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D-GLUCOSE TRANSPORT BY MEMBRANE VESICLES FROM QUIESCENT, SERUM-STIMULATED, AND SV40-TRANSFORMED MOUSE 3T3 CELLS

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Mixed membrane vesicle preparations from mouse embryo fibroblasts (Swiss 3T3) exhibited a facilitated diffusion transport system for D-glucose that showed many of the characteristics of the D-glucose transport system of whole cells: stereospecificity, counterflow, Michaelis-Menten kinetics with an apparent K_m similar to that of whole cells, and sensitivity to inhibition by cytochalasin B. Comparison of the stereospecific D-glucose transport activities of membrane vesicles from quiescent, serum-stimulated, and SV40 virus-transformed 3T3 cells showed no significant differences in rates of D-glucose uptake or efflux. This is in contrast to whole cells; quiescent 3T3 cells transported 6-deoxy-D-glucose at a significantly lower rate than serum-stimulated or SV40-transformed cells. These results indicate that D-glucose transport in quiescent vs. actively growing cells is regulated by cellular factors that are not retained in membrane vesicle preparations.

Introduction

The transport of glucose by animal cells in culture is one of numerous cellular functions that is regulated in accordance with the growth state of the cell [1,2]. Thus, when growth of untransformed fibroblast cells becomes arrested as a consequence of serum starvation or reaching confluence, the cells enter a quiescent G_0 stage of the cell cycle, which is accompanied by a number of physiological changes, which includes a decrease in hexose transport rate. The quiescent G_0 stage is reversible upon the addition of serum or mitogens, and there is an increase in glucose transport activity following release from growth arrest [3–14]. Glucose starvation also enhances glucose transport activity [15]. Transformed cells are considered to be independent of serum and density regulation of growth and transport, but there are many reports of glucose transport enhancement in mouse embryo fibroblasts (3T3) beyond that exhibited by untransformed cells [6,8,16–18]; this transformation-associated enhancement of glucose transport appears to be especially prominent in Rous sarcoma virus (RVS)-transformed chick cells [7,12,19,20].

Regardless of whether glucose transport enhancement is a specific consequence of transformation, there is wide agreement that the apparent Michaelis-Menten constant (K_m) of glucose transport does not change, while the V_{max} is increased in actively growing serum-stimulated cells, cells plated at low density, glucose-starved cells, or transformed cells, compared to quiescent cells. This has been interpreted as indicating that there are more glucose carriers in the membrane, or the carriers are more accessible in actively growing or transformed cells, or that there are cellular factors which regulate the operation of the glucose carriers. It would appear that isolated membrane vesicle systems would present promising experimental means to determine the basis of glucose

Abbreviations: EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

transport regulation. Thus, there have been a number of studies of glucose transport by membrane vesicles from untransformed and transformed fibroblasts from mouse [21,22] and chick embryo [23–25]. The results are not in agreement, however. Lever [21] found no difference in D-glucose transport in vesicles derived from subconfluent, confluent, or SV40-transformed mouse 3T3 cells, while Inui et al. [22,23] reported increased D-glucose transport in vesicles from both SV40-transformed 3T3 cells, and RSV-transformed chick cells. In agreement with the latter, increased D-glucose transport in vesicles from RSV-transformed chick cells was reported by Zala and Perdue [24] and Decker and Lipmann [25].

We report here on D-glucose transport by membrane vesicles derived from quiescent, serum-stimulated, and SV40-transformed mouse 3T3 cells. A preliminary account of this work has appeared [26].

Materials and Methods

Cell culture. Swiss 3T3 cells, and SV101, an SV40 virus transformant of 3T3, were seeded into roller bottles (Corning, 490 cm^2) containing 50 ml Dulbecco's modified Eagle's medium (DME) supplemented with 10% calf serum (Gibco) at an initial density of $1.2 \cdot 10^7$ cells per bottle. Cultures were incubated at 37°C. SV101 cells were harvested immediately upon reaching confluence, since these cells became detached upon further incubation. To obtain quiescent 3T3 cells, the cultures were grown to confluence, then incubated an additional 24 h without change of medium. To obtain serum-stimulated 3T3 cells, the cultures received a change of medium containing 20% calf serum at confluence, and then were incubated for 8 h before harvest; previous experiments have shown that this treatment yielded 3T3 cells with maximum activity of the glucose uptake system [14].

Preparation of membrane vesicles. Mixed membrane vesicles were prepared according to the method of Inui et al. [22,23]. Cells were harvested by scraping into the culture medium with a rubber policeman while the bottles were being rotated in an ice bath. All subsequent operations were carried out at 4°C. Cells were collected by centrifugation at $500 \times g$ for 10 min, and washed twice by resuspension in 0.25 M sorbitol/1 mM Tris-Hepes (pH 7.5)/0.5 mM $MgCl_2$ (buffer A). The suspensions were centrifuged at $500 \times g$ for 10 min after the first wash, and $1000 \times g$ for 20 min after the second wash. The packed cell volume was noted, and the cells were resuspended in 20 vol. of buffer A. The cell suspension was gently homogenized by five strokes in a Dounce homogenizer, then subjected to 680 lb/$inch^2$ nitrogen pressure for 10 min in a cell disruption bomb (Parr Instrument CO., Moline, IL) while being stirred with a magnetic stirrer. After dropwise collection of the ruptured cell suspension, K_2EDTA (pH 7.5) was added to a final concentration of 1 mM. The homogenate was then centrifuged successively at $750 \times g$ for 15 min to remove intact cells and nuclei, $20\,000 \times g$ for 20 min to remove mitochondria, and at $100\,000 \times g$ for 1 h to collect the mixed membrane fraction. The pellet was resuspended in 0.1 M sorbitol/1 mM Tris-Hepes (pH 7.5) (buffer S) by passing through a fine gauge needle, centrifuged again at $100\,000 \times g$ for 1 h, and resuspended at a final density of 3–5 mg protein per ml. The yield of mixed membrane material obtained by this procedure was 0.5–1.0 ml suspension from 25–30 roller bottle cultures. Suspensions were frozen quickly in a solid CO_2-acetone bath, and stored in a Revco freezer at −80°C until used, whereupon they were held in a water bath at room temperature until thawed, and kept on ice.

Membrane vesicle transport measurement. 20 µl vesicle suspension in buffer S, containing 30–100 µg protein, was placed in the bottom of a plastic tube (12 × 75 mm, Falcon Plastics) and agitated gently with a vortex mixer at room temperature. Uptake was initiated by forcefully ejecting 5 µl double label glucose solution containing 10 mM D-[^{14}C]glucose and 10 mM L-[^{3}H]glucose, each at a specific radioactivity of 10 µCi per µmol, into the droplet of vesicle suspension from a pipetman (Gilson) while gently agitating (final concentration 2 mM D-glucose and L-glucose). After appropriate incubation times, uptake was stopped by the addition of 4.5 ml ice-cold stop solution (0.8 M NaCl/0.01 M Tris buffer (pH 7.5)/0.1 mM phloretin/0.1% (v/v) ethanol (phloretin was added by diluting a 0.1 M ethanolic solution 1–1000 with buffer)). The reaction mixture was immediately filtered on a membrane filter (Millipore

HA, 25 mm, 0.45 μm porosity) and washed with 4.5 ml ice-cold stop solution. The filter was immediately removed from the assembly and placed in 10 ml Bray's scintillation fluid [27] for double label radioassay in a Packard Liquid Scintillation Spectrometer (Downer's Grove, IL). Filtration, washing, and removal from the filter assembly were completed within 15 s. All radioactivity counts were corrrected for a blank determined for each set of measurements by adding 5 μl of isotope solution to 20 μl buffer S (without membrane vesicles) and processing exactly as above. Stereospecific D-glucose uptake was calculated as nmol D-[^{14}C]glucose minus nmol D-[^{3}H]glucose (correction for adsorption and simple diffusion) per μl intravesicular volume. Results that are shown are averages of duplicate determinations made at each time point or varied substrate concentration in a given experiment. Experiments reported are representative of several carried out with two different membrane preparations from each cell type.

Determination of intravesicular volume. Intravesicular volume was determined as space occupied by [^{14}C]urea after diffusion equilibrium minus space occupied by [^{3}H]inulin. 20 μl vesicle suspension in buffer S (40–80 μg protein) was mixed by gentle vortexing with 5 μl of a double label solution of 10 mM [^{14}C]urea (2 μCi per μmol) and [^{3}H]inulin (1 μCi per ml) in buffer S (final concentration 2 mM [^{14}C]urea, 0.2 μCi/ml [^{3}H]inulin). After incubation for 1 h at room temperature to allow diffusion equilibrium, 2.5 ml ice-cold buffer S was added rapidly, the vesicles were collected by filtration (Millipore HA, 25 mm, 0.45 μm porosity), washed with an additional 2.5 ml ice-cold buffer S, and placed in 10 ml Bray's scintillation fluid for double label counting. Volume was calculated as μl per mg protein (nmol [^{14}C]urea taken up/2 nmol per μl minus nCi [^{3}H]inulin/0.2 nCi per μl, all divided by mg protein). All counts were corrected for a blank (20 μl buffer S + 5 μl [^{14}C]urea and [^{3}H]inulin solution as above, filtered and washed as above). Average values of triplicate determinations made on vesicle preparations used in this study were: quiescent 3T3, 1.4 μl per mg protein; serum-stimulated 3T3, 0.1 μl per mg protein, SV101, 0.52 μl per mg protein.

Whole cell transport measurement. Activity of the glucose transport system in whole cells was measured by incubating cells grown on coverslips with 6-deoxy-D-[^{3}H]glucose, a non-metabolizable analog of glucose, by previously described methods [28,29].

Analytical methods. Protein in whole cells and in vesicle preparations was determined by the method of Lowry et al. [30].

Materials. D-[U-^{14}C]Glucose, L-[G-^{3}H]glucose, 6-deoxy-D-[G-^{3}H]glucose; [^{14}C]urea, and [G-^{3}H]-inulin were obtained from New England Nuclear, Boston, MA. Cytochalasin B was purchased from Sigma Chemical Co., St. Louis, MO, and phloretin was from K and K Laboratories, Plainview, NY.

Results

The time courses of total glucose uptake and stereospecific D-glucose uptake by the membrane vesicle preparations are shown in Fig. 1A. The following observations are to be made: (i) uptake was rapid in all three vesicle preparations, reaching a maximum within 20–30 s; (ii) non carrier-mediated uptake of L-glucose was significant, amounting to approximately one-third that of total D-glucose uptake, and nearly one-half that of stereospecific D-glucose uptake; (iii) the rates and final steady-state levels of total glucose uptake and stereospecific D-glucose uptake were similar in vesicle preparations made from the three cell types; if anything, uptake was slowest in vesicles from the transformed cells (SV101), but the difference is probably not significant. The final steady-state levels of stereospecific D-glucose uptake approximated the external concentration in all cases.

These results are to be compared to those obtained with whole cells (Fig. 2), where glucose transport activity was measured by following the uptake of 6-deoxy-D-glucose, a homomorphic analog of D-glucose, which cannot be phosphorylated by intracellular kinases, and which shows good affinity and specificity for the D-glucose transport system [28]. It is clear that transport activity by whole cells was much slower in all cases than that shown by vesicles; moreover, the rate of 6-deoxy-D-glucose uptake by quiescent 3T3 cells was significantly lower than that shown by serum-stimulated 3T3 cells and the transformed cells (SV101). In contrast to vesicles, where steady-state levels of

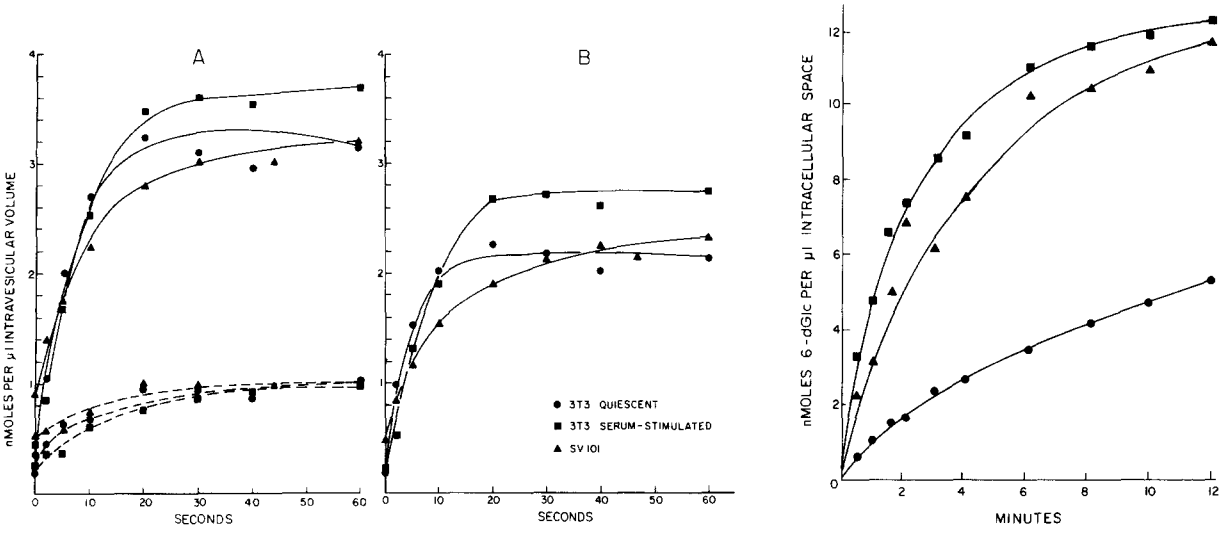

Fig. 1. Uptake of D-[^{14}C]glucose and L-[^{3}H]glucose by membrane vesicles. Vesicles were incubated with 2 mM D-[^{14}C]glucose and 2 mM L-[^{3}H]glucose (each 10 μCi/μmol). (A) Total uptake of D-glucose (solid lines) and L-glucose (broken lines) by vesicles from quiescent 3T3 (●), serum-stimulated 3T3 (■) and SV101 (▲). (B) Stereospecific D-glucose uptake (D-glucose minus L-glucose). Symbols as in A.

Fig. 2. Uptake of 6-deoxy-L-glucose (6-dGlc) by whole cells. Cells on coverslips were incubated at room temperature in the presence of 6.4 mM 6-deoxy-D-[^{3}H]glucose (4 μCi/μmol). ●, Quiescent 3T3; ■, serum stimulated 3T3; ▲, SV101.

sugar were reached in 20–30 seconds, steady-state levels of the sugar analog in whole cells were approached at 6 min in the case of serum-stimulated 3T3 cells and SV101, and were not reached at all during the 12 min duration of the experiment by quiescent 3T3 cells.

It was important to establish that stereospecific D-glucose transport took place in vesicles by carrier-mediated facilitated diffusion, as it does in whole cells. One of the characteristics of facilitated diffusion systems in whole cells, which is considered to provide strong evidence for the operation of a mobile carrier in such systems, is the phenomenon of counterflow (or countertransport): when cells are preloaded with a non-radioactive transport substrate and then transferred to a solution containing a lower concentration of the radioactive transport substrate or one of its structural analogs, there is an enhanced rate of inward flux of the radioactive species and an apparent transient accumulation again a concentration gradient, because the outward flux of the radioactive species is decreased by its displacement from the carrier by the nonradioactive species present in higher

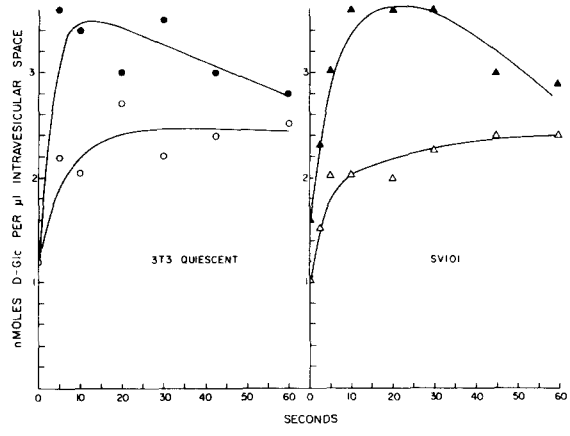

Fig. 3. Demonstration of stereospecific D-glucose (D-Glc) counterflow with membrane vesicles. Preloaded vesicles (●, ▲) were preincubated with 10 mM D-glucose for 1 min, then diluted with buffer S contaning D-[^{14}C]glucose and L-[^{3}H]glucose to give final concentration of 2 mM D-glucose and L-glucose (10 μCi/μmol each). Control vesicles (not preloaded, ○, △) were incubated with buffer S for 1 min, then diluted with buffer S containing isotopic sugars to give the same concentrations and specific activities as above (2 mM, 10 μCi/μmol).

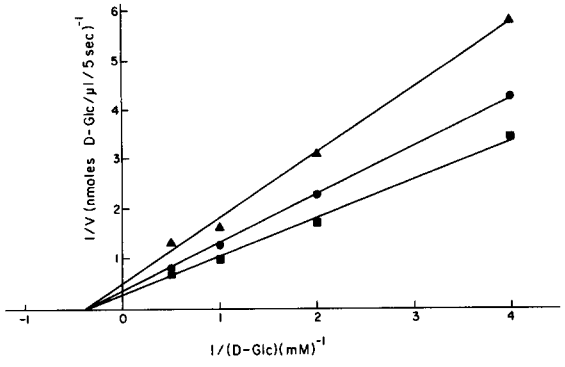

Fig. 4. Lineweaver-Burk plot of stereospecific D-glucose (D-Glc) uptake by membrane vesicles. Vesicles prepared from quiescent 3T3 (●). serum-stimulated 3T3 (■), and SV101 (▲) were incubated with D-[^{14}C]glucose and L-[^{3}H]glucose at indicated concentrations, each at 10 μCi/μmol.

concentration on the internal side of the cytoplasmic membrane. Fig. 3 demonstrates counterflow in the stereospecific uptake of D-[^{14}C]glucose by membrane vesicles from 3T3 and SV101 cells. In both cases, there was an enhanced uptake and a transient concentration in vesicles that had been preloaded with D-glucose.

Fig. 4 shows a Lineweaver-Burk plot of the stereospecific uptake of D-glucose by vesicles derived from quiescent 3T3, serum-stimulated 3T3, and SV101, measured for 5 s to obtain initial velocities. All three vesicle preparations showed the same apparent K_m of 2.7 mM, a value that is close to the value previously reported for whole 3T3 cells and a number of other animal cell types (about 2 mM) [29,31,32]. Values of V_{max} for the different vesicle preparations did not vary greatly, ranging from 0.40 nmol per μl for s for SV101 to 0.57 and 0.66 nmol per μl per s for serum-stimulated and quiescent 3T3 cells, respectively.

Similar activity of the D-glucose carrier in the three vesicle preparations was also indicated by efflux experiments, shown in Fig. 5A where first order efflux rates are shown, and compared with

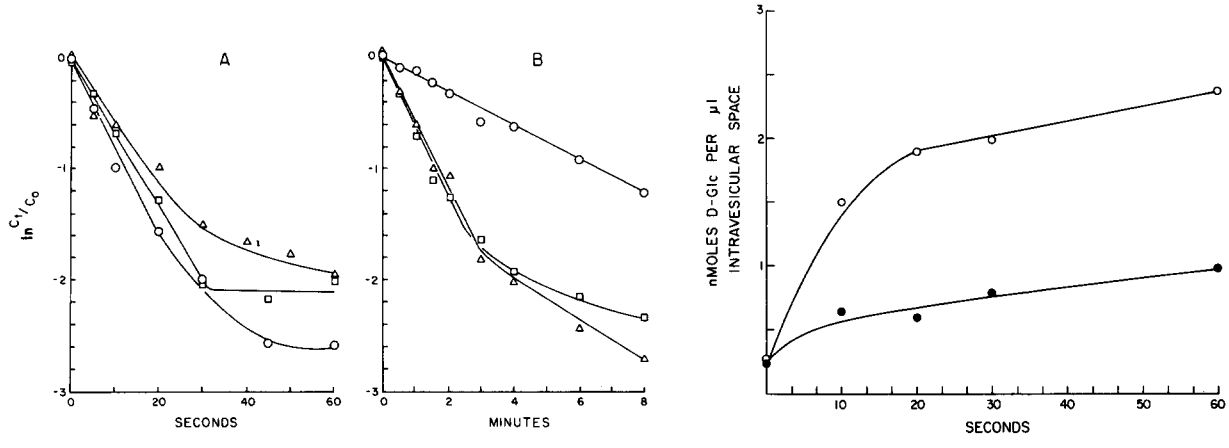

Fig. 5. Stereospecific efflux of D-glucose from membrane vesicles (A), and efflux of 6-deoxy-D-glucose from whole cells (B). (A) Vesicles: for each timepoint, 20 μl membrane vesicle suspension were mixed with 5 μl 10 mM D-[^{14}C]glucose and 10 mM L-[^{3}H]glucose (final concentration of each 2 mM, 10 μCi/μmol); uptake was allowed to proceed at room temperature for 1 min; efflux commenced on addition of 2.5 ml buffer S at room temperature, and stopped at appropriate time by filtration and immediate washing on the filter with 5 ml ice-cold stop solution (see Materials and Methods). Intravesicular concentration at zero time was determined by adding 2.5 ml ice-cold stop solution after 1 min uptake period, filtering, and washing with 5 ml ice-cold stop solution. (B) Whole cells: cells on coverslips were incubated in a petri dish with 10 ml glucose-free Hanks' solution containing 4 mM 6-deoxy-D-[^{3}H]glucose (4 μCi/μmol) at room temperature to allow preloading (quiescent 3T3, 20 min; serum-stimulated 3T3, 15 min; SV101, 5 min). The isotopic solution was aspirated off and replaced with room-temperature glucose-free Hanks' solution to start efflux. Coverslips were removed at appropriate time intervals, and were washed four times with ice-cold glucose-free Hanks' solution. Symbols: ○, quiescent 3T3; □, serum-stimulated 3T3; △, SV101. C_t and C_0 are intravesicular or intracellular concentrations of sugar at time t and time zero of efflux, respectively.

Fig. 6. Inhibition of stereospecific D-glucose (D-Glc) uptake by cytochalasin B. Vesicles from serum-stimulated 3T3 were incubated with 2 mM D-[^{14}C]glucose and L-[^{3}H]glucose (each at 10 μCi/μmol) in the absence (○) and presence of 20 μM cytochalasin B (●).

whole cells (Fig. 5B). Exit half-times (when ln $C_t/C_0 = -0.69$, where C_t and C_0 are intravesicular concentrations at time t and time zero, respectively) from vesicles were: quiescent 3T3, 9 s; serum-stimulated 3T3, 10 s; SV101, 12 s. As was the case for uptake, efflux from whole cells was slower in all cases, but the exit rate from quiescent 3T3 cells ($t_{1/2} = 4.6$ min) was about one-third of that from serum-stimulated 3T3 cells or SV101 ($t_{1/2} = 1.3$ min).

Fig. 6 confirms that cytochalasin B, a potent inhibitor of glucose transport in a wide variety of cell types, also inhibited stereospecific D-glucose uptake by our membrane vesicle preparations.

Discussion

Membrane vesicle preparations from mouse 3T3 cells used in this study showed many of the characteristics of whole cells with respect to properties of the D-glucose transport system. Thus, they exhibited a stereospecific carrier-mediated facilitated diffusion system, the phenomenon of counterflow, Michaelis-Menten kinetics with a K_m close to that shown by whole cells, and sensitivity to inhibition by cytochalasin B. However, both uptake and efflux of D-glucose was much more rapid in membrane vesicles than was the uptake and efflux of 6-deoxy-D-glucose in whole cells. Moreover, the decreased rate of sugar uptake and efflux found in quiescent 3T3 cells, as compared to growing cells that were released from density dependent growth inhibition by serum addition, or to SV40-transformed cells, was not manifest in the membrane vesicle preparations. Rates of stereospecific D-glucose uptake and efflux were essentially the same in vesicles from quiescent 3T3 cells, serum-stimulated 3T3 cells, and SV40-transformed 3T3 cells. Thus, these data indicate that there are cellular factors which modulate D-glucose transport that are not retained by membrane vesicles.

Comparison of our data with those of others does not yield a consistent picture, however. We are in agreement with Lever [21] that D-glucose transport activity was essentially the same in membrane vesicles prepared from confluent or non-confluent mouse 3T3 cells, or SV40 transformed 3T3 cells. On the other hand, Inui et al. [22] reported that the initial rate of stereospecific D-glucose uptake was more than 2-fold higher in vesicles prepared from SV40 transformed 3T3 cells than in vesicles from untransformed cells. It is difficult to reconcile differences between these latter results and those reported here. Differences are probably not due to method of membrane vesicle preparation, since we followed the procedures worked out by these authors. Differences may be due to methods of transport assay. Inui et al. [22] calculated results on the basis of intravesicular concentration of D-glucose as we have, but determined intravesicular volume from the equilibrium uptake of D-glucose or 3-*O*-methyl-D-glucose. We used urea as the permeant substance to determine intravesicular volume, feeling that is was preferable to use a substance whose permeation and concentration was independent of the system under study.

In the reports of three studies with the chick embryo fibroblast - Rous sarcoma virus (RSV) system it was concluded that membrane vesicles from RSV transformed cells transported D-glucose at higher rates than vesicles from untransformed cells [23–25]. However, in two of these studies the difference in stereospecific D-glucose uptake rate between vesicles from transformed vs. untransformed cells was small in comparison to differences that have been reported in whole cells. Inui et al. [23], using a chick embryo fibroblast line infected with a temperature-sensitive mutant of RSV (TS-68), reported that vesicles from cells grown at the temperature permissive for the transformed phenotype showed a 1.5-fold increase in stereospecific D-glucose uptake over vesicles from cells grown at the non-permissive temperature (based on intravesicular volume). The data of Zala and Perdue [24] show only a 1.3-fold difference between vesicles from untransformed cells vs. vesicles from cells transformed with RSV (based on vesicle protein). These difference are small indeed compared to difference that have been reported for whole cells, where RSV transformed chick cells have been reported to transport 2-deoxy-D-glucose or 3-*O*-methyl-D-glucose as much as 6–8-times as rapidly as growing normal cells and 24–33-times as rapidly as density inhibited cells [7,33]. Only Decker and Lipmann [25] have reported a difference in D-glucose transport rates between vesicles from RSV transformed chick cells

and vesicles from non-transformed cells that is comparable to that seen in whole cells; these workers reported a 3–5-fold increase in initial rate in vesicles from transformed cells (based on vesicle protein). However, they also found that vesicles from glucose-starved normal cells transported D-glucose essentially as rapidly as vesicles from transformed cells, and that vesicles from serum-starved cells did not transport D-glucose at a significantly lower rate than vesicles from normal subconfluent growing cells, a situation not seen in whole cells. Another feature of this latter study was the report of a putative second transport system for D-glucose with a very high K_m that was operative at glucose concentrations of 5–25 mM and was not saturable. It was not clear from the data whether adequate correction was made for simple diffusion, which would be very significant at these high concentrations of glucose. The authors stated that L-glucose uptake occurred at about 20% of the initial rate of D-glucose uptake in normal cell vesicles and was similar in other types of vesicles, but there was no indication that L-glucose uptake was incorporated as an internal control in the experiments reported, as was the case in our study and in others cited above [22–24].

Lee and Lipmann [34,35] have reported the extraction of proteins from chick embyro cells, loosely bound to normal cells and more tightly bound to transformed cells, that bind glucose and stimulate glucose transport activity upon addition to cells. The relationship of these proteins to D-glucose transport by membrane vesicles is not clear. Inui et al. [23] have speculated that partial loss of these proteins during membrane preparation may explain differences in transport properties between intact cells and membrane vesicles.

Taking all these data together, it would appear that the question of whether the enhanced D-glucose transport exhibited by actively growing normal cells and transformed cells in comparison to quiescent cells is solely a function of the number of glucose carriers in the cytoplasmic membrane and thus is an intrinsic property of the membrane itself, or whether there are cytoplasmic or other factors related to cellular organization which regulate D-glucose transport, remains an open one. More study is required to resolve this question.

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