Human Erythrocyte Sugar Transport is Incompatible with Available Carrier Models†

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ABSTRACT: GLUT1-mediated, passive D-glucose transport in human erythrocytes is asymmetric. V_{max} and $K_{m(app)}$ for D-glucose uptake at 4 °C are 10-fold lower than V_{max} and $K_{m(app)}$ for D-glucose export. Transport asymmetry is not observed for GLUT1-mediated 3-*O*-methylglucose transport in rat, rabbit, and avian erythrocytes and rat adipocytes where V_{max} for sugar uptake and exit are identical. This suggests that transport asymmetry is either an intrinsic catalytic property of human GLUT1 or that factors present in human erythrocytes affect GLUT1-mediated sugar transport. In the present study we assess human erythrocyte sugar transport asymmetry by direct measurement of sugar transport rates and by analysis of the effects of intra- and extracellular sugars on cytochalasin B binding to the sugar export site. We also perform internal consistency tests to determine whether the measured, steady-state 3-*O*-methylglucose transport properties of human erythrocytes agree with those expected of two hypothetical models for protein-mediated sugar transport. The simple-carrier hypothesis describes a transporter that alternately exposes sugar import and sugar export pathways. The fixed-site carrier hypothesis describes a sugar transporter that simultaneously exposes sugar import and sugar export pathways. Steady-state 3-*O*-methylglucose transport in human erythrocytes at 4 °C is asymmetric. V_{max} and $K_{m(app)}$ for sugar uptake are 10-fold lower than V_{max} and $K_{m(app)}$ for sugar export. Phloretin-inhibitable cytochalasin B binding to intact red cells is unaffected by extracellular D-glucose but is competitively inhibited by intracellular D-glucose. This inhibition is reduced by 13% ± 4% when saturating extracellular D-glucose levels are also present. Assuming transport is mediated by a simple-carrier and that cytochalasin B and intracellular D-glucose binding sites are mutually exclusive, the cytochalasin B binding data are explained only if transport is almost symmetric (V_{max} exit = 1.4 V_{max} entry). The cytochalasin B binding data are consistent with both symmetric and asymmetric fixed-site carriers. Analysis of 3-*O*-methylglucose, 2-deoxy-D-glucose, and D-glucose uptake in the presence of intracellular 3-*O*-methylglucose demonstrates significant divergence in experimental and theoretical transport behaviors. We conclude either that human erythrocyte sugar transport is mediated by a carrier mechanism that is fundamentally different from those considered previously or that human erythrocyte-specific factors prevent accurate determination of GLUT1-mediated sugar translocation across the cell membrane. We suggest that GLUT1-mediated sugar transport in all cells is an intrinsically symmetric process but that intracellular sugar complexation in human red cells prevents accurate determination of transport rates.

The passive glucose transport system of human erythrocytes is characterized by translocational, stereochemical, and biochemical asymmetry (Widdas, 1980). V_{max} and $K_{m(app)}$ for erythrocyte D-glucose uptake into sugar-depleted cells are 5−10-fold lower than the corresponding parameters for efflux into sugar-free saline (Baker & Naftalin, 1979; Hankin et al., 1972; Lowe & Walmsley, 1986; Miller, 1968b). The transporter shows asymmetric affinities for extracellular and intracellular sugars and unique stereochemical requirements for ligand binding at endo- and exofacial binding sites (Barnett et al., 1973, 1975; Basketter & Widdas, 1978). Erythrocyte sugar transport is inhibited by intracellular trypsin but not by extracellular trypsin (Carruthers & Melchior, 1983; Coderre et al., 1995; Masaik & LeFevre, 1977).

These asymmetries in translocation constants, ligand binding, and susceptibility to proteolysis are not unexpected of a transport system whose catalytic subunit is an integral membrane protein (GLUT1)[1] that lacks internal, primary structural repeats and spans the plasma membrane multiple times (Mueckler et al., 1985). What is surprising is the finding that GLUT1-mediated sugar transport in rat, rabbit, and pigeon erythrocytes and in rat adipocytes and CHO fibroblasts[2] is translocationally symmetric [V_{max} entry = V_{max} exit; (Helgerson & Carruthers, 1989; Naftalin & Rist, 1991; Regen & Morgan, 1964; Simons, 1983; Taylor & Holman, 1981)]. While transport in these tissues is symmetric, the stereochemistry of substrate binding at import and export sites resembles that of the human red cell sugar transporter (Helgerson & Carruthers, 1989; Holman et al., 1981b; Holman & Rees, 1982; Simons, 1983). This asymmetry in human GLUT1-mediated erythrocyte sugar translocation could result from (1) primary structural elements specific to

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[1] Abbreviations used: CHO, Chinese hamster ovary; GLUT1, human erythrocyte glucose transport protein; 2DODG, 2-deoxy-D-glucose; 3OMG, 3-*O*-methylglucose; CCB, cytochalasin B; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]; Tris-HCl, tris(hydroxymethyl)aminomethane.

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Homo sapiens GLUT1 (Asano et al., 1988; Birnbaum et al., 1986; Due et al., 1995; Mueckler et al., 1985; Wagstaff[3] et al., 1992); (2) from experimental difficulties associated with the measurement of the high rate of sugar transport in human red cells [human red cell sugar transport is 500-fold faster that rat erythrocyte sugar transport (Helgerson & Carruthers, 1989)]; or (3) from environmental factors presented uniquely by human erythrocytes. We discount the relative transport rate hypothesis as a single explanation because rates of sugar transport in rat adipocytes (basal and insulin stimulated) are similar to those observed in human red cells (Taylor & Holman, 1981).

In the present study, we examine the hypothesis that human erythrocyte-specific factors influence GLUT1-mediated sugar transport in human red cells. The action of cellular factors on protein-mediated sugar transport could be expressed in two very different ways. The ability of the transporter to translocate sugars could be affected directly (Carruthers & Melchior, 1983), or cellular sequestration/complexation (reduced self-diffusion) of glucose could prevent accurate determination of sugar translocation rates and thereby give rise to an apparent asymmetry in transport even if the transporter were intrinsically translocationally symmetric (Baker & Naftalin, 1979; Carruthers, 1991; Lieb & Stein, 1974; Naftalin & Holman, 1977; Naftalin et al., 1985). Recent studies from this laboratory (Cloherty et al., 1995) show that sugars interact with an intracellular sugar binding complex following their dissociation from the human erythrocyte sugar transporter. We therefore examined the human erythrocyte sugar transport system for internal consistencies/inconsistencies that reveal whether steady-state sugar transport measurements in red cells are adequate.

Using methods of steady-state sugar transport determination that were used previously to establish rat erythrocyte sugar transport symmetry (Helgerson & Carruthers, 1989), we confirm that human erythrocyte 3-*O*-methylglucose transport is translocationally asymmetric. We then use a methodologically independent procedure (ligand binding to the sugar export site; (Helgerson & Carruthers, 1987) to assess asymmetry in export site availability. If transport is mediated by a simple-carrier mechanism, as has been suggested recently by some groups (Walmsley & Lowe, 1987; Wheeler & Whelan, 1988), the present ligand binding studies show that the transporter must be considerably less translocationally asymmetric (1.4-fold versus 10-fold) than previously believed. Closer examination reveals systematic deviations of experimental transport data from the behavior predicted for classical mechanisms for carrier-mediated sugar transport.

These findings lead us to conclude that cell-specific factors prevent direct determination of GLUT1-mediated sugar translocation rates in human erythrocytes.

MATERIALS AND METHODS

Materials. [^{3}H]-3-*O*-Methylglucose, [^{14}C]-D-glucose, [^{14}C]-2-deoxy-D-glucose, [^{3}H]cytochalasin B, and [^{3}H]H_2O were purchased from New England Nuclear. Recently expired human blood was obtained from the University of Massachusetts Medical Center Blood Bank. Reagents were purchased from Sigma Chemicals.

Solutions. Saline consisted of 150 mM NaCl, 5 mM HEPES, 0.5 mM EDTA, pH 7.4. In some experiments, the NaCl content of saline was substituted by KCl. Stopper consisted of ice-cold saline plus 10 μM cytochalasin B, 100 μM phloretin, and 10 μM $HgCl_2$. All cytochalasin B binding solutions contained 10 μM cytochalasin D in order to inhibit cytochalasin B binding to actin and other non-GLUT1 sites (Helgerson & Carruthers, 1987).

Red Cells. Red cells were isolated from whole human blood by repeated wash/centrifugation cycles in ice-cold saline. One volume of whole blood was mixed with 3 vol of saline and centrifuged at 10 000*g* for 5 min at 4 °C. Serum and the buffy coat were aspirated, and the wash/centrifugation cycle was repeated until the supernatant was clear and the buffy coat was no longer visible. Cells were resuspended in 20 vol of saline and were allowed to rest at room temperature for 30 min in order to deplete intracellular sugar levels.

Net 3-O-Methylglucose Uptake. 3OMG, D-glucose, and 2DODG uptake were measured as described previously (Helgerson et al., 1989). Sugar-free cells (at ice temperature) were exposed to 5 vol of saline (ice temperature) containing variable concentrations of unlabeled sugar plus labeled sugar. Uptake was permitted to proceed over intervals as short as 5 s to intervals as long as 3 h, and then 50 vol (relative to cell volume) of stopper solution was added to the cell suspension. Cells were sedimented by centrifugation (14 000*g* for 30 s), washed once in stopper, collected by centrifugation, and extracted in 500 μL of 3% perchloric acid. The acid extract was centrifuged, and duplicate samples of the clear supernatant fluid were counted. Zero-time uptake points were prepared by addition of stopper to cells prior to addition of medium containing sugar and radiolabel. Cells were immediately processed. Radioactivity associated with cells at zero-time was subtracted from the activity associated with cells following the uptake period. All uptakes were normalized to equilibrium uptake where cells were exposed to sugar medium at 37 °C for 60 min prior to addition of stopper. Uptake assays were performed using solutions and tubes pre-equilibrated to 4 °C.

Net 3-O-Methylglucose Exit. Erythrocytes were incubated in 10, 5, 2.5, 1, 0.5, or 0.1 mM 3OMG for 60 min at 37 °C by which time equilibrium uptake is attained. At this time, [^{3}H]3OMG (2-5 μCi) was added to the suspension and the cells were incubated for a further 20 min at 37 °C. Sugar-loaded cells were placed on ice, and aliquots were sedimented by centrifugation (14 000*g* for 30 s). The supernatant was removed, exit was initiated by addition of 100 vol (relative to packed cell volume) of ice-cold saline, and, following the appropriate exit interval (0.5, 1, 2.5, 5, or 10 min), exit was terminated by addition of 50 vol of ice-cold stopper. Cells were sedimented by centrifugation, the supernatant was aspirated, and the cell pellet was washed once more in 150 vol of stopper. The final cell pellet was extracted in perchloric acid as above. Zero-time exit points were obtained by addition of stopper to cells prior to addition of saline.

Equilibrium Exchange 3-O-Methylglucose Uptake. In these experiments, intracellular [3OMG] = extracellular [3OMG] and the rate of cellular equilibration with extracellular tracer radiolabeled 3OMG is monitored. The uptake assay is otherwise identical to net sugar uptake measurements. Cells were pre-equilibrated with varying [3OMG] (0−60 mM) by incubation in 10 vol of equilibration medium for 1 h at 37 °C. Cells were collected by centrifugation, resuspended in 1 vol of ice-cold equilibration medium, and, following equilibration to 4 °C, uptake was initiated by

Table 1: Human Erythrocyte Sugar Transport at Ice Temperature

		transported sugar			
experiment[a]	parameter[b]	3OMG	n[c]	D-glucose	2DODG
zero-trans entry	$K_{m(app)}$	0.38 ± 0.13	3	0.46 ± 0.09	0.59 ± 0.27
	V_m	0.18 ± 0.02		0.15 ± 0.01	0.29 ± 0.04
zero-trans exit	$K_{m(app)}$	4.35 ± 0.62	3		
	V_m	1.62 ± 0.10			
equilibrium exchange	$K_{m(app)}$	22.62 ± 6.17	4		
	V_m	9.17 ± 3.44			
infinite-trans entry	$K_{m(app)}$	1.57 ± 0.10	3	2.19 ± 0.36	0.76 ± 0.19
	V_m	5.62 ± 0.11		12.19 ± 0.88	8.55 ± 0.79
infinite-cis entry	$K_{m(app)}$	0.66 ± 0.15[d]	3		
	$K_{m(app)}$	0.47 ± 0.11[e]	3		
	$K_{m(app)}$	3.35 ± 0.62[f]	2		

[a] The experimental conditions are as follows: *zero-trans* entry, external sugar levels are varied, internal sugar is absent; *zero-trans* exit, external sugar is absent, internal sugar levels are varied; equilibrium exchange, external sugar levels = internal sugar levels at all times, but both are varied; *infinite-trans* entry, external sugar levels are varied, internal sugar level is saturating; *infinite-cis* entry, external sugar level is saturating, internal sugar levels are varied. [b] $K_{m(app)}$ parameters have units of mM, V_{max} parameters have units of mmol/liter of cell water/minute. Results are shown as mean ± standard error. [c] The number of individual experiments made using 3OMG. The number of experiments made using D-glucose or 2DODG as the transported sugar was three or more. In *infinite-trans* D-glucose and 2DODG uptake experiments, the intracellular sugar was 50 mM 3OMG. [d] This result was obtained as that intracellular [3OMG] which reduced net 3OMG uptake by one-half. [e] This result was obtained by use of an integrated Michaelis−Menten equation to obtain $K_{m(app)}$ from a complete time course of saturated (20 mM) 3OMG uptake into cells which were initially free of intracellular sugar. [f] This result was obtained by determining that intracellular 3OMG level that stimulates saturated, unidirectional 3OMG uptake half-maximally.

addition of labeled 3OMG. Stopper contained sucrose at a concentration identical to intracellular [3OMG].

infinite-trans Homo- and Hetero-Exchange Sugar Uptake. Red cells were preloaded with 50 mM 3OMG by preincubation in 20 vol of 50 mM 3OMG for 1 h at 37 °C. $K_{m(app)}$ for net 3OMG exit at 4 °C is 4 mM (see Table 1). This means that exit sites are >90% saturated at 50 mM intracellular 3OMG. Sugar-loaded cells were sedimented by centrifugation for 30 s at 4 °C, 14 000*g*, and the supernatant was removed by aspiration. Uptake of sugar was initiated by adding 1 vol of loaded cells to 50 vol of ice-cold saline containing variable concentrations of unlabeled 3OMG (0.1−10 mM) plus labeled 3OMG. The osmolalities of external and media were balanced by addition of ACS-grade sucrose to the uptake medium such that [3OMG] plus [sucrose] = 50 mM. Uptake was allowed to proceed for 5, 15, 30, 60, or 120 s and was then arrested by addition of 100 vol of ice-cold stopper solution. Cells were sedimented by centrifugation (14 000*g* for 30 s), washed once in stopper, collected by centrifugation, and extracted in perchloric acid. Because 3OMG uptake into 3OMG-loaded cells is measured under these conditions, this experiment is a homo-exchange uptake experiment. Hetero-exchange uptake experiments were also made in which labeled and unlabeled D-glucose or 2DODG were substituted for extracellular 3OMG.

Zero-time uptake points were prepared by addition of stopper to cells prior to addition of medium containing sugar and radiolabel. Cells were immediately processed. Radioactivity associated with cells at zero-time was subtracted from the activity associated with cells following the uptake period. All uptakes were normalized to equilibrium uptake where cells were exposed to sugar medium at 37 °C for 120 min prior to addition of stopper. Uptake assays were performed using solutions and tubes pre-equilibrated to 4 °C.

infinite-cis Net and Unidirectional 3OMG Uptake. In these transport measurements, extracellular 3OMG levels are saturating, the intracellular 3OMG level is varied, and sugar uptake is measured. Unidirectional uptake of 3OMG is measured by addition of labeled 3OMG to the external medium only. Net uptake is measured by loading cells with variable [3OMG] plus radiolabeled 3OMG and then measuring the initial rate of 3OMG uptake from a saturating external [3OMG] of identical specific activity to that present within the cell. Net uptake can also be measured by following the time course of saturated 3OMG uptake by cells that are initially free of intracellular sugar.

In unidirectional *infinite-cis* uptake experiments, cells were loaded with 0, 0.1, 0.25, 0.5, 1, 5, and 10 mM unlabeled 3OMG by incubation in 20 cell volumes of saline containing these concentrations of sugar for 1 h at 37 °C. Cells were collected by centrifugation for 30 s at 4 °C and 14 000*g*, and the supernatant was aspirated. Uptake was initiated by addition of 50 vol of 20 mM 3OMG plus labeled 3OMG and was allowed to proceed for 30, 60, 150, or 300 s. Uptake was arrested by addition of 100 vol of ice-cold stopper solution.

In net *infinite-cis* uptake experiments, a stock 20 mM 3OMG solution containing 10 μCi of [^{3}H]-3-*O*-methylglucose per μmol of 3OMG was diluted to produce final 3OMG concentrations of 0.1, 0.25, 0.5, 1, 5, and 10 mM. Cells were equilibrated with these solutions by incubation for 1 h at 37 °C and then sedimented by centrifugation (30 s at 4 °C and 14 000*g*), and the supernatant was aspirated. Uptake was initiated by addition of 50 vol of the stock 20 mM 3OMG solution to the cells and was allowed to proceed for 15, 30, 60, 120, and 300 s. Uptake was arrested by addition of 100 vol of ice-cold stopper solution.

Net uptake of 20 mM extracellular 3OMG was also measured in cells that were initially free of intracellular sugar. The time course of uptake was analyzed according to Baker and Naftalin (1979). Cells were sedimented by centrifugation (14 000*g* for 30 s), washed once in stopper, collected by centrifugation, and extracted in perchloric acid. Radioactivity associated with cells at zero-time was subtracted from the activity associated with cells following the uptake period. All uptakes were normalized to equilibrium uptake where cells were exposed to sugar medium at 37 °C for 120 min prior to addition of stopper. Uptake assays were performed using solutions and tubes pre-equilibrated to 4 °C and were carried out in a thermostatically cooled block.

Calculation of Transport Constants. Net sugar uptake at 4 °C is characterized by pseudo-first-order rate constants

($V_{max}/K_{m(app)}$) of 0.5, 0.3, and 0.5 min^{-1} for 3OMG, DG, and 2DODG, respectively (see Table 1). These constants correspond to theoretical half-times for 3OMG, DG, and 2DODG uptake of 1.4, 2.3, and 1.4 min, respectively, which means that uptake of even the lowest concentration of sugar is linear during the first 30 s of uptake determination. This incubation time was used in all net and *infinite-trans* experiments for sugar concentrations lower than 1 mM. At concentrations of 1 mM or greater where progressive saturation of the import site causes the pseudo-first-order rate constant for uptake to fall, uptake was measured over 1 min intervals. In net uptake experiments, intracellular sugar levels never exceeded 0.15 mM (extracellular [sugar] = 5 mM), a concentration 30-fold lower than $K_{m(app)}$ for 3OMG exit.

The pseudo-first-order rate constant for equilibrium exchange 3OMG uptake was obtained as the first-order rate constant for label equilibration with cell water. This was calculated by nonlinear regression analysis of the time course of label uptake assuming mono-exponential uptake kinetics. At the 3OMG concentrations used in the present study (0.5−30 mM), this assumption is satisfied (Cloherty et al., 1995).

V_{max} and $K_{m(app)}$ for transport of sugars were computed by direct, nonlinear regression analysis of transport velocity versus sugar concentration data assuming transport follows simple saturation kinetics. Under the conditions of our experiments, net 3OMG uptake is inhibited by more than 98% by the sugar transport inhibitor cytochalasin B (50 μM). This suggests that nonmediated (nonsaturable) pathways for sugar transport contribute only a very small proportion to total sugar uptake. In *infinite-cis* net uptake experiments, time course data were analyzed using an integrated form of the Michaelis−Menten equation as suggested by Baker and Naftalin (1979). The software package used in linear and nonlinear regression analyses was KaleidaGraph 3.0 (Synergy Software, PA).

Cytochalasin B Binding. Red cells were loaded with D-glucose or depleted of endogenous sugars by incubation at 37 °C in 40 vol of saline containing or lacking 100 or 150 mM D-glucose for 2 h. Cells were then sedimented by centrifugation at 14 000*g* for 5 min and placed on ice for 20 min, and cytochalasin B binding was measured by one of three methods.

Method 1. Cells (30 μL of a 60%−80% cell suspension) were added to an Eppendorf tube and sedimented by centrifugation (1 min at 14 000*g*) and the supernatant was aspirated. The cells were resuspended by addition of 120 μL of medium containing [^{3}H]cytochalasin B. Aliquots (20 μL) of the suspension were mixed with two drops of H_2O_2 and were counted by liquid scintillation spectroscopy. This is a measure of "total" suspension [cytochalasin B]. Cells were incubated for 0.5−5 min on ice, by which time equilibrium cytochalasin B binding is achieved (Helgerson & Carruthers, 1987). The cell suspension was centrifuged at 14 000*g* for 20 s, and aliquots (20 μL) of the clear supernatant were counted by liquid scintillation spectroscopy. This is a measure of "free" [cytochalasin B]. Bound [cytochalasin B] is computed as total [cytochalasin B] − free [cytochalasin B]. When binding was measured in cells containing D-glucose, cytochalasin B binding medium also contained an equimolar concentration of mannitol (to prevent cell lysis) or D-glucose. Cytochalasin B binding was also measured in sugar-free cells in the presence of mannitol or D-glucose or in the absence of extracellular sugar.

Three circumstances could result in underestimation of inhibition of CCB binding by intracellular D-glucose. (1) Extracellular D-glucose slightly reduces inhibition of CCB binding by intracellular D-glucose. Significant contamination by extracellular D-glucose under conditions where the sugar is presumed to be absent could, therefore, result in underestimation of inhibition of CCB binding by intracellular D-glucose. (2) Intracellular D-glucose levels may fall rapidly, resulting in underestimation of intracellular sugar levels. (3) CCB binding at nonGLUT1 sites could give rise to a significant component of CCB binding that is insensitive to inhibition by D-glucose. Contamination by extracellular D-glucose was reduced by use of two additional methods for CCB binding determination.

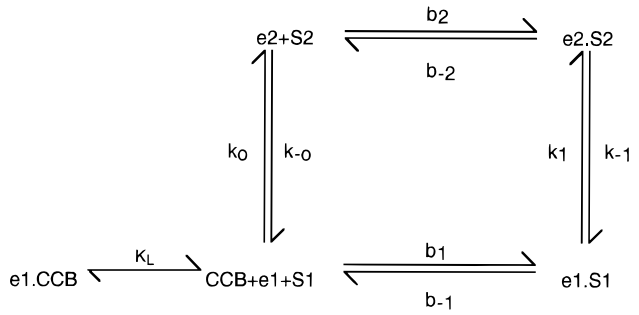
Method 2. Sugar-loaded cells were sedimented, the supernatant was aspirated, and then the cell pellet was resuspended in 1000 vol of ice-cold saline containing 150 mM mannitol and centrifuged for 5 min at 14 000*g*. The supernatant was aspirated, and cytochalasin B binding was measured immediately as described for method 1. This method avoids the initial presence of significant levels of extracellular D-glucose but does not prevent increases in extracellular D-glucose levels resulting from sugar export (rate of increase is 0.3 mM per min, assuming transport is uninhibited by cytochalasin B).

Method 3. [^{3}H]Cytochalasin B binding to erythrocytes following 1 min incubation at ice temperature (30 μL of cells in 33 mL of cytochalasin B binding medium) was measured (following cell sedimentation at 14 000*g* for 5 min) as the difference between the [^{3}H]cytochalasin B space of the erythrocyte pellet and the tritiated water space of the cell pellet measured in a parallel tube. Upon addition of cytochalasin B binding medium to the red cell pellet, the initial extracellular sugar concentration (pellet extracellular space = 10%) is 20 μM and the fractional saturation of import sites is 0.01. If all intracellular sugar were exported during the cytochalasin B binding determination, the final extracellular D-glucose level would be 0.2 mM. However, this is unlikely for binding experiments require 11 min for completion and less than 10% of intracellular sugar is lost during this interval (Lowe & Walmsley, 1986). In some experiments with D-glucose-loaded cells, cell pellets were washed in 1000 vol of D-glucose-free (100 mM mannitol-containing) saline prior to addition of D-glucose-free cytochalasin B binding medium.

We also addressed the possibility that intracellular D-glucose levels become depleted by increasing the concentration of D-glucose used to preload cells. When cells are loaded with 150 mM D-glucose, the half-time for depletion of intracellular D-glucose is increased from 18 to 27 min (V_{max} for D-glucose uptake = 2.77 mM/min; Lowe & Walmsley, 1986). Since processing of the cells requires 11 min, intracellular D-glucose levels fall to 120 mM and extracellular D-glucose levels increase to 8 μM. This means that intracellular D-glucose binding sites are never less than 98% saturated ($K_{m(app)}$ exit = 1.4 mM; Lowe & Walmsley, 1986) and extracellular D-glucose binding sites are never more than 0.4% saturated with sugar ($K_{m(app)}$ *infinite-trans* entry = 2.2 mM; see Table 1). Inhibition of CCB binding to red cells by loading with 150 mM D-glucose (82% ± 2%; n = 12) is not significantly greater than that produced by loading cells with 100 mM D-glucose (74% ± 8%; n = 9).

We examined GLUT1-independent CCB binding to red cells by blocking transporter-associated binding by addition

Scheme 1. Simple Carrier



of phloretin (a sugar transport inhibitor). Total saturable CCB binding was inhibited by addition of unlabeled CCB. In the present study, [^{3}H]CCB binding is inhibited by 99% ± 1% (n = 12) when binding is measured in the presence of 50 μM unlabeled CCB (Table 5). The sugar transport inhibitor phloretin (100 μM) inhibits [^{3}H]CCB binding to a similar extent (98% ± 1%; n = 9). The constant for nonspecific (nonsaturable) CCB binding is in the order of 0.5 μmol of CCB per 10^{13} erythrocytes per μM CCB.

An iso-osmotic control for D-glucose-loaded cells and for cells exposed to extracellular D-glucose is exposure of control cells to an equivalent concentration of mannitol. If mannitol is contaminated by transported sugars (e.g., D-glucose), this could elevate intracellular levels of sugars that compete with CCB for binding to the transporter and thereby inhibit CCB binding. This seems unlikely for two reasons: (1) CCB (50 nM) binding to red cells exposed to saline (6.1 ± 1.0 pmol per 10^8 cells; n = 3) is not greater than CCB binding to red cells exposed to 100 mM mannitol (8.0 ± 0.9 pmol per 10^8 cells; n = 3). (2) Extracellular D-glucose (100 mM) does not inhibit CCB binding (Table 5).

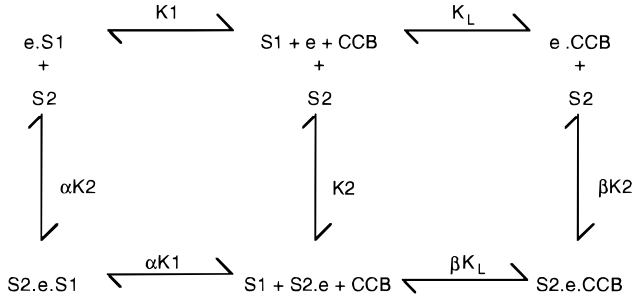
Transport Models. Two transport models are considered in the present study. The simple-carrier (see Scheme 1) exists in either of two states in the absence of sugar or CCB: e1 presents a binding site to intracellular sugar (S1) or to CCB, while e2 presents a binding site to extracellular sugar (S2). k_o, k_{-o}, k_1, k_{-1}, b_{-1} and b_{-2} are first-order rate constants describing rates of conformational change or sugar dissociation from the carrier, and b_1 and b_2 are second-order rate constants describing the interactions of carrier with S1 and S2, respectively. The dissociation constants for sugar binding to e1 and e2 are $K1$ and $K2$, respectively, which are given by b_{-1}/b_1 and b_{-2}/b_2, respectively. CCB binding to e1 is characterized by a dissociation constant, K_L.

Cytochalasin B binding to the simple-carrier in the presence of transported sugar is described by

$$[\mathrm{CCB}][\mathrm{GLUT}]/\left\{K_L\left[1+\frac{S1}{K1}\left(1+\frac{k_1}{k_{-o}+k_{-1}\frac{S2}{K2}}\left(1+\frac{S2}{K2}\right)\right)+\frac{k_o}{k_{-o}+k_{-1}\frac{S2}{K2}}\left(1+\frac{S2}{K2}\right)\right]+[\mathrm{CCB}]\right\}$$

(Helgerson & Carruthers, 1987), where S1 and S2 are intra- and extracellular D-glucose concentrations, respectively, [CCB] is the free cytochalasin B concentration, and [GLUT] is the concentration of glucose transporters. This analysis (Helgerson & Carruthers, 1987) assumes that CCB binds only to the e1-form of the transporter and that binding of CCB and D-glucose to e1 is mutually exclusive. If human erythrocyte sugar transport is indeed mediated by a simple-carrier, then this requirement appears to be satisfied (Carruthers & Helgerson, 1991).

Scheme 2. Fixed-Site Carrier



The fixed-site carrier (see Scheme 2) presents binding sites for extracellular sugar (S2) and intracellular sugar (S1) simultaneously, which, when occupied by S1 and S2, interact with the cooperativity constant α. The carrier can also bind CCB. Binding of CCB and S1 is mutually exclusive, while binding of S2 and CCB is permitted and is characterized by the cooperativity constant β. Dissociation constants for S1, S2, or CCB interaction with the carrier in the absence of other ligands are $K1$, $K2$, and K_L, respectively. CCB binding to the fixed-site carrier is described by the following (Helgerson & Carruthers, 1987):

$$[\mathrm{e.CCB}]+[\mathrm{S2.e.CCB}]=\frac{[\mathrm{GLUT}][\mathrm{CCB}]}{K_L\left(\frac{1+\frac{S2}{K2}+\frac{S1}{K1}+\frac{S1S2}{\alpha K1K2}}{1+\frac{S2}{\beta K2}}\right)+[\mathrm{CCB}]}$$

RESULTS

3-O-Methylglucose Transport Is Asymmetric. Table 1 summarizes the net 3OMG uptake, net 3OMG exit, and equilibrium exchange 3OMG uptake properties of human erythrocytes at 4 °C. As with erythrocyte D-glucose transport (Carruthers & Melchior, 1983; Hankin et al., 1972; Lowe & Walmsley, 1986; Regen & Tarpley, 1974; Widdas, 1980), erythrocyte 3OMG transport is also asymmetric. $K_{m(app)}$ and V_{max} for 3OMG exit are 10-fold greater than the corresponding parameters for 3OMG entry.

The steady-state velocity equations for sugar transport mediated by an asymmetric simple-carrier or by a fixed-site carrier are characterized by one affinity (K) and four resistance (R) constants (Carruthers, 1991; Lieb & Stein, 1974). These constants can be computed directly from V_{max} and $K_{m(app)}$ parameters for net 3OMG uptake, exit, and 3OMG exchange transport and are summarized in Table 2.

Is Transport Compatible with Existing Models for Sugar Transport? Using the constants listed in Table 1 or 2, we can compute theoretical $K_{m(app)}$ for carrier-mediated 3OMG efflux into saline containing saturating levels of extracellular 3OMG. The classical, asymmetric simple-carrier (Widdas, 1952) predicts that 3OMG exit is half-maximal when the intracellular 3OMG concentration is

$$\frac{V^{ee}K^{io}}{V^{oi}V^{io}\left(\frac{1}{V^{io}}+\frac{1}{V^{oi}}-\frac{1}{V^{ee}}\right)}=\frac{KR_{oi}}{R_{ee}}$$

Table 2: Macro Constants for Simple-Carrier-Mediated Erythrocyte Sugar Transport

constant[a]	30MG[b]	D-glucose[b]	2DODG[b]
R_{oi}	5.65 ± 0.68	6.49 ± 0.46	3.48 ± 0.55
R_{io}	0.62 ± 0.04		
R_{ee}	0.11 ± 0.03	0.032 ± 0.001[c]	0.037 ± 0.002[c]
R_{oo}	6.16 ± 0.79		
K^d	0.39 ± 0.03	0.48 ± 0.10	0.34 ± 0.15

[a] *R* constants have units of minutes per mM. The *K* constant has units of mM. R_{oi} is computed as the reciprocal of V_{max} for *zero-trans* entry. R_{io} is computed as the reciprocal of V_{max} for *zero-trans* exit. R_{ee} is computed as the reciprocal of V_{max} for equilibrium exchange transport. $R_{oo} = R_{io} + R_{oi} - R_{ee}$. [b] The transport data used to compute these constants are obtained from Table 1. [c] R_{ee} computed as reciprocal of D-glucose exchange and reciprocal of 2DODG exchange at 200 mM (see Table 3). [d] *K* was computed from $K_{m(app)}$ for *zero-trans* uptake as $K = K_{m(app)}R_{oi}/R_{oo}$; from $K_{m(app)}$ for *zero-trans* exit as $K_{m(app)}R_{io}/R_{oo}$ and from $K_{m(app)}$ for equilibrium exchange as $K_{m(app)}R_{ee}/R_{oo}$.

[see Lieb and Stein (1974)]. Because equilibrium exchange 3OMG transport does not deviate systematically from simple saturation kinetics [Table 1; but see Holman et al. (1981a)], $K_{m(app)}$ for fixed-site carrier-mediated 3OMG efflux into saturating extracellular 3OMG is also given by this expression (Carruthers, 1991). Thus, for these two hypothetical transport mechanisms, 3OMG exit into saturating 3OMG should be half-maximal at 21.7 ± 2.7 mM intracellular 3OMG.

We measured this half-saturation constant by three different methods. Figure 1 summarizes an *infinite-cis* net uptake experiment in which the time course of saturated net sugar uptake is measured at varying initial intracellular 3OMG levels. In this type of experiment, the specific activities of intracellular and extracellular [^{3}H]-3-*O*-methylglucose are identical. A Figure 1 panel shows that net uptake is inhibited by more than 50% when intracellular [3OMG] is <1 mM. In this experiment, $K_{m(app)}$ for inhibition of net uptake by intracellular 3OMG is 0.50 ± 0.06 mM (Figure 1B). Table 1 summarizes the results of three similar experiments. Figure 2 summarizes the effects of loading erythrocytes with varying unlabeled 3OMG levels on the rate of unidirectional 3OMG uptake from saline containing 20 mM 3OMG. Uptake is half-maximally stimulated when $[3OMG]_i = 3.4 \pm 0.6$ mM. Figure 3 shows the time course of 20 mM 3OMG uptake by cells that were initially free of intracellular sugar. Net uptake is reduced by 50% when intracellular [3OMG] is 0.47 ± 0.01 mM.

Homo- and Hetero-Exchange Transport Tests of Carrier Mechanisms. Table 1 also summarizes V_{max} and $K_{m(app)}$ parameters for D-glucose and 2DODG net uptake by human red cells at 4 °C. The transport parameters for D-glucose uptake are in close agreement with those reported previously for outdated blood (Jacquez, 1983; Lacko et al., 1973; Lowe & Walmsley, 1986; Wheeler, 1986).

The availability of V_{max} and $K_{m(app)}$ parameters for D-glucose and 2DODG net uptake together with the estimate of R_{oo} obtained from measurements of 3OMG transport (Table 2) permit computation of the resistance parameter R_{oi} and *K*, the intrinsic affinity constant of the asymmetric simple-carrier for D-glucose and 2DODG uptake at 4 °C. These constants are summarized in Table 2 and, in combination with measurements of V_{max} for unidirectional uptake of 3OMG, D-glucose, and 2DODG into cells loaded with saturating unlabeled 3OMG, can be used to predict the effects of preloading erythrocytes with saturating 3OMG on unidirectional uptake of 3OMG, D-glucose, and 2DODG.

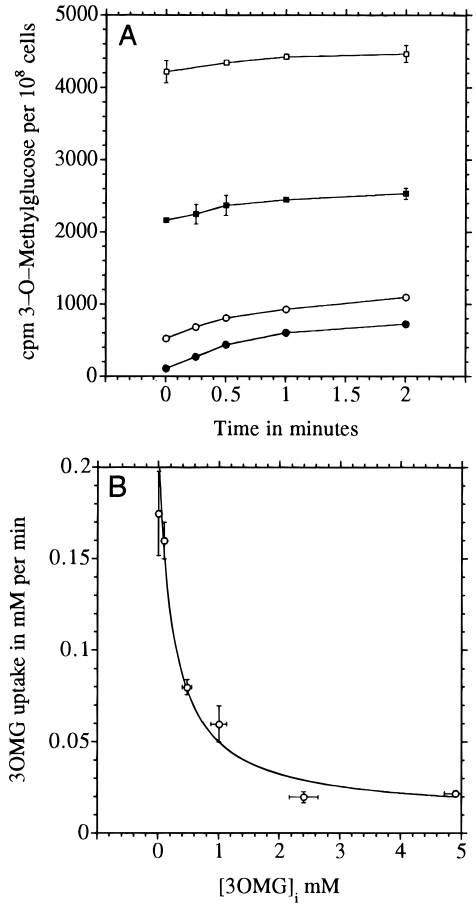


FIGURE 1: Concentration-dependence of net [^{14}C]-3-*O*-methylglucose (20 mM) uptake by erythrocytes preloaded with varying 3OMG concentrations (0−5 mM) of identical specific activity. (A) Raw uptake data. Ordinate, cpm 3OMG per 10^8 cells; abscissa, time in minutes. Results are shown as mean ± standard error of one experiment made in quadruplicate. Data are shown for 0 (●), 0.1 (○), 0.5 (■), and 1 mM (□) intracellular 3OMG. Curves are drawn through the points by eye. (B) Initial rates of net 3OMG uptake (computed from the first 30 s of uptake in A) expressed as a function of intracellular [3OMG]. Ordinate: rate of 3OMG uptake in mmol of sugar per liter of cell water per minute. Abscissa, intracellular [3OMG] in mM. The curve drawn through the points was computed by nonlinear regression assuming that net uptake falls in a simple saturable manner with increasing intracellular [3OMG] and has the following constants: uptake in the absence of $3OMG_i = 0.17 \pm 0.01$ mM/min; uptake is reduced to 50% when $[3OMG]_i = 0.50 \pm 0.06$ mM.

Table 3 summarizes experiments in which erythrocytes were loaded with 200 mM 3OMG, D-glucose, or 2DODG. These cells were then resuspended in 200 mM radiolabeled 3OMG, D-glucose, or 2DODG, and the rate of sugar uptake was monitored. Homo-exchange uptake of 3OMG is slower than homo-exchange D-glucose and 2DODG uptake. Uptake of 3OMG is accelerated when the intracellular sugar is D-glucose or 2DODG. If we assume that transport at ice temperature is saturated at 200 mM sugar, then we can use these results to predict the concentration dependence of 3OMG, D-glucose, or 2DODG uptake into cells loaded with 50 mM 3OMG. Figure 4 summarizes the results of such experiments and contrasts experimental findings with results predicted for an asymmetric simple carrier. The presence of saturating intracellular 3OMG stimulates unidirectional 3OMG, D-glucose, and 2DODG uptake by 31-, 81-, and 30-fold, respectively.

Cytochalasin B Binding to Human Erythrocytes. Erythrocytes present 3.8 μmol of D-glucose inhibitable CCB

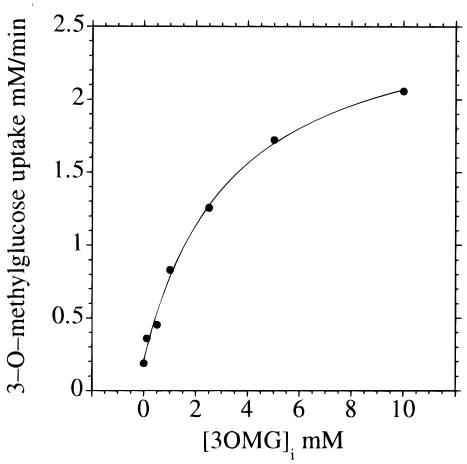


FIGURE 2: Concentration dependence of unidirectional [^{14}C]-3-*O*-methylglucose (20 mM) uptake by erythrocytes preloaded with varying concentrations (0−10 mM) of unlabeled 3OMG. Ordinate: rate of 3OMG uptake in mmol of sugar per liter of cell water per min. Abscissa: intracellular [3OMG] in mM. Results are shown as mean ± standard error of three experiments made in quadruplicate. The curve drawn through the points was computed by nonlinear regression assuming that uptake increases in a simple saturable manner with increasing intracellular [3OMG] and has the following constants: uptake in the absence of $3OMG_i = 0.22 \pm 0.04$ mM/min; maximum increase in uptake produced by $3OMG_i = 2.47 \pm 0.15$ mM/min; uptake is increased half-maximally when $[3OMG]_i = 3.35 \pm 0.62$ mM.

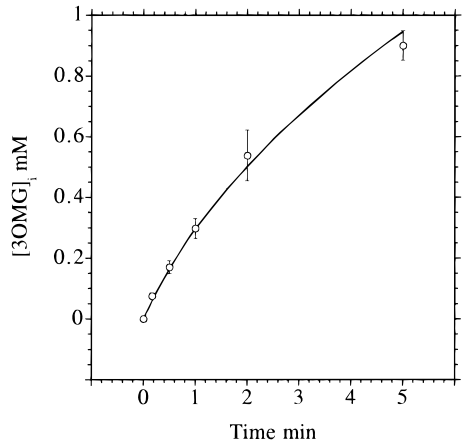


FIGURE 3: Time course of 20 mM 3OMG uptake by erythrocytes initially free of intracellular sugar. Ordinate: intracellular [3OMG] in mmol of sugar per liter of cell water. Abscissa: time in minutes. The curve drawn through the points was computed by fourth-order Runge-Kutta numerical integration for a transport system with $K_{m(app)} = 0.47$ mM and $V_{max} = 0.40$ mmol/liter of cell water per min.

binding sites per liter of cell water, and CCB binding to these sites is half-maximal at approximately 150 nM ligand (Coderre et al., 1995; Hebert & Carruthers, 1992; Helgerson & Carruthers, 1987; Zottola et al., 1995). Sogin and Hinkle (1980) and Gorga and Lienhard (1981) have shown that analysis of sugar inhibition of CCB binding to the transporter at low CCB concentrations may be used to determine whether the transporter behaves as a simple-carrier or as a fixed-site carrier. If the carrier behaves as a simple-carrier, then this method can also be used to estimate the relative rates of isomerization of the carrier between import- and export-competent states.

We have used this approach to estimate translocational asymmetry in human erythrocyte sugar transport. Table 4 summarizes the effects of extra- or intracellular D-glucose on cytochalasin B binding to human red cells at [CCB] = 50 nM. External D-glucose (150 mM) reduces CCB binding to red cells by 9% ± 4%. Intracellular D-glucose (150 mM) competitively inhibits cytochalasin B binding to GLUT1 5-fold. $K_{i(app)}$ for D-glucose$_i$ inhibition of 50 nM cytochalasin B binding to erythrocytes is 12.8 ± 1.2 mM ($n = 3$). Table 4 shows that cytochalasin B binding to erythrocytes in the presence of 150 mM intra- and extracellular D-glucose is (1.7 ± 0.2)-fold greater than that measured in the presence of intracellular D-glucose alone.

Table 3: Homo- and Hetero-Exchange Sugar Transport in Human Red Cells at 0 °C

		sugar influx[a]	
internal sugar[b]	external sugar[c]	observed[d]	predicted[e]
3OMG	3OMG	9.20 ± 0.60	7.60
D-glucose	3OMG	7.79 ± 0.11	9.64
2DODG	3OMG	7.54 ± 0.02	9.64
3OMG	D-glucose	17.12 ± 0.03	16.69
D-glucose	D-glucose	31.21 ± 0.71	31.16
2DODG	D-glucose	35.51 ± 0.29	31.16
3OMG	2DODG	15.29 ± 2.32	16.69
D-glucose	2DODG	34.60 ± 0.40	31.16
2DODG	2DODG	26.80 ± 1.54	31.16

[a] Sugar influx is in mmol of sugar per liter of cell water per min. [b] The intracellular sugar was 200 mM 3OMG, 200 mM D-glucose, or 200 mM 2DODG. [c] The extracellular sugar was 200 mM 3OMG, 200 mM D-glucose, or 200 mM 2DODG. [d] The measured rate of sugar uptake is shown as the mean ± standard error of three separate quadruplicate determinations. [e] Simulated assuming transport is mediated by a simple carrier and that saturated exchange predominates. V_{max} (saturated) exchange is $nk_1k_{-1}/(k_1 + k_{-1})$ where n is the concentration of cellular glucose transporters (3.8 μmol per liter of cell water) and k_1 and k_{-1} are first-order rate constants describing the rate of e.S1 → e.S2 and e.S2 → e.S1 conversion, respectively. The values used were, for 3OMG, $k_1 = 50$ s^{-1} and $k_{-1} = 100$ s^{-1}; for D-glucose and 2DODG, $k_1 = k_{-1} = 273.33$ s^{-1}.

DISCUSSION

The present study asks two questions: (1) Are human erythrocyte steady-state sugar transport data compatible with classical models for sugar transport? (2) Does transport truly display asymmetry in V_{max} and $K_{m(app)}$ parameters for net uptake and exit? Our findings lead us to conclude that the answer to both questions is no.

Transport Mechanisms. Although the subunit composition (Hebert & Carruthers, 1991, 1992; Zottola et al., 1995) and subunit membrane topography (Deziel et al., 1985; Hresko et al., 1994; Mueckler et al., 1985; Preston & Baldwin, 1993) of the human erythrocyte glucose transporter are well-characterized, the molecular mechanism of GLUT1-mediated sugar transport is uncertain.

The earliest studies on erythrocyte sugar transport led to the development of the *simple-carrier* model for protein-mediated sugar transport (Widdas, 1952). This model describes sugar transport via a membrane complex that alternately presents sugar import and sugar export pathways. A rather different model—the fixed-site carrier—was later suggested in which the transporter presents import and export pathways simultaneously (Baker & Widdas, 1973). Both models account for many features of passive sugar transport (Carruthers, 1991; Lieb & Stein, 1974; Widdas, 1980). The most striking features include transport asymmetry ($K_{m(app)}$ and V_{max} entry ≠ $K_{m(app)}$ and V_{max} exit) and accelerated exchange transport where unidirectional sugar movements are accelerated by the presence of sugar at the opposite, *trans*

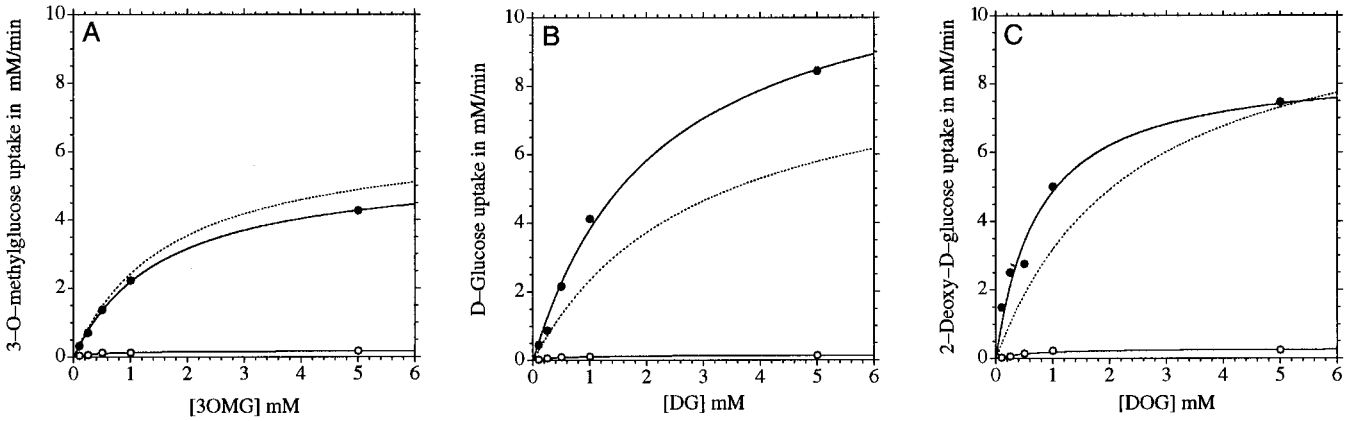


FIGURE 4: Concentration dependence of 3-*O*-methylglucose (A), D-glucose (B), and 2-deoxy-D-glucose (C) uptake by erythrocytes lacking (○) or containing (●) 50 mM 3-*O*-methylglucose. Ordinate, rate of sugar uptake in mmol per liter of cell water per min; abscissa, extracellular sugar concentration in mM. The solid curves drawn through the points were computed by nonlinear regression assuming Michaelis–Menten kinetics and have the following constants: (A) ○, $K_{m(app)} = 0.38$ mM, $V_{max} = 0.18$ mM/min; ●, $K_{m(app)} = 1.57$ mM, $V_{max} = 5.62$ mM/min; (B) ○, $K_{m(app)} = 0.46$ mM, $V_{max} = 0.15$ mM/min; ●, $K_{m(app)} = 2.19$ mM, $V_{max} = 12.19$ mM/min; (C) ○, $K_{m(app)} = 0.59$ mM, $V_{max} = 0.29$ mM/min; ●, $K_{m(app)} = 0.76$ mM, $V_{max} = 8.55$ mM/min. The dashed curves were computed for transport in the presence of 50 mM intracellular 3OMG by using the constants in Table 2 and the steady-state velocity equation for simple-carrier-mediated transport (Stein, 1986). This figure summarizes three separate measurements made in quadruplicate for each data point (shown as mean ± standard error).

Table 4: Cytochalasin B Binding to the Glucose Transporter[a]

[glucose] mM		pmol of CCB bound			
			simple-carrier		fixed-site
inside	outside	experimental	asymmetric[b]	symmetric[c]	carrier[d]
0	0	4.93 ± 0.11	4.91	4.01[e]	5.00
0	150	4.48 ± 0.05	5.24[e]	4.97[e]	4.50
150	0	0.93 ± 0.11	0.06[e]	0.93	0.93
150	150	1.59 ± 0.19	0.5[e]	1.51	1.59

[a] Effects of extracellular and intracellular D-glucose on cytochalasin B binding to erythrocytes. Cytochalasin B binding was measured using Method 3 (see Materials and Methods). Cells were exposed to 150 mM mannitol (control), to 150 mM extracellular D-glucose, to 150 mM intracellular D-glucose, or to 150 mM extracellular D-glucose plus 150 mM intracellular D-glucose. Binding is expressed as pmol of CCB bound. Experimental results are shown as mean ± SEM ($n = 3$ or more). Number of cells per assay, $\sim 8 \times 10^7$. [b] Results are predicted for an asymmetric simple carrier mechanism. Constants (see carrier schema) were chosen to best mimic the D-glucose transport properties of erythrocytes at ice temperature (Lowe & Walmsley, 1986). These are as follows: $k_o = 1.4$ s^{-1}, $k_{-o} = 14.5$ s^{-1}, $k_1 = k_{-1} = 273.3$ s^{-1}, $K1 = 23.6$ mM, $K2 = 2.4$ mM, $K_L = 130$ nM, $n = 3.8$ μmol per liter of cell water, [CCB] = 50 nM, amount of transporter in solution = 20 pmol. Results are also predicted for a symmetric simple-carrier mechanism[c] and a fixed-site carrier mechanism.[d] Constants for the symmetric simple carrier are as follows: $k_o = 62.5$ s^{-1}, $k_{-o} = 117.5$ s^{-1}, $k_1 = 208.3$ s^{-1}, $k_{-1} = 391.6$ s^{-1}, $K1 = 65.3$ mM, $K2 = 4.9$ mM, $K_L = 130$ nM, $n = 3.8$ μmol per liter of cell water. Predicted V_{max} parameters for sugar uptake into sugar-free cells, sugar exit into sugar-free saline, and equilibrium exchange transport are 12.3, 17.1, and 31 mmol of D-glucose per liter of cell water per min, respectively. Constants used to compute CCB binding to the fixed-site carrier (see carrier schema) are as follows: $K1 = 25.75$ mM, $K2 = 2.57$ mM; $\alpha = 2.53$, $\beta = 1.15$, $K_L = 150$ nM, [CCB] = 50 nM, [GLUT] = amount of transporter in solution = 20 pmol. [e] The probability that this value is the same as the experimental value is less than 1% (two-tailed *t*-test).

side of the membrane (Baker & Naftalin, 1979; Lacko et al., 1973; Levine et al., 1965; Wheeler, 1986).

Following the development of the simple-carrier hypothesis, a body of evidence accumulated that led to the rejection of this model as an adequate theoretical description of human erythrocyte sugar transport (Baker & Naftalin, 1979; Carruthers & Melchior, 1983, 1985; Ginsburg & Stein, 1975; Hankin et al., 1972; Harris, 1964; Lieb & Stein, 1977). Subsequent analyses of sugar transport in the absence of transport inhibitors have either accepted the simple-carrier hypothesis without critical evaluation (Lowe & Walmsley, 1986; Walmsley & Lowe, 1987) or have resulted in findings that are consistent with this hypothesis (Wheeler, 1986; Wheeler & Whelan, 1988).

Which transport hypothesis (if any) is correct? The adequacy of the simple-carrier hypothesis has been challenged by the results of steady-state hetero-exchange sugar transport studies (Miller, 1968a,b) and by results that suggest a high-affinity sugar export site under conditions where the extracellular sugar levels are saturating (Baker & Naftalin, 1979; Carruthers & Melchior, 1983; Carruthers & Melchior, 1985; Ginsburg & Stein, 1975; Hankin et al., 1972; Harris, 1964; Lieb & Stein, 1977). Close examination of these arguments reveals internal inconsistencies in human erythrocyte sugar transport data.

Hetero-Exchange Transport. Miller (1968a,b) concluded that human erythrocyte hetero-exchange sugar transport data are inconsistent with the simple carrier hypothesis for sugar transport because the extents of *trans*-acceleration of D-glucose exit produced by extracellular mannose or galactose are inconsistent with the observed rates of self-exchange of mannose, D-glucose, or galactose and with net mannose or galactose exits. Miller's simple-carrier analysis assumes

(1) that the rates of translocation of any given sugar are identical in both directions ($k_{-1} = k_1$);

(2) that the rate of relaxation is independent of direction ($k_o = k_{-o}$);

(3) that transport is symmetric (V_{max} exit = V_{max} entry), and

(4) that hetero-exchange transport is saturated at the sugar concentrations used (130 mM).

The latter assumption is incorrect. $K_{m(app)}$ for exchange D-galactose transport in human red cells is 140 mM (Ginsburg & Ram, 1975). We also know that transport is asymmetric [see Table 1 and Lowe and Walmsley (1986)] which indicates that $k_o \neq k_{-o}$ (Stein, 1986). Miller observed that glucose exit at 20 °C into mannose or galactose is significantly faster than into glucose. He reasoned that this cannot be due to mannose and galactose translocations occurring more rapidly, since if this were the case, then mannose–mannose and galactose–galactose exchanges would be faster than glucose–glucose exchange, and this is not

Table 5: Bounds for the Rate Constants of the Simple-Carrier

constant[a]	computed as[b]	3OMG[c]	D-glucose[c]	DODG[c]
k_1, k_{-1}, b_{-1}, b_{-2}	$\frac{1}{nR_{ee}}$	39.9	137.1	118.5
k_o	$\frac{1}{nR_{oi}} < k_o < \frac{1}{n(R_{oi} - R_{ee})}$	$0.78 < k_o < 0.79$	$0.68 < k_o < 0.68$	$1.26 < k_o < 1.27$
k_{-o}	$\frac{1}{nR_{io}} < k_{-o} < \frac{1}{n(R_{io} - R_{ee})}$	$7.07 < k_{-o} < 8.60$		
b_1	$\frac{1}{nKR_{oi}}$	1.99×10^3	1.41×10^3	3.71×10^3
b_2	$\frac{1}{nKR_{io}}$	1.81×10^4		

[a] The first-order constants, k_1, k_{-1}, k_o, k_{-o}, b_{-1}, and b_{-2}, have units of s^{-1} and describe the following reactions, respectively: e.S1 → e.S2, e.S2 → e.S1, e1 → e2, e2 → e1, e.S1 → e1 + S1, and e.S2 → e2 + S2. The second-order rate constants, b_1 and b_2, have units of M^{-1} s^{-1} and describe the reactions, respectively: e1 + S1 → e.S1 and e2 + S2 → e.S2. [b] Constants were calculated assuming n (glucose transporter concentration) = 3.8 μmol per liter of cell water per min and by using the data of Table 2 as suggested in Stein (1986). [c] Constants were computed for 3OMG, D-glucose, and 2DODG transport.

observed. However, this conclusion is negated when $k_{-1} \neq k_1$. Assuming only saturated exchange exit occurs, V_{max} for exchange exit is given by

$$\frac{nk_1k_{-1}}{k_1 + k_{-1}}$$

where n is the concentration of erythrocyte glucose transporters (Carruthers, 1991). When $k_{1(glucose)} = k_{-1(glucose)} > k_{1(mannose)} > k_{1(galactose)}$ but $k_{-1(glucose)} < k_{-1(galactose)} < k_{-1(mannose)}$, exofacial mannose and galactose will stimulate glucose exit more than will exofacial glucose. Because Miller measured self-exchange of galactose at a subsaturating concentration (130 mM ≈ $K_{m(app)}$), galactose−galactose self-exchange will be slower than saturated glucose−glucose exchange. Net sugar transport rates are dominated by the slower relaxation rate constants k_o and k_{-o}. If relaxation (k_{-o}) is 3-fold slower than $k_{1(glucose)}$, V_{max} for net exit [$nk_1k_{-o}/(k_1 + k_{-o})$] increases in the order galactose < mannose < glucose ≤ nk_{-o}. These predictions broadly match the results obtained by Miller (1968a) and thus cannot be used to refute the simple-carrier mechanism for sugar transport.

Naftalin and Rist (1994) have examined hetero-exchange sugar transport in rat erythrocytes where 3-*O*-methylglucose transport is symmetric and where V_{max} for 3-*O*-methylglucose transport is greater than that for uptake of mannose or 2-deoxy-D-glucose or for exit of mannose. This observation confirms earlier measurements of rat red cell sugar transport (Helgerson & Carruthers, 1989), demonstrating that unlike sugar transport in human erythrocytes, V_{max} for net sugar uptake in rat cells is strongly dependent on the transported sugar. This result means that if sugar transport in this tissue is mediated by a simple-carrier, it is not rate-limited just by substrate-independent relaxation (k_o or k_{-o}) but also by translocation. Knowing that reduced mannose uptake and exit reflect reduced translocation (k_{-1} and k_1 for mannose are less than k_1 and k_{-1} for 3-*O*-methylglucose), Naftalin and Rist were at a loss to explain how V_{max} for mannose self-exchange is almost as great as V_{max} for 3-*O*-methylglucose self-exchange. They conclude correctly (Naftalin & Rist, 1994) that simple-carrier kinetics cannot account for this behavior and show that a hypothetical carrier mechanism that exposes import and export sites simultaneously is more successful in predicting this result.

Because rat red cell hetero-exchange sugar transport shows such clear deviation from simple-carrier kinetics while human red cell exchange transport does not, we re-examined human erythrocyte hetero-exchange fluxes under conditions (low temperature) where transport rates can be measured accurately.

Table 5 summarizes bounds for the first- and second-order rate constants describing simple-carrier-mediated sugar transport in red cells at 0 °C. These were computed as suggested by Lieb (1982) and by Stein (1986). We used these values as a starting point for simulating the saturated homo- and hetero-exchange fluxes of Table 3. Our experimental findings are simulated adequately by the simple-carrier hypothesis (Table 3) provided $k_{1(glucose)} \approx k_{-1(glucose)} \approx k_{1(deoxy-D-glucose)} \approx k_{-1(deoxy-D-glucose)} = 3k_{-1(3OMG)} = 2k_{1(3OMG)}$. These simple-carrier simulations are less successful in accounting for the concentration dependence of heteroexchange transport in red cells (Figure 4) where, just as with simple-carrier simulations of rat erythrocyte hetero-exchange (Helgerson & Carruthers, 1989), the affinity of the transport system for external sugar is systematically underestimated when the internal sugar level is saturating. The parameters used in these simulations (Tables 2 and 5) are in close agreement with those measured in previous studies (Lowe & Walmsley, 1986; Walmsley & Lowe, 1987; Wheeler, 1986).

High-Affinity Export Site. Table 1 lists the results of *infinite-cis* 3-*O*-methylglucose uptake experiments measured by three independent methods. $K_{m(app)}$ for intracellular 3OMG inhibition of net sugar uptake is approximately 0.5 mM, while $K_{m(app)}$ for intracellular 3OMG stimulation of sugar uptake is 3.4 ± 0.6 mM. The predicted result for simple- and fixed-site carrier-mediated transport (KR_{oi}/R_{ee} = 21 mM) is significantly greater than the experimental observation. The probability that this experimental result is accounted for by the simple-carrier and fixed-site carrier hypotheses is less than 1 in 200. These findings are consistent with previous studies demonstrating a high-affinity sugar efflux site when extracellular sugar levels are saturating (Baker & Naftalin, 1979; Carruthers & Melchior, 1983, 1985; Ginsburg & Stein, 1975; Hankin et al., 1972; Harris, 1964; Lieb & Stein, 1977).

A single analysis is consistent with the predictions of the asymmetric simple-carrier hypothesis reporting a low-affinity

$K_{m(app)}$ for sugar exit into media containing saturating sugar levels (Wheeler & Whelan, 1988). This result was obtained by applying a correction algorithm to transport measurements obtained at time points far exceeding times normally used to obtain initial transport rates. The central assumption of the correction algorithm used is that transport is mediated by an asymmetric simple carrier. We conclude that previous demonstrations of a high-affinity intracellular sugar export site under conditions of saturating extracellular sugar levels are accurate. If this site is an intrinsic property of the transport system, there is currently no existing transport model that can account for this result.

Cytochalasin B Binding. The failure of extracellular D-glucose to inhibit CCB binding to red cells is consistent with three possibilities: (1) Transport is mediated by a strongly asymmetric simple-carrier ($k_{-o}/k_o = 10$) in which most of the carrier (>90%) exists in the e1 state in the absence of substrate (see Table 4). (2) Transport is mediated by an almost symmetric simple-carrier (asymmetry = 1.4) that displays weak *trans*-acceleration ($k_o = 0.3k_1$; see Table 4). (3) Transport is mediated by a fixed-site carrier that can bind intracellular CCB and extracellular D-glucose simultaneously (see Table 4). In the simple-carrier simulations, constants for the symmetric simple-carrier were constrained to produce a maximum exchange rate close to that observed in red cells. This is important because theoretical analyses show that, unlike net fluxes, this rate is unaffected by the presence of unstirred layers of sugar or sugar binding sites within the cell (Carruthers, 1991; Lieb & Stein, 1974).

The inhibition of CCB binding by intracellular D-glucose and its weak reversal by extracellular D-glucose are incompatible with the strongly asymmetric simple-carrier (Table 4). However, this result is predicted for a simple-carrier that displays 1.4-fold asymmetry (Table 4) or for a fixed-site carrier that can bind sugars simultaneously at import and export sites that interact cooperatively (Table 4). We therefore conclude that although the observed steady-state sugar transport properties of human erythrocytes at ice temperature support the notion that transport is mediated by a strongly asymmetric simple-carrier mechanism, this is rebutted by the equilibrium cytochalasin B binding properties of the erythrocyte sugar transport system which suggest either an almost symmetric simple carrier mechanism or a transport mechanism that presents import and export sites simultaneously to substrate.

This is further supported by the results of GLUT1-reconstitution studies (Appleman & Lienhard, 1989; Carruthers & Melchior, 1984) and by the results of cell lysis/resealing studies (Carruthers, 1986; Carruthers & Melchior, 1983) which demonstrate that GLUT1-mediated sugar transport asymmetry (V_{max} exit/V_{max} entry) ranges from 1 (symmetric) to 1.7 in the absence of other cellular components. These findings are also consistent with previous demonstrations of symmetry in GLUT1-mediated sugar transport in avian, rat, and rabbit erythrocytes and in basal adipocytes (Helgerson & Carruthers, 1989; Naftalin & Rist, 1991; Regen & Morgan, 1964; Simons, 1983; Taylor & Holman, 1981).

CONCLUSION

Our findings are consistent with either of two possibilities. (1) Human erythrocyte sugar transport is mediated by a carrier mechanism with properties fundamentally different to those of mechanisms previously considered for passive sugar transport. (2) The reported steady-state sugar transport properties of human erythrocytes do not accurately reflect the intrinsic translocational properties of the red cell transporter. Our findings further suggest that if the transporter functions as a simple-carrier type mechanism, the degree of translocational asymmetry is severely overestimated by steady-state sugar transport determinations. If the transporter functions as a fixed-site carrier type mechanism, then transport may indeed by translocationally asymmetric. The data presented in this study do not distinguish between these possibilities.

WORKING HYPOTHESIS

It has been suggested (Baker & Naftalin, 1979; Carruthers, 1991; Helgerson & Carruthers, 1989; Naftalin & Holman, 1977) that erythrocyte sugar transport is mediated by a translocationally symmetric (simple- or fixed-site) carrier but that slow, reversible sugar complexation by intracellular molecules in close proximity to the glucose transporter rate-limits net transport. If true, this would negatively impact the ability of the experimentalist to accurately determine sugar transport rates over intervals as short as even 1 ms (Carruthers, 1991). Measurable cellular transport properties under these circumstances would reflect the sum of both sugar translocation and sugar complexation processes.

Cloherty et al. (1995) have demonstrated the existence of sugar binding sites within the erythrocyte in functional proximity to the glucose transporter. Sugar binding to these sites strongly impacts net sugar transport into bulk cytosol (Cloherty et al., 1995). The present study demonstrates that transport is not mediated by a simple-carrier with 10-fold translocational asymmetry but rather (if transport is mediated by a simple-carrier) is catalyzed by a carrier with 1.4-fold asymmetry. These results are consistent with the conclusions of studies of sugar transport in other cell types indicating that GLUT1-mediated sugar transport is a translocationally symmetric process and support the hypothesis of Baker and Naftalin (1979) that the steady-state sugar transport properties of human erythrocytes are the sum of two serial processes: translocationally symmetric, protein-mediated sugar transport and slow, reversible intracellular sugar binding.

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