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GLUCOSE TRANSPORT IN THYMOCYTE PLASMA-MEMBRANE VESICLES

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Calf-thymocyte membrane vesicles, prepared by hypotonic lysis and homogenization, were isolated by standard centrifugal techniques designed for enrichment of plasma membrane. At 20°C, these vesicles equilibrated with D-glucose and 3-*O*-methyl-D-glucose more rapidly than with L-glucose. About 25% of the equilibrium D-sugar space (6 μl/mg protein) was very slowly penetrated by L-glucose ($t_{1/2} \simeq 6$ h). The time course of D-sugar accumulation in excess of L-glucose accumulation indicated that this space equilibrated with D-glucose and 3-*O*-methyl-D-glucose with half-times of approximately 0.2–0.4 min. The remainder of the equilibrium D-sugar space (about 75%) appeared equally accessible to both glucose isomers ($t_{1/2} \simeq 2$ to 5 min). This was confirmed in studies of efflux from preloaded vesicles, where the D-glucose space fell with a short half-time (0.2 min) to the L-glucose space, after which the two isomers exited with the same half-time. Addition of sucrose to increase osmolarity reduced both spaces (specific and non-specific) in a manner which indicated that little if any of the vesicle sugar was bound. This was confirmed by the fact that equilibrium glucose space was independent of glucose concentration and by the fact that vesicles immediately lost their sugar when diluted with water at 0°C. These data indicate the presence of two vesicle types, discriminant and indiscriminant as regards transport of the glucose isomers. Entry of D-glucose into the discriminant (stereospecific) vesicles was temperature sensitive ($Q_{10} > 2$), saturable ($K_m \simeq 2$ mM), and was inhibited by phloretin ($K_i < 200$ μM), *N*-ethylmaleimide ($K_i < 10$ mM) and cytochalasin B ($K_i < 2$ μM), suggesting that these vesicles contain the plasma-membrane glucose carrier. Entry of L- and D-glucose into the indiscriminant vesicles showed none of these properties. The equilibrium-exchange K_m and V were about five times the entry K_m and V, indicating the substrate loading greatly facilitates carrier translocation, at least in the outward direction.

Introduction

The successful solubilization and reconstitution of glucose transport activity from erythrocytes [1,2] has opened the way for purification and characterization of the glucose carrier. This approach has been extended to the adipocyte [3], where the native carrier is regulated by insulin and other factors. Studies with adipocyte membrane vesicles [4,5] and liposomes derived from these vesicles [6,7] have provided important insights into the character and regulation of adipocyte glucose transport. These studies have demonstrated insulin-induced transfer of glucose carrier from an organelle sedimenting with the golgi to the plasma membrane [5,7] and insulin-induced increase in fluidity of the membranous environment of the carrier [4]. The mechanisms of these effects are not yet known. As a first step toward application of these procedures to the regulable glucose carrier of thymocytes [8–10], we have investigated the glucose transport properties of

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Pipes, piperazine-*N,N'*-bis(2-ethanesulfonic acid).

thymocyte plasma-membrane preparations. The results are presented here.

Materials and Methods

Preparation of plasma membrane. Calf thymus was obtained from a local slaughter house, packed in crushed ice, and either used within 2 h or frozen at −20°C and used upon thawing. Plasma membrane was purified from cells disrupted by homogenization in hypotonic medium followed by differential [11,12] and discontinuous sucrose gradient centrifugation [13]. The calf thymus (100–200 g), trimmed free of fat and fascia, was minced. About 1 volume of ice-cold Tris-buffered saline (150 mM NaCl in 10 mM Tris-HCl, pH 7.3) was added. Tissue fragments were crushed by one stroke of a loose-fitting Dounce homogenizer, and the cell suspension filtered through one layer of nylon net. The suspension was diluted 1 : 3 with ice-cold 10 mM Tris-HCl (pH 7.3), and allowed to stand on ice for 10 min. Portions (120 ml) were homogenized with 15 up and down strokes of a loose-fitting glass-Teflon homogenizer; the homogenates were centrifuged at $300 \times g_{av}$ for 15 min (2°C). The resulting supernatants were pooled, and readjusted to 150 mM Na^+ with 1.5 M NaCl, 10 mM Tris-HCl (pH 7.3). After centrifugation at $5000 \times g_{av}$ for 15 min (2°C), the resulting supernatant was centrifuged at $30\,000 \times g_{av}$ for 45 min (2°C). The resulting pellet was resuspended in 200 ml ice-cold 10 mM Tris-HCl, and repelleted at $30\,000 \times g_{av}$ as described. The pellet was resuspended in 100 ml ice-cold 10 mM Tris-HCl, homogenized with 5 gentle strokes (Dounce A), and combined with 100 ml ice-cold 80% (w/v) sucrose in 10 mM Tris-HCl (pH 7.3). This mixture was homogenized with 5 gentle strokes of the Dounce A, divided equally into 20 Beckman 30 rotor tubes (approx. 26 ml capacity), overlayed with 30% sucrose in 10 mM Tris-HCl (15 ml/tube) and centrifuged at 25 000 rev./min ($54\,000 \times g_{av}$) 8 h (2°C). The 'top' [13] portion of the 30% sucrose layer was aspirated, resuspended in 3 vol of ice-cold 10 mM Tris-HCl, and centrifuged at $30\,000 \times g_{av}$ for 60 min (2°C). The resulting pellet was resuspended in Tris-buffered saline (incubation medium) and was used as purified plasma membrane. The 'middle' portion (between 30% and 40%) was treated likewise.

Enzyme assays. We assayed 5′-nucleotidase by the method of Gentry and Olsson [14]. The 5′-AMP concentration was 5 μM (0.1 μCi/μmol) and the inhibitor (α,β-methylene ADP) concentration was 25 μM. Glucose-6-phosphatase was assayed by a modification of the method of Kitcher et al. [15]. We used 50 mM Pipes buffer, pH 6.5, and added 1 mM KF (inhibitor of acid phosphatase [16]) and 0.1% Triton X-100 to all tubes. We used a low substrate concentration (25 μM Glc-6-*P*, 0.01 μCi/μmol). Monoamine oxidase was assayed by the method of Wu and Dyck [17] with 1 μM tyramine as substrate.

Assay of glucose transport in membrane vesicles. Membrane suspension in incubation medium (14–15 μl, approx. 3 μg protein/μl) was added to 12 × 100 mm glass test tubes. To initiate transport, 1 μl of a mixture of L-[1-^{3}H]glucose (0.5 μCi) and D-3-*O*-[*Me*-^{14}C]methylglucose or D-[U-^{14}C]glucose (0.08 μCi) in incubation medium was added to the membranes. The final concentration of D-glucose was 0.1 mM. The contents of the tubes were mixed, and incubated at room temperature (20–25°C) for the desired times. Transport was terminated by addition of 1 ml ice-cold stop solution (Tris buffered saline containing 1 μM cytochalasin B and 0.2% ethanol). This mixture was transferred onto a prewetted 25 mm Gelman fiber-glass filter (Type A-E) in a Millipore filtration apparatus, filtered immediately, and washed immediately thereafter with 5 ml ice-cold stop solution. Filters were transferred to scintillation vials, and 5 ml of scintillation fluid (ACS, Amersham) was added. Vials were allowed to stand for 30 min. Subsequently, 0.4 ml distilled water was added to each vial, the contents mixed, and measurements of radioactivity performed.

Glucose influx was the difference between total radioactivity associated with filters and non-specific radioactivity adsorbed to membranes and filters. Non-specific adsorption was measured as follows. Radioactive D- and L-glucose (1 μl) was added to a 12 × 100 mm tube, diluted with 1 ml ice-cold stop solution, and added to the appropriate volume of vesicle suspension. This mixture was then rapidly filtered, washed, and determinations of radioactivity performed as described.

Glucose efflux experiments were performed as follows. Membrane suspension (75 μl) was placed in a 15 × 120 mm glass tube and equilibrated 5 min at

room temperature. A mixture (5 μl) of D-[^{14}C]- and L-[^{3}H]glucose (0.4 μCi and 2.5 μCi, respectively) was added, and the tube incubated 5 min at room temperature. At this time, a 16-μl sample was removed, diluted with 1 ml ice-cold stop solution, and filtered as described. The remaining suspension was diluted with 4 ml room temperature Tris-buffered saline (with or without 200 μM phloretin), and 1-ml samples filtered at the appropriate times. Radioactivity determinations were performed as described for glucose influx.

Data are expressed as cpm in vesicles per mg protein divided by cpm per μl in the incubation mixture. This expression is distribution 'space', the volume (μl) of the incubation mixture containing the amount of permeant in vesicles equivalent to one mg protein.

Representative experiments will be presented. All experiments have been repeated with confirming results. Most have been repeated numerous times with confirming results. One can appreciate the modest point-to-point variability from the pattern of data on a given plot. There was little day-to-day variation in the pattern of data in a given kind of experiment. There was however considerable day-to-day and preparation-to-preparation variation in absolute space values, owing to the numerous sources of variation (vesicle purity, protein assay, assay of radioactivity added to vesicles, each of which had a proportional effect on all data in a given experiment).

Other methods. Protein was estimated by the method of Bradford [18] using cyrstalline bovine serum albumin (Sigma) as the standard.

Reagents. Radiochemicals were purchased from New England Nuclear. Phloretin was obtained from Mann, cytochalasin B from Aldrich, *N*-ethylmaleimide from Sigma, and Triton X-100 from Research Products International. All other chemicals were of the highest purity commercially available.

Results

Characterization of membrane preparation

Table I shows the marker enzyme activities at various stages in the membrane purification. Most transport experiments were carried out with the membranes which floated to the top in the sucrose gradient centrifugation. This fraction was 32-times enriched in 5′-nucleotidase, 46-times enriched in glucose-6-phosphatase, and 2-times enriched in monoamine oxidase, by comparison with the cell suspension. The final centrifugation removed a substantial amount of contaminating protein from this fraction. The results are essentially as expected from the original description of the preparation, which was shown by electron microscopy [12,13] to yield plasma-membrane vesicles.

TABLE I

MARKER-ENZYME ACTIVITIES IN MEMBRANE PREPARATION

Stage	5′-AMPase (nmol/min per mg)	G-6-Pase (nmol/min per mg)	MAO (pmol/min per mg)
Cell suspension	2.3	0.21	0.24
Post-nuclear supernatant	4.4	0.34	0.38
Mitochondrial pellet	9.3	2.02	3.45
Crude membranes	37.6	5.18	1.03
Sucrose gradient			
Top	74.2	9.69	0.41
Middle	49.4	4.85	0.78
Pellet	7.8	0.77	2.33

Data are averages from two different preparations. 5′-AMPase, 5′-nucleotidase; G-6-Pase, glucose-6-phosphatase; MAO, monoamine oxidase.

Equilibration time courses

As seen in Fig. 1, thymocyte membrane vesicles took up both D- and L-glucose, but at all time points, D-glucose space exceeded L-glucose space. The fold difference (D-Glc Space/L-Glc Space) was most pronounced in the early samples, the D-glucose time course being distinctly biphasic and the L-glucose time course being approximately monophasic. The difference curve (D-Glc Space minus L-Glc Space) designated 'Specific D-Glc Space' exhibited a very short half-time (less than 0.4 min in this experiment) by comparison with the L-glucose curve, which exhibits a half-time of about 4 min. These data indicate that the vesicle preparation contains two compartments, one of which is highly accessible to D-glucose but virtually inaccessible to L-glucose, the other of which is about equally accessible to both isomers.

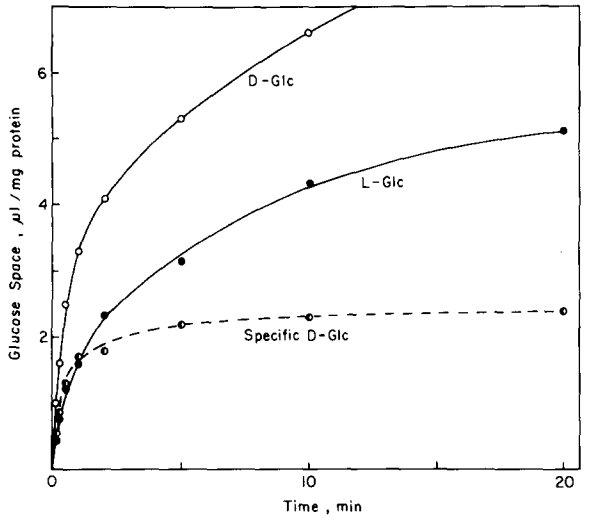
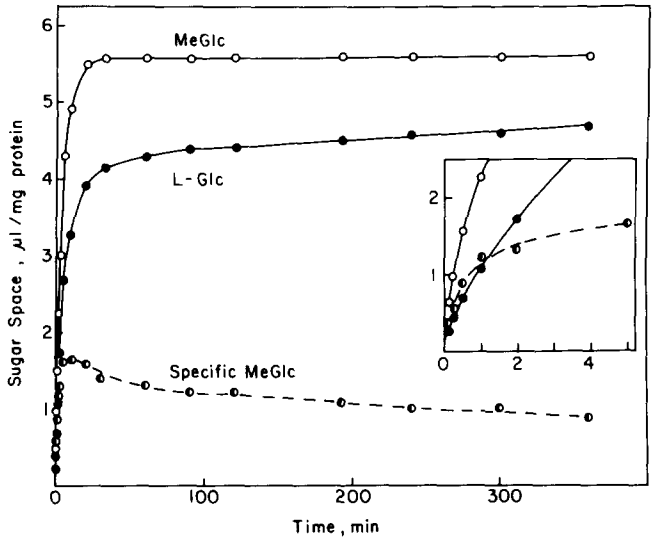


Fig. 1. Time course of D- and L-glucose influx into thymocyte membrane vesicles. To initiate sugar entry, a mixture of (1 µl) of D-[^{14}C]glucose (0.08 µCi) and L-[^{3}H]glucose (0.5 µCi) was added to 15 µl (50 µg protein) of membrane suspension at room temperature. Non-radioactive D-glucose was present in the radioactive sugar mixture to give a final glucose concentration of 100 µM. Vesicle radioactivity was assayed at the times indicated as described in Methods.

If the selective compartment is a vesicular volume (rather than a reaction product or bound form) it should equilibrate with L-glucose given enough time. Fig. 2 shows a very long time course of 3-*O*-methyl-D-glucose and L-glucose uptake. Methylglucose was like D-glucose in that it equilibrated faster than L-glucose. As seen in the inset, the difference curve approached its maximum with half-time of about 0.4 min. The similarity between methylglucose and glucose in this regard indicates that the specific space is not due to a metabolic conversion. The difference curve declined over the next 350 min as the L-glucose space gradually approached the methylglucose space. In this experiment, the L-glucose space approached the D-glucose space with a half time of about 6 h. In other experiments, the convergence occurred with a half-time of about 3 h. It appears that L-glucose



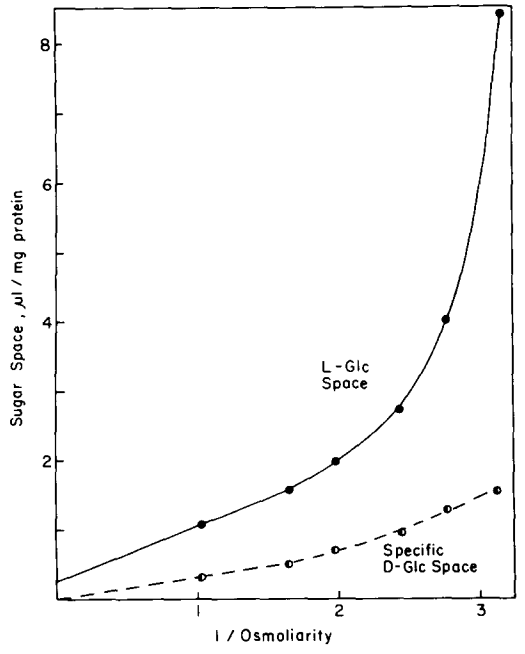


Fig. 2. (left). Time course of 3-*O*-methyl-D-glucose and L-glucose influx into thymocyte membrane vesicles. Transport was initiated by addition of 1 µl of a mixture of 3-*O*-methyl[^{14}C]glucose (0.08 µCi) and L-[^{3}H]glucose (0.5 µCi) to 15µl of membrane suspension (45 µg protein) at room temperature. The final methylglucose concentration was 100 µM. Transport was terminated at the times indicated and vesicle radioactivity assayed as described.

Fig. 3. (Right). Effects of osmolarity on 'equilibrium' sugar space. At 20°C, 10 µl of vesicle suspension was added to 10 µl Tris-buffered saline containing various concentrations of sucrose, and 1 µl of D-[^{14}C]glucose/L-[^{3}H]glucose mixture added. Reactions were stopped 20 min later for analysis of vesicular radioactivity. The sugar spaces at this time reflect equilibrium spaces rather well (Fig. 1). The 'specific D-glucose space' was calculated as the total D-glucose space (not shown) in excess of the L-glucose space.

does gain access to the specific D-glucose compartment, consistent with the view that this is a vesicular volume. This was also confirmed by the fact that the equilibrium β-hydroxybutyrate space was essentially the same as the D-glucose space and exceeded the L-glucose space (data not shown).

Effects of increased osmolarity on sugar space

To determine whether a significant fraction of vesicle sugar was bound, 20-min sugar spaces were measured in the presence of various concentrations of sucrose, which itself permeates very slowly (data not shown). As seen in Fig. 3, the expected linear dependence of sugar space on reciprocal external osmolarity was not found. Instead, modest amounts of sucrose reduced both L-glucose space and specific D-glucose space markedly, whereas further increases of sucrose concentration reduced permeant sugar space less dramatically. The relation was such that little if any sugar space would reside at infinite external osmolarity, indicating that very little L-glucose or D-glucose was bound to the vesicles, unless of course sucrose were a competitor for the binding site. The unusual shape of the space vs. 1/osmolarity curves may be due to salt permeability of the vesicles in their usual dimensions. If the vesicles were rather salt permeable, then they would assume dimensions which minimize potential energy (membrane stress and electrostatic). The shrinkage following modest sucrose addition would be followed by outward diffusion of salt, resulting in much more shrinkage than would occur if salt were impermeable. That modest and higher sucrose concentrations do not obliterate space completely might be due to: (a) reduced salt permeability in shrunken vesicles, (b) entry of some sucrose into vesicles, or (c) recoil of vesicles from their shrunken (deformed, high energy) state.

Effects of hypotonic lysis on vesicle contents

In the usual glucose uptake assay, uptake was stopped by addition of 1 ml ice-cold Tris-buffered saline containing 1 μM cytochalasin B. This was filtered and the filter washed with 5 ml of the same stop solution. Omission of cytochalasin B from the stop solution did not result in significant loss of sugars during the dilution and filtration which was completed in a few seconds. However, omission of the saline resulted in 80% loss of vesicle sugars. Omission of both saline and Tris-HCl from the stop solution resulted in total loss of vesicle sugars (leaving only that sugar which would be in the filter with no vesicles present). Since the vesicles were in isotonic medium prior to the dilution with stop solution, the hypotonic solutions would swell and lyse the vesicles. That this caused a loss of vesicle-associated sugar confirms the view that the vesicle sugar was dissolved in vesicle cavities rather than bound to vesicle components.

Effects of cytochalasin B

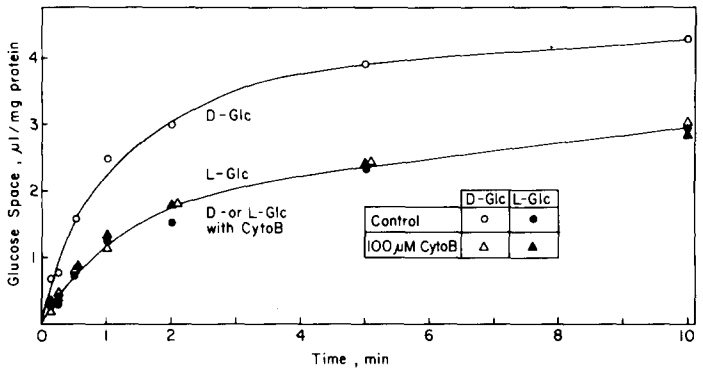
As seen in Fig. 4, a high dose of cytochalasin B (100 μM) obliterated the uptake advantage of D-glucose over L-glucose without affecting L-glucose uptake. With this classical glucose-transport inhibitor, D-glucose spaces were virtually the same as L-glucose spaces. This confirms the view that L-glucose uptake (or space) is an adequate measure of non-specific D-glucose uptake (or space). Fig. 5 shows the effects of cytochalasin B on 0.2-min D-glucose space in excess of L-glucose space. Although 0.2-min D-glucose uptake may not exactly reflect initial rate, this interval was operationally comfortable and provided enough signal to noise ratio (vesicular radioactivity relative to blank) for reliable measurements of specific D-glucose space. To the extent that the uptake is nonlinear, the data underestimate effects of agents. We would, therefore, conclude from Fig. 5 that the K_i of cytochalasin B inhibition at 20°C is less than the apparent K_i of 2 μM. These data support the view that specific D-glucose uptake is carrier-mediated entry into vesicles.

Effects of other agents on sugar uptake

As shown in Fig. 6A, phloretin inhibited specific D-glucose entry into thymocyte membrane vesicles. The apparent K_i of this agent was approximately 200 μM. *N*-Ethylmaleimide (Fig. 6B) likewise inhibited sugar entry, 50% inhibition occurring at approx. 10 mM. These findings provide additional support for carrier-mediated sugar entry into the vesicular lumen.

Sugar efflux

If sugar uptake is indeed entry into vesicle cavities and if the D-glucose space in excess of L-glucose space is due to carrier mediated transport, then the time



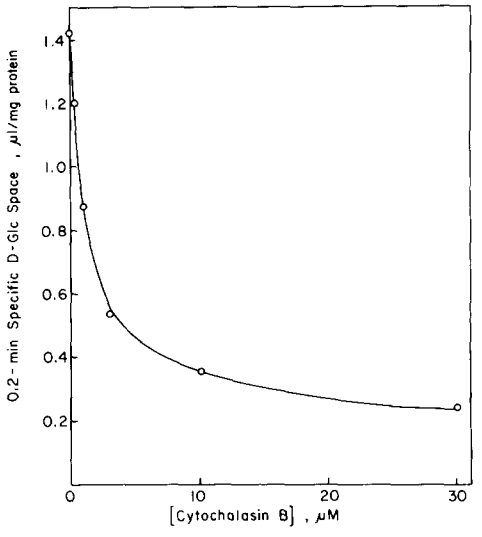


Fig. 4. (Left). Effects of cytochalasin B on time course of D- and L-glucose entry into thymocyte membrane vesicles. Cytochalasin B in ethanol or ethanol alone (6 μl) was added to the membrane suspensions (270 μl) to give the following final concentrations: ethanol (2%), cytochalasin B (100 μM). After 10 min at room temperature, 15 μl samples (50 μg protein) were removed and sugar uptake determined as described in Fig. 1.

Fig. 5. (Right). Dose dependence of cytochalasin B inhibition of D-glucose entry into thymocyte membrane vesicles. Cytochalasin B in ethanol or ethanol alone (1 μl) was added to the membrane suspensions (44 μl) to give final concentrations of ethanol (2%) and cytochalasin B as indicated. After incubation for 10 min at room temperature, duplicate 15-μl samples (38 μg protein) were removed and 0.2-min sugar uptake measured as described.

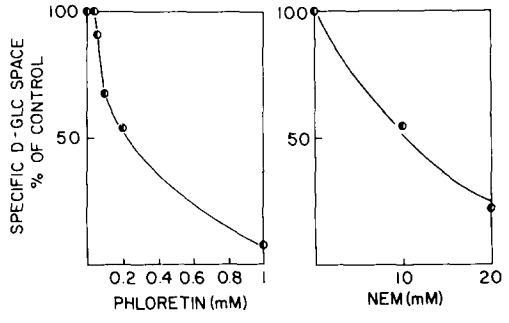


Fig. 6. Effects of phloretin and *N*-ethylmaleimide (NEM) on specific D-glucose entry into thymocyte membrane vesicles. (A) Ethanol (1 μl), which contained or lacked phloretin at the final concentrations indicated, was added to membrane suspension (44 μl). After 10 min at room temperature, portions (15 μl, 35 μg protein) were used to assay sugar transport. (B) One μl of incubation medium 10% in ethanol (with or without *N*-ethylmaleimide at the final concentrations indicated) was added to 14 μl of membrane suspension (40 μg protein). The mixture was incubated 10 min at room temperature at which time sugar transport was initiated. In both instances, sugar entry was terminated at 0.2 min, and the specific D-glucose space determined as described in Methods.

courses suggest the presence of two very different kinds of vesicles, sealed vesicles equipped with the glucose carrier and 'leaky' vesicles functionally lacking the carrier. To examine this proposition further, we observed sugar exit from 5-min preloaded vesicles (Fig. 7). This loading time sufficed for equilibration of the specific D-glucose space but not for equilibration of the non-specific space, judging from Fig. 1. It is seen that D-glucose space of control vesicles (open circles) was initially higher than the L-glucose space and fell with a half-time of about 0.2 min to the L-glucose space (filled circles), after which D-glucose and L-glucose spaces were similar as exit continued. This shows that the compartment permeable to L-glucose was equally permeable to D-glucose. This equality would not obtain if the glucose carrier contributed to D-glucose transport into and out of this compartment. Exit of D- and L-glucose from the non-specific compartment is much too fast (half-time of few minutes) for this compartment to be enclosed by the specific compartment (L-glucose

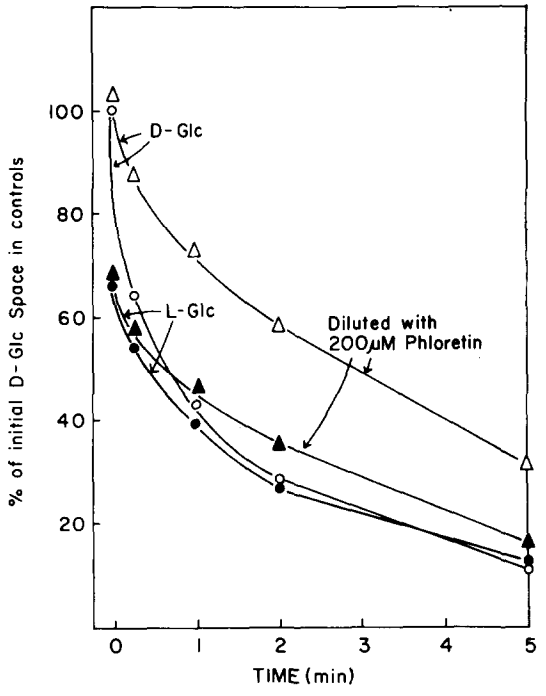


Fig. 7. Time course of glucose release from thymocyte membrane vesicles. Membranes (160 μg protein) in 75 μl were incubated with radioactive D- and L-glucose for 5 min at room temperature. Efflux was initiated by dilution and vesicle radioactivity was assayed as described in Methods.

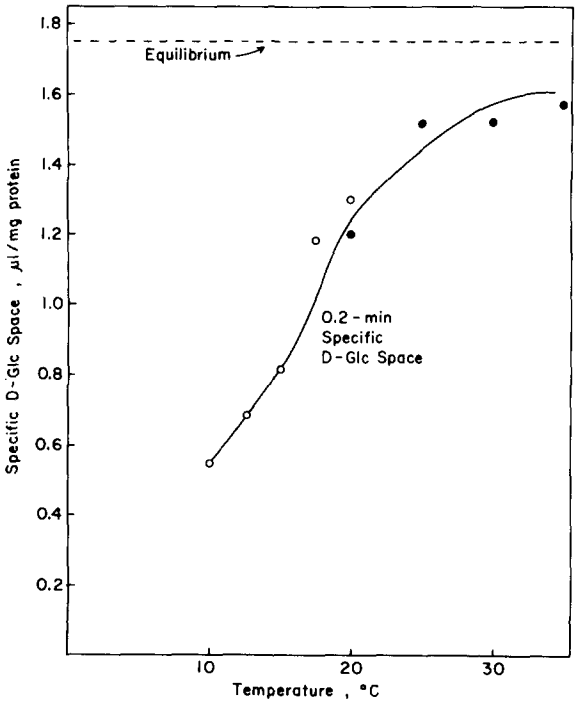


Fig. 8. Temperature dependence of specific D-glucose uptake in thymocyte membrane vesicles. Membrane suspension (15 μl, 35 μg protein) was equilibrated 5 min at the appropriate temperature. Specific D-glucose entry (0.2 min) was assayed essentially as described.

half-time of several hours). Likewise, the exit of D-glucose from the specific compartment was much too fast (half-time of 0.2 min) for this compartment to be enclosed by the non-specific compartment (half-time of few minutes for both isomers). The two compartments then communicate with the medium directly, as expected if they are two kinds of vesicles.

The figure also shows that phloretin in the dilution medium prevented the rapid fall of D-glucose space to the L-glucose space, again supporting the view that the glucose carrier accounts for the selectivity of the fraction of vesicles taking up D-glucose and virtually excluding L-glucose.

Temperature dependence

Fig. 8 shows 0.2-min specific D-glucose space at several temperatures. The discriminant vesicles at 20°C were more than half equilibrated by 0.2 min, so the increased initial rates with higher temperature do not result in comparably increased 0.2-min spaces. The increased transport at higher temperature is better reflected in the reduction of yet unequilibrated space which was diminished by half by a increase of temperature from 20 to 30°C (filled circles). The 0.2-min spaces at temperatures below 20°C (open circles), reflect more directly the temperature sensitivity of initial rate. Increasing temperature from 10 to 20°C more than doubled transport rate. Since we could not comfortably and reliably assay transport over intervals less than 0.2 min, we used temperatures below 25°C for most experiments.

Kinetic properties

The dependence of 0.2-min specific D-glucose space on glucose concentration is shown in Fig. 9. In these S/v vs. S plots, the half saturation concentration is that which raises S/v to twice the y intercept. Each of the data plots is bent concave up, suggesting cooperativity. For example, in the experiment where

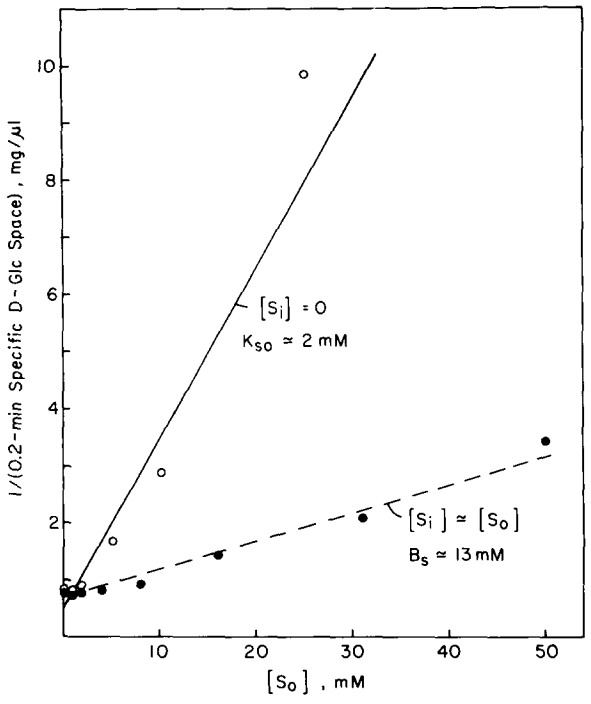


Fig. 9. Dependence of influx on external and internal glucose concentration. Unlabelled glucose was either mixed with the D-[^{14}C]glucose/L-[^{3}H]glucose mixture prior to addition to the vesicle suspension (○, $[S_i] = 0$) or was equilibrated with the vesicles 30 min prior to addition of the D-[^{14}C]-glucose/L-[^{3}H]glucose mixture (●, $[S_i] = [S_o]$). In all cases, vesicular radioactivity was measured 0.2 min after labelled glucose addition. The initial slope of a glucose-space vs. time plot is v/S (μmol in per min per mg protein divided by μmol/μl external concentration); so the reciprocal of 0.2-min space is proportional to S/v, if uptake is linear for 0.2 min.

labelled and unlabelled glucose were added simultaneously ($[S_i] = 0$) the general trend of the data indicates a K_m of about 2 mM, but the apparent influx coefficient (v/S) was greater at 1 mM than 0.1 mM and was essentially the same at 2 mM as at 0.1 mM. Had 2 mM glucose actually reduced influx coefficient 50% (relative to 0.1 mM), the 0.2-min space should have been substantially reduced (relative to that of 0.1 mM).

The other interesting feature of this experiment is that higher external sugar concentrations (5 mM to 25 mM) reduced influx coefficient (increased reciprocal 0.2-min space) much more in vesicles lacking glucose than in vesicles containing glucose at the external concentration, so the apparent equilibrium-exchange K_m ($B_s \simeq 13$ mM) was much higher than the apparent entry K_m ($K_{so} \simeq 2$ mM). This striking difference between entry K_m and exchange K_m is not an artifact attributable to the fact that 0.2-min uptake underestimates initial uptake. It can be shown with integrated transport equations that, if 0.2 min is the half time for equilibration with low substrate concentration (0.1 mM), then our experiment (0.2-min samples) would overestimate entry K_m about by 1.11-times and overestimate equilibrium-exchange K_m about 1.46-times. The apparent exchange K_m/entry K_m ratio would be about 1.3, if the true ratio were 1.0. The observed ratio of 6.5 could, therefore, not be attributed to our inability to measure initial rates. The true ratio of exchange $K_m$to entry K_m must be about 5, as would be the ratio of exchange V to entry V [19,20]. These properties have been seen in rat thymocytes [19] and human erythrocytes [20]. They indicate that maximal sugar entry rate is limited by outward movement of empty carrier and that loading of inward-facing carrier facilitates its reorientation to the outside. This language is not meant to imply that the sugar-binding site necessarily moves from face to face. The translocation might involve opening and closing of pores between the binding site and bulk phases.

Although the glucose influx coefficient was inversely related to glucose concentration, the equilibrium glucose space was not. At 10, 20 and 30 min, D-glucose and L-glucose spaces were independent of glucose concentration between 0.2 mM and 10 mM. This confirmed the earlier conclusion that no significant fraction of vesicle sugar was bound.

Discussion

These studies demonstrate that calf-thymocyte-plasma-membrane preparations contain vesicles capable of taking up glucose and retaining it during filtration. There appear to be two kinds of vesicles as regards glucose transport. Nondiscriminant vesicles equilibrate with D- and L-glucose at the same rate ($t_{1/2} \simeq 2$ to 5 min). Discriminant vesicles equilibrate with D-glucose rapidly ($t_{1/2} \simeq 0.2$ to 0.4 min) and with L-glucose slowly ($t_{1/2} \simeq 3$ to 6 h). The discriminant vesicles exhibit the usual properties of plasma-membrane glucose transport: stereospecificity,

high temperature sensitivity, saturation entry kinetics, and inhibition by phloretin, *N*-ethylmaleimide and cytochalasin B. Internal sugar enhances influx of external sugar in these vesicles as in whole rat thymocytes. We presume that they are indeed plasma membrane vesicles. Attempts to separate the two kinds of vesicles by sucrose-density-gradient centrifugation have met with little success, suggesting that the density dispersions of the vesicle types are similar. Similar results with plasma-membrane-enriched vesicles from cultured chick-embryo fibroblasts have been reported [21].

The thymocyte plasma-membrane preparation, then, appears to contain the plasma-membrane glucose carrier and is an appropriate material for solubilization-reconstitution-purification studies. While the preparation is contaminated by vesicles which take up D- and L-glucose indiscriminantly, glucose transport activity can be adequately studied by use of double-label techniques. This preparation may, therefore, be useful for testing hypotheses regarding transport regulatory signals. Rat-thymocyte membranes, prepared by a scaled down version of the bovine-thymocyte membrane method, behaved like the bovine membranes in all respects examined (equilibrium D-glucose space, equilibrium L-glucose space, uptake half-times, saturability, inhibitability, etc.).

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