogenized in 10 mM Tris containing 0.25 M sucrose and recentrifuged as above. The pellet from the last centrifugation was homogenized in 20 ml of phosphate/saline/azide buffer and designated the low-speed pellet. The supernatants from the latter two centrifugations were then combined and centrifuged for $3 \cdot 10^6 \times g \cdot$ min. The vesicles in the resulting pellet were then hypotonically lysed by homogenization of the pellet in 10—15 ml of 10 mM Tris-

HCl; this suspension was designated the high-speed pellet. The suspension was fractionated by layering it onto 3 ml of 10 mM Tris-HCl containing 10% dextran (w/v), and centrifuging for $2 \cdot 10^6 \times g \cdot$ min. The pellet, designated the 10% dextran pellet (or endoplasmic reticulum fraction) was homogenized in 3—5 ml of phosphate/saline/azide buffer. The material at the interface was removed, diluted to 15 ml with phosphate/saline/azide buffer, and centrifuged for $3 \cdot 10^6 \times g \cdot$ min. The resulting pellet, designated the 10% dextran interface (or plasma membrane fraction), was homogenized in 1—2 ml phosphate/saline/azide buffer. All fractions were stored on ice until assay.

Transport assay. Measurement of stereospecific D-glucose uptake was performed according to the following standardized procedure. Sufficient volumes of membrane suspension and phosphate/saline/azide buffer to give 50 μg protein in 80 μl were transferred to 10 X 75 mm tubes in a water bath held at 20°C. Uptake was started by the addition of 20 μl of the differentially labelled D- and L-glucose solution; the final concentration of these hexoses was 1 mM. After incubation for the desired time (normally 0.30 min), uptake was stopped by rapid addition of 2 ml of ice-cold hypertonic phosphate/saline/azide buffer. The suspension was then filtered through a Gelman 0.45 μm membrane filter, which was washed once with 2 ml cold hypertonic phosphate/saline/azide buffer; filtration and washing were complete 0.3 min after uptake was stopped. Multiple washes resulted in a slow decrease in the apparent value for stereospecific uptake (approx. 25% lost after five washes). Zero-time samples were obtained by adding the phosphate/saline/azide buffer prior to the radioactively labelled stock solution followed by filtration and washing. Replicates were either transferred to vials for liquid scintillation counting to determine values for D- and L-glucose uptakes, or extracted overnight with 1.5 ml of 5% SDS, from which 0.2 ml aliquots were removed for protein analysis. Blanks omitting membrane protein were run in parallel. Protein recovery ranged from 50 to 70% of that added to the filter. Uptakes (nmol/mg protein) or uptake rates (nmol/mg protein per min), from which zero time values have been subtracted, are expressed as mean ± S.E. Stereospecific D-glucose uptake is the difference between D-glucose and L-glucose uptake. L-glucose serves as an internal control for completeness of washing and as a measure of non-carrier-mediated (diffusional) entry of sugar.

Extraction of plasma membranes with dimethylmaleic anhydride. A volume of plasma membrane suspension containing 1 mg protein was diluted to 10 ml with distilled water. To this, 5 mg dimethylmaleic anhydride were then added, with continuous stirring, and the pH was maintained between 7.2 and 7.5 with 0.01 N NaOH until evolution of acid ceased (normally 20 min). Control membranes were diluted to 10 ml with 10 mM Tris buffer. The suspensions were then centrifuged for $3 \cdot 10^6 \times g \cdot$ min and the supernatants set aside for ultrafiltration. The pellets were homogenized in a further 10 ml of distilled water and again centrifuged for $3 \cdot 10^6 \times g \cdot$ min. These pellets were homogenized in 0.2 ml of 10 mM Tris. The above supernatants were first ultrafiltered to 1.5 ml in an Amicon Model 52 ultrafiltration cell fitted with a PM-10 membrane. These supernatants were further concentrated to 0.2 ml using an Amicon Minicon macrosolute concentrator. Membrane suspensions were assayed immediately for stereospecific uptake activity, and all fractions were stored at —20°C until electrophoresis.

Analysis of membrane polypeptide composition. Membranes were analyzed by SDS-polyacrylamide gel electrophoresis in 7.5% acrylamide slab gels 1.5 mm thick, according to the method of Laemmli [42].

Enzyme assays and biochemical determinations. NADH-cytochrome *c* reductase was assayed by using the method of Mackler and Green [43]; succinic dehydrogenase, by the method of Ziegler and Rieske [44]; sialic acid, by the method of Jourdian et al. [45], and protein by the method of Lowry et al. [46].

Results

Characterization of the mixed vesicle preparation

Biochemical properties of the fractions obtained using the mixed vesicle preparation procedure. Relative to the homogenate, the specific activities of the membrane markers NADH-cytochrome *c* reductase (endoplasmic reticulum), succinic dehydrogenase (mitochondria), and sialic acid (plasma membrane) are increased in the mixed membrane vesicle fraction by 40, 90 and 117%, respectively (Table I). The specific activities of each marker in the low-speed pellet fraction are decreased to approx. 50% of the values for the homogenate.

In parallel with the above data, mixed vesicles show a 69% increase in stereospecific D-glucose uptake rate relative to the homogenate, while the activity in the low-speed pellet is decreased to 53% of that of the homogenate (Table I). The recovery of total uptake activity in the membrane vesicles is 39%.

Uptake of D- *and* L-*glucose.* D-Glucose enters the membrane vesicles rapidly, reaching an equilibrium value at 2 min (Fig. 1A); this value remains constant for 1 h and then slowly decreases, perhaps as a result of vesicle lysis (Fig. 1B). Unlike the plasma membrane of the intact cell, vesicles are highly permeable to

TABLE I

BIOCHEMICAL ANALYSIS OF MEMBRANE FRACTIONS PREPARED FROM CHICK EMBRYO FIBROBLASTS USING THE MIXED VESICLE PREPARATION PROCEDURE

Assays were performed as described in Methods. Results from two to four separate preparations are expressed as means ± S.E.

Component	Activity of component in		
	Homogenate	Low-speed pellet	Membrane vesicles
Protein * (mg)	100	34.6 ± 3.5	23.0 ± 3.9
NADH cytochrome *c* reductase (nmol/min per mg protein)	25 ± 3	12 ± 2	35 ± 6
Succinic dehydrogenase (nmol/min per mg protein)	2.9 ± 0.6	1.5 ± 0.2	5.5 ± 1.9
Sialic acid (nmol/mg protein)	35 ± 7	18 ± 5	76 ± 16
Stereospecific D-glucose uptake rate (nmol/min per mg protein)	1.60 ± 0.13	0.84 ± 0.20	2.70 ± 0.16
Total stereospecific D-glucose uptake activity ** (nmol/min)	160 ± 13	29 ± 7.5	62 ± 11

* Expressed as per 100 mg of homogenate protein.

** Product of protein and stereospecific D-glucose uptake rate.

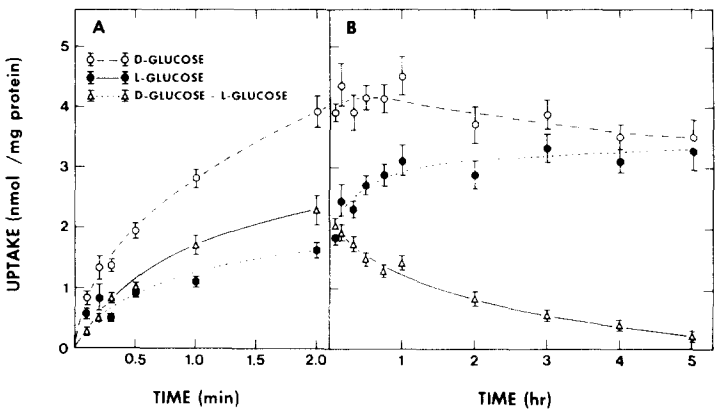

Fig. 1. D- and L-glucose uptake into membrane vesicles as a function of time. At time zero, 20 μl of the stock D-[^{14}C]-glucose (5 mM, 1 mCi/mmol) and L-[^{3}H]-glucose (5 mM, 2.5 mCi/mmol) solution were added to 80 μl of membrane suspension containing 50 μg protein. After incubation at 20°C for the times indicated, uptake was stopped by addition of cold hypertonic buffer, and the mixture filtered and washed. D- and L-glucose on the filters were determined by liquid scintillation counting. The difference between D-glucose uptake (○) and L-glucose uptake (●) is the stereospecific uptake (△). Results are from six separate preparations; individual values are the means ± S.E. from between 8 and 19 replicate uptakes: (A) time scale, minutes; (B) time scale, hours.

L-glucose; uptake of this isomer proceeds rapidly during the first minutes and then slows, gradually approaching the value for accumulated D-glucose during the next several hours. The intravesicular spaces accessible to D- and L-glucose are thus probably identical, and correspond to a value of 3—4 μl/mg protein.

Stereospecific uptake, i.e., D-glucose uptake minus L-glucose uptake, increases to a maximum at 2—5 min, followed by a gradual decline to zero as L-glucose uptake continues (Fig. 1). Stereospecific uptake appears linear for 0.3 min, and this time was therefore chosen as the standard time for measurement of uptake rate. At shorter time intervals, errors of time estimation and of D- and L-glucose accumulation due to reduced counts become large; at longer time intervals, uptake is definitely non-linear. Even at 0.3 min, however, the intravesicular concentration of D-glucose (approx. 0.3 mM) has reached one-third of the equilibrium value of 1 mM, and the measured rates are underestimates of the true initial rates. For these reasons, it was not possible to obtain estimates of K_m and V.

Stereospecific D-glucose uptake by the vesicles is directly proportional to the amount of vesicular protein retained by the filter up to at least 60 μg per filter (Fig. 2). Above this value, flow rates slowed to the point where the filters could not be rapidly washed. Under routine assay conditions, where 50 μg were added to the filters, 25—30 μg were recovered; this is well within the linear range of the graph.

Properties of the vesicular transport system. The D- or L-glucose taken up by the vesicles is chemically unchanged after 20 min of uptake. The ^{3}H- and ^{14}C-labelled accumulated sugars extracted from filters with water show mobilities identical to those of the isotopes in the stock solution and those of unlabelled D- and L-glucose, following paper chromatography with methanol/ethanol/water (45/45/10, v/v) (data not shown).

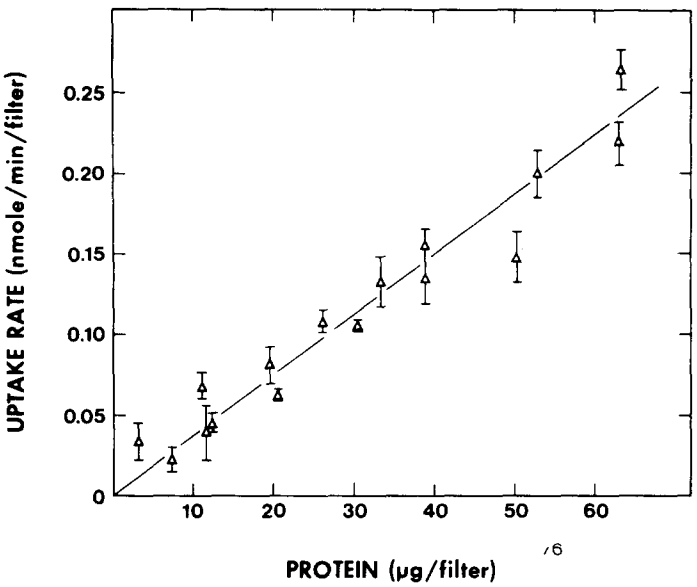

Fig. 2. Stereospecific D-glucose uptake as a function of membrane vesicle protein. Stereospecific uptake rates expressed as nmol/min per filter were determined for 80 μl of membrane vesicle suspension containing 5—120 μg of protein. The protein concentration is expressed as μg per filter; approx. 60% of that present in the suspension was retained by the filter. Results are pooled from three separate preparations; each point is the mean ± S.E. of three to five replicates. The slope of the regression line is 3.71 nmol/min per mg protein.

Stereospecific uptake activity of the membrane vesicles is stable during storage at 0°C for at least 1 week and at 20°C for at least 90 min; measurements of uptake were performed at daily and 15-min intervals, respectively, and were in no case different from the control values with a significance of less than 0.05 (data not shown).

A demonstrable change in the level of accumulated D-glucose with an increase or decrease in osmotic pressure may be considered as evidence that accumulation of sugar is the result of its entry into a vesicular compartment and not to binding. There is an inverse relation between D-glucose uptake at equilibrium and osmolarity (Fig. 3A); a plot of the reciprocal of uptake vs. osmolarity yields a straight line passing through the origin, indicating that all uptake is osmotically sensitive (Fig. 3A, inset). In addition, stopping and washing the vesicles with solutions of lower osmolarity than that of the hypertonic phosphate/saline/azide buffer (360 imosM) results in progressively less retention of D-glucose on the filter (Fig. 3B). This finding is consistent with osmotic lysis of vesicles.

Cytochalasin B, a potent inhibitor of glucose transport in intact fibroblasts with a K_i of 0.145 μM [47], inhibits stereospecific transport in membrane vesicles with a K_i of 0.13 μM (Fig. 4).

A number of structural analogs of D-glucose inhibit stereospecific uptake (Table II). Added with the radioactively labelled D- and L-glucose solution, 10 mM D-glucose, D-mannose, 2-deoxy-D-glucose and 3-*O*-methyl-D-glucose inhibit stereospecific uptake rates by approx. 40% while D-galactose and maltose show a weaker inhibition. Neither sucrose nor L-glucose inhibits stereo-

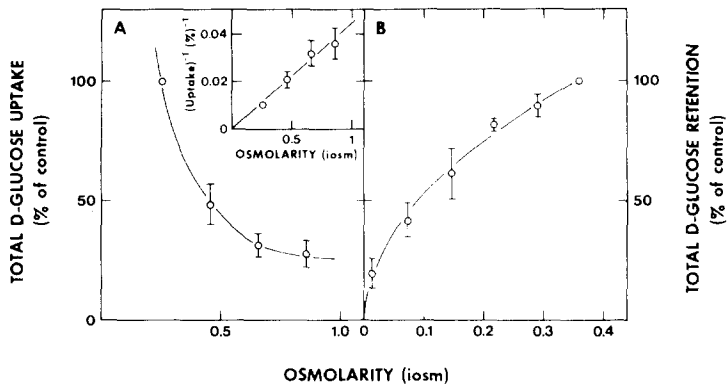

Fig. 3. Effect of osmolarity on total D-glucose uptake. (A) Equilibrium D-glucose uptake under hypertonic conditions. Membrane vesicles were combined with various volumes of 1 M sucrose dissolved in phosphate/saline/azide buffer to give a range of osmolarities between 0.26 and 0.86 iosM. Radioactively labelled D- and L-glucose uptake was then determined for 30 min at 20°C. Uptake was stopped and the filters washed with solutions of hypertonic phosphate/saline/azide buffer containing the same concentration of sucrose as that present during uptake. Uptake of D-glucose (nmol/mg protein) is expressed as the percent of the control value observed under the usual conditions of incubation osmolarity (0.26 iosM). Inset: replot of the data in A, where the reciprocal of D-glucose uptake is plotted vs. osmolarity. The y-intercept of the regression line is not significantly different from zero. (B) Total D-glucose retention on the filter as a function of decreasing osmolarity of the stop and wash solution. Uptake by membrane vesicles incubated for 0.3 min at 20°C was stopped by addition of 2 ml of one of a series of hypotonic solutions prepared by dilution of the hypertonic phosphate/saline/azide buffer normally used. Membranes on the filter were washed once with the corresponding buffer prior to liquid scintillation counting. Retention of D-glucose (nmol/mg protein) is expressed as the percent of the control value observed using the hypertonic phosphate/saline/azide buffer (0.36 iosM) to stop and wash.

specific uptake, consistent with use of the latter as a marker of nonspecific diffusion.

Uphill counterflow is a special case of competitive exchange diffusion; it can be demonstrated by inducing the formation of a concentration gradient of one substrate initially at equilibrium by addition of a high concentration of a competing substrate to one face of the membrane. Its demonstration is considered to provide strong evidence for carrier-mediated transport of the substrate [48]. In the experiment shown in Fig. 5, membrane vesicles incubated for 30 min with 1 mM D- and L-glucose were combined with concentrated D- or L-glucose solutions to give an extravesicular concentration of 25 mM. The osmolarity is thereby increased by 10%, so that a rapid decrease of approx. 10% in intravesicular D- or L-glucose would be anticipated. No further decrease would be seen in the absence of counterflow. The data show that counterflow of labelled D-glucose out of the vesicles is specifically driven by extravesicular D-glucose. Addition of D-glucose does not drive counterflow of L-glucose and addition of L-glucose drives neither.

Stereospecific and nonspecific (L-glucose) uptake were further characterized by measurement of their temperature dependencies over the range 0—40°C at a concentration of 1 mM (Fig. 6). Nonspecific uptake has a markedly lower dependence on temperature than stereospecific uptake, with apparent activation energies of 10.4 and 18.1 kcal/mol, respectively. This latter value is identical to that observed for intact cells in the range 20—40°C [37]. Below 11°C, the slope becomes abruptly steeper for stereospecific transport, with an appar-

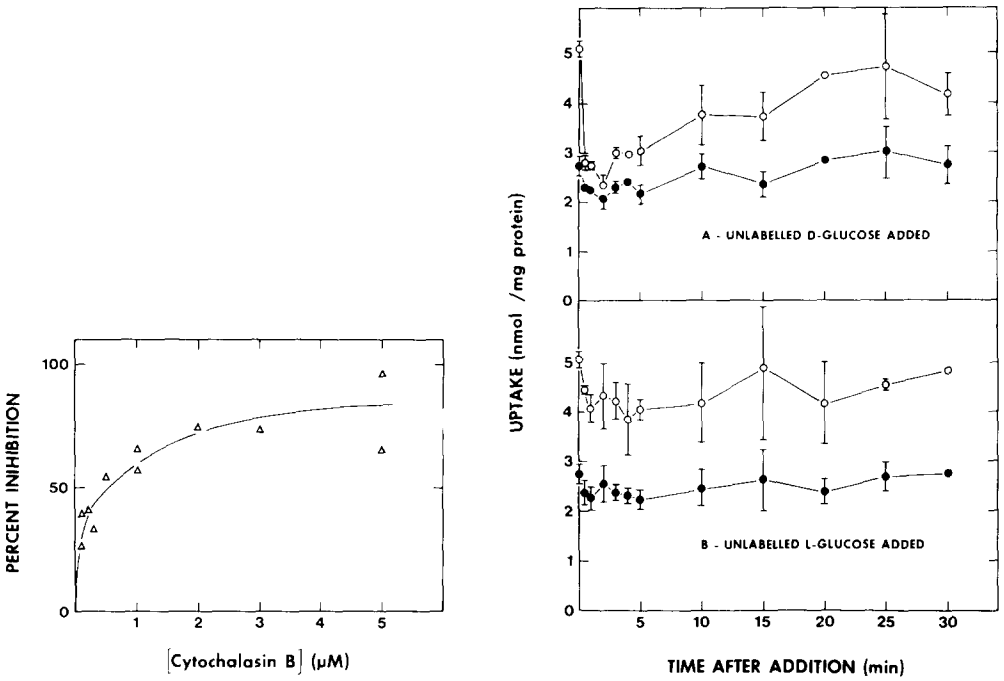

Fig. 4. Inhibition of stereospecific D-glucose uptake by cytochalasin B. Dilutions of cytochalasin B in ethanol were added to membrane vesicles to give final concentrations of between 0.1 and 5 μM (concentration of ethanol was 1%). Stereospecific uptake (0.3 min, 20°C) rates were measured 15 to 30 min after addition of cytochalasin B.

Fig. 5. Demonstration of countertransport in membrane vesicles. Membrane vesicles were combined with 1 mM radiolabelled D- and L-glucose stock solution and incubated at 20°C for 30 min. Then a sufficient volume of 1 M D-glucose (A) or L-glucose (B) was added to give a final extravesicular concentration of 25 mM. Samples were removed at various times up to 30 min and values for intravesicular D-glucose (○) and L-glucose (●) determined. Results are means ± S.E. of single values obtained from three separate preparations.

TABLE II

INHIBITION OF STEREOSPECIFIC D-GLUCOSE UPTAKE BY VARIOUS SUGARS AND SUGAR DERIVATIVES

Solutions (50 mM) of the designated sugars were made up in the radioactively labelled D- and L-glucose solution, so that the concentration during uptake was 10 mM. Each value is the mean ± S.E. of 12 replicate uptakes at 0.3 min.

Sugar (10 mM)	Stereospecific uptake rate (% of control)
D-Mannose	53.9 ± 6.9
D-Glucose	58.3 ± 7.3
2-Deoxy-D-glucose	58.6 ± 4.8
3-*O*-Methyl-D-glucose	63.8 ± 4.5
D-Galactose	75.5 ± 10.4
Maltose	79.9 ± 7.2
Sucrose	98.8 ± 5.4
L-Glucose	106.5 ± 6.0

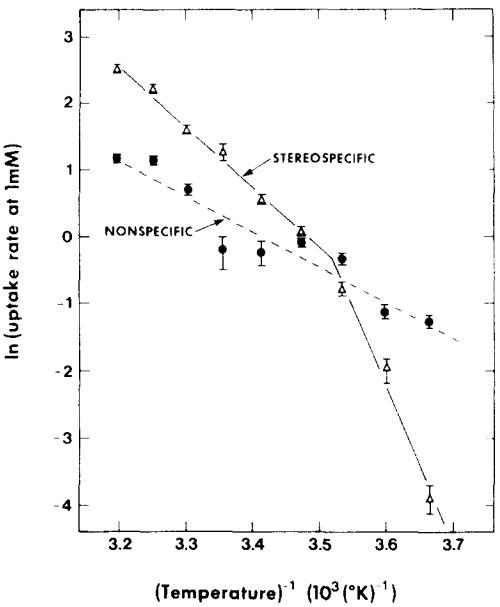

Fig. 6. Arrhenius plot of temperature dependence of stereospecific and nonspecific (L-glucose) uptake rates. Radioactively labelled D- and L-glucose stock solutions and membrane vesicles, both pre-equilibrated in a water bath at the temperatures indicated, were combined and incubated at those temperatures for a time sufficient to allow stereospecific entry of between 0.4 and 0.8 nmol/mg protein. These times ranged from 0.1 min (40°C) to 20 min (0°C). Results are expressed as means ± S.E. from triplicates from each of two separate preparations.

ent activation energy of 47.7 kcal/mol. No such change is observed for L-glucose.

Effect of viral transformation and glucose starvation on stereospecific uptake activity. Membrane vesicles prepared from cells transformed by the B_{77} strain of Rous sarcoma virus display a 30% increase in stereospecific uptake activity relative to uninfected control cells (Table III).

Vesicles prepared from cells incubated in glucose-free medium 24 h prior to harvest show a 66% increase in stereospecific uptake activity, relative to controls fed with the complete medium (Table III).

Characterization and extraction of plasma membrane vesicles

Choice of dextran cushion method of plasma membrane preparation. In initial attempts to obtain a plasma membrane fraction substantially enriched in stereospecific D-glucose uptake activity, we evaluated both a series of differential centrifugations and a 10 to 50% sucrose gradient centrifugation procedure. In both cases, little or no enrichment of stereospecific D-glucose transport could be demonstrated in any fraction, and in the latter procedure, most of the total activity was lost (data not shown).

However, in preliminary experiments involving centrifugation of membranes on a step-wise dextran gradient (10 to 30%, in 5% increments) we found that material collected at the 0%/10% interface was considerably enriched in stereospecific D-glucose uptake activity. In addition, the bulk of the membrane protein was found as a pellet at the bottom of the 30% dextran layer, with only

TABLE III

EFFECT OF ROUS SARCOMA VIRUS TRANSFORMATION AND 24 h GLUCOSE STARVATION ON STEREOSPECIFIC D-GLUCOSE UPTAKE RATES OF MEMBRANE VESICLES

Membrane vesicles were prepared from fibroblasts which were either infected with the Bratislava 77 strain of Rous sarcoma virus at the time of plating, or fed with glucose-free medium 24 h prior to harvest. Control cells were uninfected, or fed with the complete medium, respectively. Results from 20—30 replicates from four separate preparations each are expressed as means ± S.E.

Treatment	Stereospecific uptake rate (nmol/min per mg protein)
Control	2.18 ± 0.19
Transformed	2.84 ± 0.21
Control	2.70 ± 0.16
Glucose-starved	4.47 ± 0.30

very small amounts collected at any of the intervening interfaces. These observations led to the adoption of the standard procedure described in Methods for the preparation of transport-competent plasma membranes.

Biochemical properties of fractions obtained using the plasma membrane vesicle procedure. Table IV shows the protein content of the various fractions and the specific activities of stereospecific D-glucose transport and the marker components: succinic dehydrogenase for mitochondria, NADH cytochrome *c* reductase for endoplasmic reticulum and sialic acid for plasma membrane. Most of the mitochondrial marker is removed by low-speed centrifugation, and its specific activity in the plasma membrane fraction is reduced to one-fifth of that of the homogenate. The endoplasmic reticulum marker is enriched 3-fold in the

TABLE IV

BIOCHEMICAL ANALYSIS OF MEMBRANE FRACTIONS PREPARED USING THE PLASMA MEMBRANE VESICLE PREPARATION PROCEDURE

Assays were performed as described in Methods. Triplicate values from two separate preparations were pooled and expressed as means ± S.E.

Component	Activity of component in				
	Homogenate	Low-speed pellet	High-speed pellet	10% dextran pellet	10% dextran interface
Protein * (mg)	100	48	16.7	6.7	2.8
NADH cytochrome *c* reductase (nmol/min per mg protein)	7.10 ± 1.18	—	14.7 ± 2.1	25.7 ± 4.1	5.50 ± 0.36
Succinic dehydrogenase (nmol/min per mg protein)	4.65 ± 0.76	7.78 ± 0.11	1.76 ± 0.10	2.48 ± 0.51	0.87 ± 0.06
Sialic acid (nmol/mg protein)	23.6 ± 0.1	—	97.1 ± 1.8	22.5 ± 2.7	87.8 ± 3.1
Stereospecific D-glucose uptake rate (nmol/min per mg protein)	1.07 ± 0.53	—	2.04 ± 0.45	—0.51 ± 0.35	9.04 ± 0.38

* Expressed as mg per 100 mg of homogenate protein.

TABLE V

EXTRACTION OF PLASMA MEMBRANES WITH DIMETHYLMALEIC ANHYDRIDE

Sufficient distilled water was added to a volume of plasma membranes containing 1 mg protein to give a final volume of 10 ml. To this, 5 mg dimethylmaleic anhydride were added and the pH maintained at 7.2—7.5 until evolution of acid ceased. Control membranes were diluted to 10 ml with 10 mM Tris. After centrifugation and washing as described in Methods, pellets were assayed for protein and stereospecific D-glucose uptake, at a time point of 0.1 min. Results of three to five replicates from each of four separate experiments were pooled and expressed as mean ± S.E. Protein expressed as mg per mg recovered in the control. Total activity expressed as the product of protein and stereospecific D-glucose uptake rate.

Treatment	Stereospecific D-glucose uptake rate (nmol/min per mg protein)	Protein (mg)	Total activity (nmol/min)
Control	9.57 ± 1.33	1.00	9.57 ± 1.33
Dimethylmaleic anhydride-extracted membranes	12.49 ± 1.64	0.75	9.37 ± 1.23

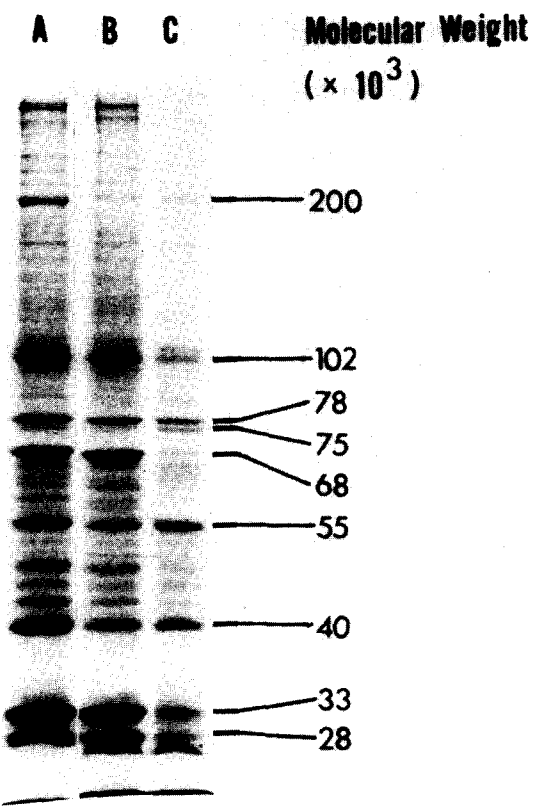

Fig. 7. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of fractions following dimethylmaleic anhydride extraction of plasma membranes. (A) Plasma membranes. (B) Residue following extraction of plasma membranes with 5 mg dimethylmaleic anhydride/mg protein as described in Methods. (C) Extract, concentrated by ultrafiltration, of plasma membranes treated with dimethylmaleic anhydride.

10% dextran pellet but is not enriched in the plasma membrane fraction. Sialic acid is enriched 4-fold in the plasma membrane fraction. Stereospecific D-glucose transport activity, however, is enriched 9-fold in the plasma membrane fraction, whilst the dextran pellet is devoid of this activity. An overall recovery of 24% of D-glucose uptake activity is seen for the plasma membranes, while recoveries of mitochondrial, endoplasmic reticulum, and plasma membrane markers are 0.5, 2.2 and 10.4%, respectively.

Glucose uptake and polypeptide composition of plasma membranes following extraction with dimethylmaleic anhydride. The stereospecific D-glucose uptake rates of control plasma membranes and plasma membrane extracted with 5 mg dimethylmaleic anhydride/mg protein are shown in Table V. Treatment with dimethylmaleic anhydride removed 25% of the membrane protein with no loss of total uptake activity. Polypeptide analysis of the plasma membrane extracts and residues by SDS-polyacrylamide gel electrophoresis is shown in Fig. 7. The polypeptides of molecular weights 40 000, 55 000, 75 000, 78 000, 102 000 and 200 000 are substantially or completely removed by dimethylmaleic anhydride treatment. The predominant proteins remaining in the membrane residue have molecular weights of 28 000, 33 000 and 68 000. The use of greater quantities of dimethylmaleic anhydride, while extracting more total protein, did not improve the selectivity of the extraction; in addition, the membrane residue displayed a partial loss or inactivation of D-glucose transport activity.

Discussion

In initiating studies directed at purification of the D-glucose transport protein of the chick embryo fibroblast plasma membrane and determination of molecular mechanisms of transport activity regulation, we have prepared mixed and plasma membrane vesicle fractions capable of stereospecific D-glucose transport. The vesicular nature of the preparations is confirmed by the sensitivity of uptake to osmotic conditions and by electron microscopy (not shown). Stereospecific D-glucose uptake activity is stable for up to 1 week at 0°C, and sensitive to inhibition by cytochalasin B ($K_i = 1.3 \cdot 10^{-7}$ M) and several structural analogues of D-glucose, including 2-deoxy-D-glucose and 3-*O*-methyl-D-glucose, but not L-glucose. Counterflow of labelled D-glucose can be demonstrated upon addition of unlabelled D-glucose and the apparent activation energy of stereospecific uptake is the same as that of the intact cells in the range 20—40°C. On the basis of this data, we conclude that the cellular and vesicular transport systems are similar.

In contrast to intact fibroblasts, the vesicles are quite permeable to L-glucose; however, evidence for carrier-mediated transport of L-glucose was not found, in that L-glucose did not inhibit D-glucose uptake, nor did it participate in counterflow. Considerable uptake of L-glucose has been found by other workers using other systems [15,16,18,19] including purified plasma membranes; this indicates that during the homogenization and purification procedures, plasma membranes may be structurally altered to a leaky state in the vesicular form, and take up L-glucose at faster rates than in the intact cell. The

present results underline the necessity for control of non-carrier-mediated monosaccharide uptake.

The temperature dependence of stereospecific D-glucose uptake at 1 mM indicates a break in the Arrhenius plots, with values of apparent activation energies of 18.1 and 47.7 kcal/mol above and below 11°C, respectively, while L-glucose uptake has a constant apparent activation energy of 10.4 kcal/mol. Such breaks have been observed in vesicular glucose transport [6] and membrane ATPase [49] reactions and have been interpreted as indicating a change in protein conformation or in the rate-limiting step [50]. However, these results must be cautiously interpreted, as rates assayed at a fixed substrate concentration can yield Arrhenius plot artifacts, dependent on the temperature variation of K_m [51]. Because true initial rates were not obtainable, we were unable to measure K_m and V in the vesicular system and hence to estimate true activation energies. However, the much greater value of activation energy for stereospecific D-glucose transport compared to L-glucose transport confirms that stereospecific and nonspecific uptake occur by different mechanisms.

It has been shown for amino acid [23,26,27] and nucleoside [31] transport in cultured cells that differences in cellular uptake rates between quiescent, actively growing, and transformed cells are due to plasma membrane alteration, as they persist in isolated membrane vesicles. In the present report, vesicles from transformed and glucose-starved cells are shown to have 30 and 66% increases in stereospecific D-glucose uptake rates, respectively, over control vesicles. The transport rate increases in vesicles derived from the intact cells are not as great as these observed previously for intact cells [36,38]; this may be at least partially explained by the fact that true initial rates are underestimated by the present procedure and the higher the rate, the greater the amount by which it is underestimated. The demonstration that these rate increases persist in vesicles supports evidence obtained for intact chick embryo fibroblasts that increased transport activity and not phosphorylation is responsible for the increases in glucose uptake brought on by transformation [36,38] and glucose starvation [40].

Our results with chick embryo fibroblast vesicles agree closely with those recently reported by Inui et al. [52] for mouse fibroblasts and Cheng et al. [53] for rat sarcolemma, but differ in two respects from those presented by Lever [35] for mouse fibroblasts. In particular, Lever did not observe transport increases in membrane vesicles from simian virus 40-transformed cells relative to control, and did not observe inhibition of glucose uptake by cytochalasin B. A possible explanation for these differences is that Lever apparently used total D-glucose uptake as a measure of carrier-mediated transport whilst we and the other workers used stereospecific uptake. A considerable portion of the total D-glucose uptake is nonspecific, and therefore actual differences in rates of carrier-mediated transport might be obscured by using total rather than stereospecific uptake as a measure of transport rates.

The increased transport activity following glucose starvation has been used as the basis for a method of identifying putative glucose carrier protein(s) by pulse-labelling with [^{35}S]methionine followed by SDS-polyacrylamide gel electrophoresis [54,55]. It was found that the rates of synthesis of two polypeptides of molecular weights 75 000–78 000 and 95 000 are increased follow-

ing glucose starvation. Whether these proteins are in fact carrier proteins, regulatory proteins, or proteins otherwise involved in the altered cell metabolism in the absence of glucose is not known. To date, the only studies on purification of the D-glucose transport protein in the chick embryo fibroblast membrane have focussed on an aqueous extract capable of binding D-glucose and increasing 2-deoxy-D-glucose uptake in quiescent fibroblasts [56,57]. Several distinct regions possessing either binding or transport-stimulating activity were seen on fractionation of the extract on Sepharose and aminoethyl cellulose columns. However, the relation between the binding proteins, the transport-stimulating activities, and the plasma membrane glucose transport protein(s) is still unclear.

Partial purification of glucose transport proteins from human red blood cells [58] and rat [18] and human [19] adipocytes has been achieved in several laboratories by specific extraction of peripheral membrane proteins with protein perturbants (notably dimethylmaleic anhydride) under conditions where transport activity is retained by the extracted residue. In the present study, major bands not substantially or completely extracted by dimethylmaleic anhydride (5 mg/mg protein) have molecular weights of 68 000, 33 000 and 28 000; numerous minor bands are also visible in the extracted membranes. The involvement of any of these polypeptides in glucose transport is possible; however, it is more likely that the transport proteins are present in quantities too small to produce a visible band. If it is assumed that the hexose carrier has a molecular weight of 50 000 and a molecular activity of 1000 molecules of D-glucose/min when saturated at 20°C, it can be calculated that this hypothetical system would exhibit a V value of 20 μmol/mg protein per min. The transport rate observed for plasma membrane vesicles at a D-glucose concentration of 1 mM is approx. 10 nmol/mg protein per min; assuming a K_m of 5 mM, then V calculated for the vesicle system is equal to 60 nmol/mg protein per min. In this example, only approx. 0.3% of the plasma membrane protein is sufficient to account for the observed uptake rates; such an amount might not be detectable as a discrete band on conventionally stained gels following electrophoresis in SDS.

A plasma membrane preparation maximally enriched in D-glucose transport activity is indispensible for the purification and isolation of a functional D-glucose transport protein. The dimethylmaleic anhydride-extracted plasma membrane preparation described here is highly suitable for this purpose and is now being used in studies directed at reconstituting the D-glucose transport system in liposomes and at the identification of the protein(s) involved by the use of a tritiated maleimide derivative of glutathione.

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