Kinetic Properties of the Reconstituted Glucose Transporter from Human Erythrocytes*

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The kinetic parameters of D-glucose transport in liposomes reconstituted with the purified glucose transport protein were determined. Net uptake and efflux both had $K_m$ values of 0.7 to 1.2 mM and $V_{max}$ values of 1.6 μmol/mg of protein/min. Equilibrium exchange had a $K_m$ of 35 mM and a $V_{max}$ of 60 μmol/mg of protein/min. By separating the liposomes from unreconstituted protein using density centrifugation, the $V_{max}$ of exchange was increased to 86 μmol/mg of protein/min, about 3 times that of the erythrocyte membrane.

Trypsin, which inhibits erythrocyte glucose transport only from the cytoplasmic side, inhibited reconstituted transport activity about 40% when added externally. With internal treatment as well, the inhibition was about 80%. This suggests that the reconstituted transporter is oriented about equally in both directions. Antibody prepared against the purified transporter inhibited transport to a maximum of about 40%, also indicating a scrambled orientation. External trypsin treatment decreased the $K_m$ for uptake and increased the $K_m$ for efflux, consistent with asymmetric kinetic parameters for the two faces of the transporter. However, the calculated $K_m$ values are lower than those reported for erythrocytes.

Phloretin and diethylstilbestrol inhibit the reconstituted transporter. However, they bind to liposomes, producing anomalous results under some experimental conditions. When this binding is taken into account, phloretin inhibits completely and symmetrically. The binding accounts for the apparent asymmetric effects of phloretin reported by others. The inhibitory effects of mercuric ions are consistent with action at two classes of binding sites. Treatment with trypsin increases the sensitivity to HgCl₂, indicating that the more sensitive site is on the external face of the transporter.

The glucose transporter from human erythrocytes has been reconstituted into liposomes (1, 2) and purified (3, 4). Studies on the chemical characterization (5), cytochalasin B-binding activity (6, 7), and immunological identification (8, 9) of the transporter have been reported.

The kinetic studies presented here were undertaken with the purified transporter from Dr. Yi-Teh Li, Department of Biochemistry, Tulane University School of Medicine.

**Experimental Procedures**

Materials—Oxidated human blood was a gift from the American Red Cross, Syracuse, NY. U-[¹⁴C]Glucose and phloretin were obtained from ICN; soybean lipid (1,2-O-phosphatidylcholine), trypsin, and diethylstilbestrol from Sigma; and L-[¹⁴C]glucose from New England Nuclear. Endo-β-galactosidase from Escherichia freundii was a gift from Dr. Yi-Teh Li, Department of Biochemistry, Tulane University School of Medicine.

Preparation of the Transporter—Erythrocyte ghosts (10) and alkaline-washed vesicles (5) were prepared as described. The glucose transporter was prepared as described (5 except that the éuète from the Bio-Beads column was concentrated by repeated additions of dry Sephadex G-50, desalted on a column (150 ml) of Sephadex G-50 pre-equilibrated with 1 to 2 mM Na/MOPS buffer, pH 7.5, and lyophilized.

Reconstitution of D-Glucose Transport—The freeze-thaw reconstitution procedure described previously (3) was used with minor modifications. Ten mM MOPS buffer, pH 7.5, was used rather than Tris-HCl, and MgCl₂ was omitted. Acetone washing of the soybean lipids and flushing with N₂ before sonication were found to be unnecessary. Reconstitution was performed using 10 mg of lipid/ml and 170 μg of purified transport protein/ml. We found that more reproducible activity was obtained using a shorter sonication time (about 5 s) after the freeze-thaw step than was used previously (20 to 30 s) (3). In some of the experiments reported here (those involving phloretin and diethylstilbestrol), a 15-s sonication was used.

Transport Assays—For net uptake experiments, an aliquot of reconstituted liposomes (containing 0.56 mg of lipid and 3 to 5 μg of protein in 50 μl) was placed in a test tube (13 X 100 mm). The tube was placed in a Vortex mixer and radioactive glucose solution (200 μl) was added to initiate the assay. At the desired time, the assay was stopped with 5 ml of an ice-cold stopping solution containing 0.5 mM HgCl₂, 10 mM MOPS buffer, pH 7.5, and NaCl giving the same osmolality as the assay mixture. The mixture was filtered on a 0.22-μm filter (Millipore type GSWP) and washed with 10 ml of the cold stopping solution. Filters were soaked in H₂O and washed with 5 ml of stopping solution prior to filtration. For equilibrium exchange uptake, the same procedure was followed except that liposomes were pre-equilibrated at least 15 min with nonradioactive glucose at the

1 The abbreviation used is: MOPS, morpholinepropanesulfonic acid.
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same concentration as the added radioactive glucose. The equilibrated volume in 2 min of exchange was about 0.2 μl/μg of protein for 10 to 40 mM glucose and decreased to about 0.1 μl/μg at 80 mM. Experiments examining the net uptake of glucose at high concentrations over several hours indicated that this was due not to a failure to pre-equilibrate completely, but due to the fact that the liposomes do not regain their original volume under such conditions. For efflux experiments, 10 μl of vesicle suspension pre-equilibrated with radioactive glucose (containing about 0.2 mg of lipid and 1 to 2 μg of protein) was mixed with 3 to 4 ml of medium by blending on a Vortex mixer. The assay was stopped with 1 ml of stopping solution containing sufficient HgCl₂ to make the mixture 0.5 mM, and filtered and washed with 0.5 mM HgCl₂ solution as above. Filters were counted in 10 ml of scintillation liquid (Liquiscint, National Diagnostics). All assays contained 10 mM MOPS buffer, pH 7.5, and were run at about 23°C. Zero-time controls were obtained by adding stopping solution before initiation of reactions; the resulting counts were subtracted from the experimentally obtained counts. In addition, nonspecific transport was determined at each time point using L-glucose or D-glucose plus 0.5 mM HgCl₂. This was subtracted to obtain the specific transport. In some experiments, D- and L-glucose uptake were measured simultaneously using D-[14C]glucose and L-[3H]glucose. The double label measurements gave transport rates similar to those obtained in separate measurements of D- and L-glucose. All determinations were done in duplicate or triplicate.

In some uptake experiments, radioactive glucose was placed in a test tube containing a 1-cm stirring bar, and liposomes were added to initiate the reaction. It was found that at glucose concentrations above the Km, higher rates were obtained using the Vortex mixer method described above, indicating inadequate mixing with the stirring bar method. We verified that the mixing using the Vortex mixer method was adequate in two ways. First, increasing the speed of mixing did not affect the specific uptake rate at 30 mM glucose. Second, we measured the hydrolysis of 0.2 mM dibenzylphosphoryl acid using the same mixing procedure. A half-time of about 9 s, giving a rate constant of about 40 m⁻¹ s⁻¹, was obtained using both mixing speeds, in good agreement with previous determinations (11).

In the experiments reported here, the specific uptake of d-glucose into reconstituted liposomes was approximately 1000 times the calculated binding of glucose to the purified transporter protein, using the Kr for glucose inhibition of cytochalasin B binding and the number of cytochalasin B binding sites per mg of purified protein (7). Therefore, it is very unlikely that binding of glucose to the transporter is contributing significantly to the observed transport.

In the experiments described below, we measured the apparent initial rates of transport at various glucose concentrations. These were by far the most sensitive method for measuring transport rates, and were required to describe the corrected uptake in Fig. 1 (open circles and dashed line). This is to be expected, since the liposomes are probably heterogeneous in both size and the number and activity of reconstituted transporters. The two-component fit (dashed line) is therefore only an approximation of the sum of many different equilibration processes having a range of rate constants and extents.

In the experiments described below, we measured the apparent initial rates of transport at various glucose concentrations. These will be somewhat lower than the true initial rates, which binds to lipids (15), so that each increment of binding results in a spectral shift toward the low pH form. By comparing the relative absorbances at 284 and 322 nm after each liposome addition to the relative absorbances of phloretin at various pH values, the amounts of ionized and neutral forms could be determined. From this, the free and bound phloretin concentrations were calculated.

**RESULTS**

Zero-trans Uptake Kinetics—We examined the kinetic parameters of glucose transport in the reconstituted system under conditions of net uptake, net efflux, and equilibrium exchange. The net flux experiments were performed in the absence of glucose on the trans side of the membrane (zero-trans experiments). A typical time course for the net uptake of 0.2 mM glucose is shown in Fig. 1. d-Glucose shows a rapid uptake into the liposomes which is complete within 2 min and which has a half-time of less than 10 s. L-Glucose uptake is much slower.

The time course of equilibration of glucose at a concentration well below its Km into a liposome is expected to follow the equation:

\[ U_t = U_o(1 - e^{-Kt}) \]

where \( U_t \) is the extent of uptake at time \( t \), \( U_o \) is the uptake at complete equilibration, and \( K \) is the rate constant. It was found that at least two pairs of rate constants and extents were required to describe the corrected uptake in Fig. 1 (open circles and dashed line). This is to be expected, since the liposomes are probably heterogeneous in both size and the number and activity of reconstituted transporters. The two-component fit (dashed line) is therefore only an approximation of the sum of many different equilibration processes having a range of rate constants and extents.

In the experiments described below, we measured the apparent initial rates of transport at various glucose concentrations. These will be somewhat lower than the true initial rates,
since transport is not linear in the time range measured. For example, in the experiment shown in Fig. 1, the calculated time course fit to two rate constants (dashed line) has an initial rate of 39.5 pmol/s, while the apparent initial rate (assuming the uptake to be linear for 3 s) is 31.0 pmol/s. In the design of experiments, one must compromise between using early time points, which give calculated rates closer to the true initial rates, and longer time points, which give extents that can be determined more accurately. In the experiments reported below, the time points were selected to give 15 to 35% equilibration.

Fig. 2A shows the results of a zero-trans uptake experiment. The specific uptake was measured at times ranging from 4 s (0.5 mM glucose) to 14 s (8 mM); in each case, 18 to 29% equilibration was obtained, based on the extent of uptake of 0.5 mM glucose in 2 min. The kinetic parameters obtained in this experiment are listed in Table I. We also estimated the parameters using the uptake of 0.5 to 3 mM glucose in 3 s. From the apparent initial rates, the values of $K_m$, $V_{max}$ for the purified transporter was previously estimated as 1.0 pmol/mg of protein/min based on this $K_m$ of 2.2 mM and the uptake of 0.2 mM glucose in 5 s (3).

Zero-trans Efflux Kinetics—The time course for the efflux of 0.2 mM glucose from pre-equilibrated liposomes is similar to that shown in Fig. 1 for uptake. In the efflux experiments, the liposomes were diluted 300- to 400-fold such that the external glucose concentration was negligible. Fig. 2B shows the results of a zero-trans efflux experiment. The specific efflux was measured at times ranging from 3 s (0.2 mM glucose) to 8 s (4 mM), with 17 to 33% equilibration being obtained in each case. The values of $K_m$ and $V_{max}$ obtained in this experiment are listed in Table I.

Equilibrium Exchange Kinetics—The uptake of radioactive glucose into liposomes pre-equilibrated with the same concentration of nonradioactive glucose shows a time course similar to that shown in Fig. 1 for net uptake (20). The results of an equilibrium exchange experiment are shown in Fig. 2C. The specific exchange was measured at 4 s (10 to 20 mM glucose) or 5 s (40 to 80 mM); in addition, the extent of equilibration at 2 min was measured for each glucose concentration. At the early time points, 26 to 33% equilibration was observed.

The calculated values of $K_m$ and $V_{max}$ are listed in Table I. Both $K_m$ and $V_{max}$ are much higher than the parameters for net uptake and efflux. The value for $K_m$ of 35 mM is similar to $K_m$ values observed with erythrocytes (20 to 38 mM) (21-24). At high glucose concentrations, the decrease in liposome volume produced greater errors in the rate measurements. This has produced some variability when extrapolations to $V_{max}$ were made. We have obtained values for $K_m$ of 20 to 50 mM in experiments such as that shown in Fig. 2C, with a similar variation in $V_{max}$. A more reliable measurement is the rate at a single intermediate glucose concentration. At 20 mM glucose, the exchange activity is about 20 pmol/mg of protein/min.

Fractionation of Reconstituted Liposomes by Centrifugation—We found that it was possible to separate reconstituted liposomes from unreconstituted protein using centrifugation. Three ml of a reconstituted liposome suspension was layered on top of 5 ml of a solution containing 0.4 mM sucrose and 10 mM MOPS buffer, pH 7.5, and centrifuged at 25,000 rpm for 30 min in a Beckman 40 rotor. Four fractions were characterized (Fig. 3): a clear upper layer, a cloudy layer at the original interface containing most of the lipid, a clear layer containing small amounts of protein and lipid, and a pellet containing about 40% of the protein. The two upper fractions were passed through a column of Sephadex G-50 to remove the sucrose, and equilibrium exchange of 20 mM glucose was assayed. The upper fraction (fraction 1 in Fig. 3), although it contained significant amounts of protein and lipid, showed no detectable transport activity. The cloudy fraction (fraction 2) showed a specific activity of about 50 pmol/mg/min, 2.5 times that of the original reconstituted liposomes. The protein of the pellet was passed through Sephadex and used in a second freeze-
Control liposomes were prepared as described under “Experimental Procedures.” The trypsin treatment and sucrose purification are described in the text. The reconstituted activity was only about 10% of the original liposomes. The \( V_{\text{max}} \) and \( K_m \) of the active fraction are shown in Table I; the procedure increased the specific activity under conditions of thaw reconstitution. The reconstituted activity was only about 86 pmol/mg/min, about 2.2 pmol/mg/min, when the trypsin was added after reconstitution. When trypsin was present during the original formation of the liposomes to be used in the freeze-thaw reconstitution, so that the reconstituted transporter was exposed to its action on both sides of the membrane, the uptake activity was inhibited an average of 7% (three experiments). The effect of externally added trypsin was time-dependent, with a half-time of about 5 min when \( 2 \mu \text{g} \) of trypsin/ml and 25 \( \mu \text{g} \) of transporter/ml was used. Additional trypsin added at the completion of the reaction or much larger amounts used initially did not result in any further inactivation.

The simplest explanation for these results is that trypsin cleavage reduces the activity of the transporter by about 80%, and that the reconstituted transporter is oriented to approximately the same extent in both directions. Treatment of the liposomes with trypsin should therefore produce a population in which most of the activity is due to transporters having the same orientation as erythrocytes.

We measured the net flux parameters of trypsin-treated liposomes (Table I), using about 1 \( \mu \text{g} \) of trypsin/3 \( \mu \text{g} \) of transporter protein. Rates at 0.2 to 0.5 mM glucose were also compared to those for untreated liposomes; 30 to 50% inhibition was observed. In the case of uptake, \( K_m \) was decreased about 3-fold and \( V_{\text{max}} \) about 5-fold. In the case of efflux, a slight but not statistically significant increase in \( K_m \) was observed; \( V_{\text{max}} \) was decreased 30%, much less than in the case of uptake.

**Inhibition by Antibody**—We tested the effect of antibody prepared against the transporter and purified using an affinity column of transporter linked to agarose (9). The net uptake of 0.2 mM glucose in 5 s was determined. At a ratio of 10 \( \mu \text{g} \) of antibody/\( \mu \text{g} \) of transporter, 28 ± 6% inhibition was observed. At a ratio of 50, 40 ± 7% inhibition was observed. The same levels of control IgG produced no inhibition. These results are consistent with a maximum of 50% inhibition, which would be expected if the antibody were to one face of the protein and both orientations were present equally. In previous studies, a component of a rabbit antiserum against human erythrocytes was shown to act on the glucose transporter at the cytoplasmic face (3).

**Inhibition by Phloretin and Diethylstilbestrol**—It was previously shown that two inhibitors of erythrocyte glucose transport, mercuric ions and cytochalasin B, were also effective inhibitors of our reconstituted system (1, 3). However, two other inhibitors, phloretin and diethylstilbestrol, did not inhibit under the conditions tested (3), although inhibition by phloretin was reported in other reconstituted systems (4, 28). We re-examined the effect of these inhibitors.

Verkman and Solomon (29) reported that phloretin binds to liposomes with a dissociation constant of 8 \( \mu \text{M} \) and an extent of one binding site/4 phosphatidylcholine molecules. Such binding in the earlier studies would have reduced the free phloretin concentration to such a low level that inhibition would not have been expected. We measured the binding of phloretin to liposomes under the conditions used in the kinetic experiments (0.2% ethanol, 10 \( \mu \text{M} \) MOPS buffer, pH 7.5) by a spectral titration of a phloretin solution with liposomes and obtained a dissociation constant of 15 \( \mu \text{M} \) and about 150 nmol binding sites for 1 mg of soybean lipid.

Fig. 4 shows the inhibition of the stereospecific uptake of 0.2 mM glucose by 20 to 200 \( \mu \text{M} \) phloretin in assays containing 2.25 mg of lipid/ml (solid circles). Using the above parame-

![Figure 3. Fractionation of reconstituted liposomes by centrifugation.](https://example.com/figure3)

![Figure 4. Inhibition of \( \alpha \)-glucose uptake by phloretin and diethylstilbestrol.](https://example.com/figure4)

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**Table I. Kinetic parameters for glucose transport in reconstituted liposomes**

<table>
<thead>
<tr>
<th>Liposomes</th>
<th>Procedure</th>
<th>( K_m ) (mM)</th>
<th>( V_{\text{max}} ) (μmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Zero-trans uptake</td>
<td>1.2 ± 0.1</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>Control</td>
<td>Zero-trans efflux</td>
<td>0.73 ± 0.07</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>Trypsin-treated</td>
<td>Zero-trans uptake</td>
<td>0.37 ± 0.01</td>
<td>0.34 ± 0.00</td>
</tr>
<tr>
<td>Control</td>
<td>Equilibrium exchange</td>
<td>1.0 ± 0.4</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>Sucrose purified</td>
<td>Equilibrium exchange</td>
<td>35 ± 4</td>
<td>48 ± 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>27 ± 2</td>
<td>86 ± 4</td>
</tr>
</tbody>
</table>
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The calculated concentrations of free phloretin are 0.9 to 18 \( \mu M \). The inhibition data was replotted versus the calculated free phloretin concentration (open circles), giving a value for \( K_i \) of about 3 \( \mu M \). This is in good agreement with the value of 2.4 \( \mu M \) reported for red cells (30). In efflux experiments containing a much lower concentration of lipid (0.04 mg/ml) such that the free phloretin concentration is nearly equal to the total concentration, 50% inhibition was produced by a total phloretin concentration of 5 \( \mu M \).

We compared the effects of 20 \( \mu M \) phloretin on uptake and efflux of 0.2 mM D-glucose using 0.2 mg of lipid/ml and a total assay volume of 1 ml. Both uptake and efflux were inhibited approximately 50% (not shown), indicating no asymmetry in the effects of phloretin.

The reported lack of inhibition by diethylstilbestrol (3) was also probably due to binding of most of the inhibitor to lipids. We measured the binding of diethylstilbestrol to liposomes in the presence of 0.2% ethanol and 10 mM MOPS buffer, pH 7.5, by equilibrium dialysis. The binding was nonsaturable in the range relevant to our inhibition studies (0 to 10 \( \mu M \) free diethylstilbestrol) with a ratio for bound/free of 18.6 ± 0.6 at 1 mg of lipid/ml. An inhibition experiment similar to that done with phloretin gave a \( K_i \) of about 3 \( \mu M \) when we correct for the binding of diethylstilbestrol to lipids (Fig. 4). This is in good agreement with the value of 4.7 \( \mu M \) reported for ghosts (31).

**Inhibition by Merccuric Ions**—We tested the effects of HgCl\(_2\) on net uptake and efflux and found that the \( K_i \) appeared much lower in the case of efflux. This result is similar to that seen with phloretin and suggested that mercuric ions could also be binding to the liposomes.

The binding of Hg\(^{2+}\) to liposomes was measured by equilibrium dialysis. It was found that mercuric ions bound with a dissociation constant of 30 \( \mu M \) and an extent of about 270 \( \mu M \) binding sites at 1 mg of lipid/ml. In addition, a nonsaturable component of the binding was observed, with a ratio for bound/free of 0.5 at 1 mg of lipid/ml.

Fig. 5 shows the inhibition data from both uptake and efflux experiments plotted versus the calculated free mercury concentration. These results indicate that when the binding of Hg\(^{2+}\) to lipids is taken into account the inhibition is similar for both directions of transport. In these experiments, the transporter was exposed to Hg\(^{2+}\) at the beginning of the assay. However, the inhibition is time-dependent; with a 1-min preincubation with HgCl\(_2\), 50% inhibition of uptake was produced by less than 3 \( \mu M \) free (0.5 \( \mu M \) total) HgCl\(_2\) (not shown). This is 30-fold lower than the concentration required to inhibit 50% under the conditions shown in Fig. 5. Thus, the apparent inhibition constants referred to below are related to the rate of reaction of HgCl\(_2\) with the transporter under the assay conditions.

Although complete inhibition of specific transport is produced by mercuric ions, the curve rises more slowly than would be expected for a single apparent inhibition constant (indicated by the solid curve, drawn using a \( K_i \) of 1 \( \mu M \)). The broken curve, drawn using two apparent \( K_i \) values (0.1 and 10 \( \mu M \)) and assuming reaction at each of two sites results in a maximum of 50% inhibition, seems to give a better fit to the efflux data. However, the data are not sufficiently precise to distinguish for certain between these alternatives.

If the two inhibitory sites suggested above are on opposite faces of the transporter, treatment of liposomes with trypsin should alter the degree of inhibition produced by Hg\(^{2+}\). At low levels of Hg\(^{2+}\), trypsin-treated liposomes would be expected to be either insensitive or twice as sensitive as untreated liposomes, depending on whether the more susceptible site is on the cytoplasmic or external face of the transporter, respectively. We compared the net uptake of 0.2 mM glucose into control and trypsin-treated liposomes (Table II). The treated liposomes were inhibited more than the control liposomes, with 90% more inhibition seen at the lowest HgCl\(_2\) concentrations used. This indicates that the external site is the more susceptible site.

**Effect of trypsin treatment on inhibition by mercuric ions**

Specific uptake of 0.2 mM glucose was measured for 5 s (control liposomes) or 10 s (trypsin-treated liposomes). The apparent initial rate of uptake for the treated liposomes was 51% of the control rate.

![Table II](image)

**Effect of Endo-β-galactosidase**—Treatment of the purified glucose transporter with endo-β-galactosidase results in a loss of carbohydrate and a sharpening of the broad band seen in sodium dodecyl sulfate polyacrylamide gels, but does not diminish the binding of cytochalasin B (32). We tested the effects of such treatment on the reconstituted transport activity. Two hundred eighty \( \mu G \) of purified transporter was incubated with 10 \( \mu L \) of endo-β-galactosidase stock solution (33) for 6 h at 37 °C. A control sample was incubated without enzyme. The protein was then analyzed by gel electrophoresis and reconstituted into liposomes. Although the expected sharpening of the band on gels occurred, no difference was
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found in the reconstituted transport activity (net uptake of 0.2 mM glucose in 5 s).

Effect of Dithiothreitol—The binding of cytochalasin B to the purified transporter was reported to be inhibited by dithi-othreitol, with a $K_i$ of 1 mM (7). We tested the effects of dithiothreitol on transport, but the combination of dithiothreitol and the mercury of the standard stopping solution resulted in aggregation of the liposomes and a large increase in filtration time. A stopping solution containing 20 mM phloretin instead of HgCl$_2$ was therefore used; controls showed similar rates were obtained for the two assay systems. Using the phloretin stopping solution, no effect on either uptake or efflux of glucose was produced by 1 to 5 mM dithiothreitol at pH 6.0. Moreover, these concentrations of dithiothreitol had no effect on the inhibition of transport (30 to 60%) produced by 0.25 to 0.5 mM cytochalasin B. Thus, there appears to be a discrepancy between these kinetic results and the binding experiments.

DISCUSSION

Earlier measurements of the net uptake of D-glucose in the reconstituted system reported a specific activity of only 4 to 13% of that calculated for erythrocyte membranes (3). This led to the criticism that the observed activity could be due to a minor component of the preparation (6). We have found that the rate of equilibrium exchange is much faster than that of net uptake in reconstituted liposomes. The specific activity for the equilibrium exchange at 20 mM glucose is about 20 pmol/mg/min (Fig. 2C). When the liposomes are separated from unreconstituted protein by centrifugation in sucrose, this rate is increased to 50 pmol/mg/min. This is about 5 times the specific activity calculated for erythrocyte membranes (8 to 13 pmol/mg/min, using $K_m = 38$ mM and $V_{max} = 260$ mmol/cell unit/min (21) or $K_m = 32$ mM and $V_{max} = 357$ mmol/cell unit/min (22), $1.6 \times 10^6$ cells/cell unit (34), and 0.66 pg of protein/ghost (35)). If it is assumed that the transporter is a dimer of 45,000-dalton subunits (20), and that the $1.2 \times 10^5$ (36) to $2.5 \times 10^5$ (37) glucose-reversible cytochalasin B binding sites/cell correspond to these dimers, the calculated specific activity for the transporter at 20 mM glucose is 150 to 500 pmol/mg/min. Thus, the reconstituted system has a specific activity that is 10 to 33% of that expected for a fully active purified preparation. Since the active liposome fraction probably contains some inactive protein and some protein that is not reconstituted in an active manner, this value is a reasonable one. The possibility that a minor contaminant of the preparation is responsible for the transport activity therefore appears unlikely.

One possible reason that the activity is not higher than 10 to 33% of the expected value is that proteolysis has occurred during the purification procedure, resulting in a less active protein. Mullins and Langdon have presented evidence that the 45,000-dalton protein of our preparation is a fragment of a 100,000-dalton native transporter (38). However, immunological evidence against this hypothesis has also been presented (8, 9).

The value of 500% for the specific activity of the reconstituted transporter relative to the specific activity of the erythrocyte membrane calculated above is much higher than the value of 4 to 13% reported previously (3). This large increase can be attributed to several factors. First, we have observed approximately a 3-fold increase in the rate of net uptake at 0.2 mM glucose. This is probably due mainly to improvements in the purification procedure, with smaller contributions from changes in the reconstitution and assay procedures. Second, in the earlier calculation the rate of zero-trans uptake was compared to erythrocyte rates for infinite-cis exit and equilib-

rium exchange; making the comparison to red cell zero-trans uptake would have increased the percentage about 4-fold. Third, the $V_{max}$ for exchange in the reconstituted system is higher relative to the $V_{max}$ for zero-trans uptake (about 30-fold) than is the case for erythrocytes (7- to 10-fold), giving a more favorable comparison by a factor of about three when exchange rates are used. Finally, the separation of liposomes from unreconstituted protein by centrifugation gives a 2-fold increase in specific activity. Together, these four factors result in a 70-fold increase in the ratio of specific activities.

The kinetic parameters for glucose transport in red cells depend on the type of transport being measured. Zero-trans entry has a $K_m$ of 1.6 mM (23), similar to the $K_m$ for infinite-cis exit (39) and entry (40), and a $V_{max}$ of 36 mmol/cell unit/min. Zero-trans efflux has much higher values of $K_m$ and $V_{max}$, 25 mM and 130 mmol/cell unit/min, respectively (41). The parameters for equilibrium exchange, 20 to 38 mM and 260 to 360 mmol/cell unit/min (21-24), are higher still.

We determined parameters for the reconstituted system using apparent initial rates of transport measured after at least 3 s. These rates are somewhat slower than the true initial rates. We estimated the initial rates using integrated rate equations for uptake (42), efflux (modified for the osmotic conditions of our experiments (41), and exchange (equation 1). Since these equations were derived for populations of cells having uniform transport activity and internal volume, they do not strictly apply to our heterogeneous liposome population. However, use of the equations probably results in calculated initial rates that are closer to the true rates than the apparent initial rates. Since the contributions from rapidly equilibrating liposomes are underestimated by this method, these calculated rates are still slightly lower than the true rates. For example, applying equation 1 to the 3-s uptake in Fig. 1 results in an initial rate of 37 pmol/s, 93% of the two-component fit (dashed line) and 18% faster than the apparent initial rate.

For all of the experiments listed in Table I, the parameters were also determined using the initial rates calculated with the integrated rate equations. The resulting parameters were not greatly different, with the largest change being a 30% increase in the uptake $V_{max}$. In addition, the calculations concerning the asymmetry of the transporter (see below) were not significantly affected by this refinement in the calculations. Thus, the conclusions presented here are not dependent upon the fact that initial rates have been somewhat underestimated.

The results with trypsin and antibody indicate that the transporter is oriented randomly in the reconstituted system. Therefore uptake and efflux should show the same kinetic parameters. However, we observe a 1.6-fold higher value for $K_m$ in the case of uptake (Table I). This may be due to the different experimental procedures used in measuring the two directions of flux.

Treatment of the liposomes with trypsin should inactivate those molecules oriented with the cytoplasmic face on the outside, leaving most of the activity from those molecules oriented in the same direction as in the red cell. Using the parameters listed above for erythrocytes, one would expect this to greatly decrease the $K_m$ and $V_{max}$ for uptake, moderately increase the $K_m$ for efflux, and moderately decrease the $V_{max}$ for efflux. This is in fact observed (Table I), although the change is not statistically significant in the case of the efflux $K_m$.

Using the parameters observed for uptake before and after trypsin treatment, one can estimate the values for the param-

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1 If it is assumed that the observed rate of uptake before trypsin
eters on the two faces of the transporter (Table III). This calculation indicates a 4-fold asymmetry in the parameters. A more straightforward method of doing the calculation would be to compare the results of uptake and efflux experiments after trypsin treatment (Table I); this method gives about a 3-fold asymmetry. However, as discussed above, the $K_m$ values for uptake and efflux are somewhat different in the control liposomes, and we felt it was more valid to do the calculation using results obtained from a single experimental procedure. For the asymmetric carrier model the ratio of $K_m$ and $V_{\text{max}}$ values for the two faces must be equal. Our data are consistent with this constraint, and the observed ratios are similar to the ratio of $V_{\text{max}}$ values in erythrocytes (Table III). However, both the $K_m$ values reported for erythrocytes and their ratio are much larger than we calculated for the reconstituted transporter.

A likely physical model for transport proteins such as the glucose transporter involves conformational changes of a transmembrane protein, as discussed in Ref. 43. The substrate binding site, initially accessible from one side of the membrane, becomes accessible from the other side after the conformational change. Kinetic schemes originally derived for mobile carrier models are also applicable to such a conformational change model (42). Since the protein is asymmetric with respect to the plane of the membrane, the structure of the binding site is likely to be different in the two conformations, and the conformations are likely to differ in energy. Thus, asymmetric kinetic parameters for transport from the two sides of the membrane are expected.

It is interesting to compare our results to those predicted by models for glucose transport. A test of the asymmetric carrier model described in Ref. 42 is whether the same ratio of $K_m$ to $V_{\text{max}}$ is obtained from different types of experiments. Using the parameters listed in Table III, a ratio of 0.54 to 0.66 mg min/ml is obtained for zero-trans experiments. Using the parameters for exchange listed in Table I, a value of 0.73 is obtained, in reasonable agreement. A more stringent test of the model would involve using parameters for infinite-cis experiments. On the basis of such criteria and results from erythrocytes, some workers have rejected this model (40).

The ratios of the $V_{\text{max}}$ for exchange (Table I) to the $V_{\text{max}}$ values calculated for the external and internal face of the transporter (Table III) are 71 and 19, respectively. In the erythrocyte, these ratios are 7 to 10 and 2 to 3, respectively, using the values listed above. Thus, the reconstituted transporter has a much greater ratio of exchange to net flux than treatment is due equally to the two orientations of the transporter (equation 2), and that the rate after treatment is due only to the right-side-out orientation (second term in equation 2), then the parameters for the two faces are given by equations 3 to 6.

$$v_B = \frac{1}{2} V_e \left( \frac{S}{S + K_e} \right)^{1/2} V_e \left( \frac{S}{S + K_i} \right)$$

$$V_e = 2V_A$$

$$K_e = K_A$$

$$V_i = 2(V_0 - V_A)$$

$$K_i = K_C \left[ \frac{V_e}{V_0 - V_e/(1 + K_e/K_i)} - 1 \right]$$

where the subscripts $B$ and $A$ refer to the apparent $K_m$ and $V_{\text{max}}$ for zero-trans uptake before and after trypsin treatment, respectively, and $e$ and $i$ refer to the parameters for the external and internal faces, respectively, of the transporter. Equation 6 comes from setting $S = K_i$ and $v_B = \frac{1}{2} V_e$ in equation 2 and solving for $K_i$. The terms "external" and "internal" as used here refer to the orientation of the transporter in the erythrocyte membrane and not to their orientation in the liposomes, which is assumed to be scrambled.

### Table III

<table>
<thead>
<tr>
<th>Reconstituted liposomes</th>
<th>$K_m$ (μM)</th>
<th>$V_{\text{max}}$ (nmol/mg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>External</td>
<td>0.37 ± 0.01</td>
<td>0.68 ± 0.01</td>
</tr>
<tr>
<td>Internal</td>
<td>1.66 ± 0.32</td>
<td>2.52 ± 0.16</td>
</tr>
<tr>
<td>Internal/external</td>
<td>4.5 ± 0.9</td>
<td>3.7 ± 0.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Erythrocytes</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>External (23)</td>
<td>1.6 ± 0.2</td>
<td>36 ± 1.2</td>
</tr>
<tr>
<td>Internal (41)</td>
<td>25 ± 3</td>
<td>129 ± 11</td>
</tr>
<tr>
<td>Internal/external</td>
<td>15.6 ± 2.7</td>
<td>3.6 ± 0.3</td>
</tr>
</tbody>
</table>

The external $K_m$ and $V_{\text{max}}$ were assumed to be the $K_m$ and twice the $V_{\text{max}}$ observed after trypsin treatment, and the internal parameters were calculated according to Equations 5 and 6 in Footnote 2. The external $K_m$ and $V_{\text{max}}$ was not observed, since at high concentrations of lipids the free inhibitor concentration is much lower than the total concentration.

Two groups working with reconstituted glucose transport systems have reported asymmetric effects of phloretin. Goldin and Rhoden (28) observed inhibition of glucose uptake into liposomes formed in the presence of phloretin, but no inhibition when phloretin was added externally. However, since phloretin is very permeant (15), it should be present on both sides of the membrane and no asymmetry is expected. The likely explanation for the apparent asymmetry is that liposomes formed in the presence of phloretin bind enough phloretin to provide an inhibitory level in the assay mixture. However, when phloretin is added only to the assay mixture, most of it binds to the liposomes and the remaining free does the red cell. Because of this difference, a comparison of the net flux activity to that expected for the pure transporter gives a lower result than was calculated above for exchange. Using the $V_{\text{max}}$ values in Table III and assuming the reconstituted activities would be increased 2.5-fold upon separation of unreconstituted protein by centrifugation gives values of about 2% the expected activity for both directions of flux. These changes in relative activities could be real differences in kinetic properties, due to an alteration of the protein during purification or to its different membrane environment. Alternatively, it is possible that our measured rates of net flux at high glucose concentrations are underestimated, resulting in low values for $V_{\text{max}}$ and $K_m$. As noted above, our calculated $K_m$ values for net flux are lower than those reported for erythrocytes.

In order to determine the orientation of the reconstituted transporter we examined the effects of three agents reported to act asymmetrically: trypsin, phloretin, and HgCl2. The experiments with trypsin indicate that the transporter is reconstituted with equal amounts of both orientations. During the purification procedure, the removal of Triton results in vesicles and fusion with liposomes in the freeze-thaw procedure preserves the random orientation. Baldwin et al. (26) examined the effect of trypsin on the binding of cytochalasin B by transporter reconstituted using different procedures. These observed either a 50:50 or a 75:25 distribution of orientations depending upon the method of reconstitution.

The binding of phloretin and diethylstilbestrol to lipids probably accounts for the failure to see inhibition by these two compounds in early studies of the reconstituted system (3). The experiments presented here indicate that the results obtained with such compounds will depend strongly on the conditions used, since at high concentrations of lipids the free inhibitor concentration is much lower than the total concentration.
concentration is not inhibitory. Kahlenberg and Zala (4), using a gel filtration efflux assay, observed inhibition only by externally added phloretin. In this case, internally trapped phloretin would be expected to leak out of the liposomes and be retarded on the column, resulting in little inhibition. Earlier results claiming to demonstrate asymmetric phloretin inhibition with ghosts (44) are probably also due to the effects of phloretin binding to lipids.

Goldin and Rhoden (28) also reported that externally added HgCl₂ inhibits glucose uptake to a maximum of about 50% in liposomes prepared by hollow fiber dialysis. Formation of the liposomes in the presence of HgCl₂ resulted in complete inhibition. This was attributed to reconstitution of both right-side-out and inside-out orientations, with only the former inhibited by externally added HgCl₂.

We have found that externally added HgCl₂ can completely inhibit glucose transport in either direction (Fig. 5). However, the inhibition curve rises very slowly at high concentrations, and is consistent with at least two classes of sites. The conclusion by Goldin and Rhoden of a maximum of 50% inhibition may have been due to the slow rise of the curve at high concentrations. Trypsin-treated liposomes are about twice as sensitive to low concentrations of Hg²⁺ as untreated liposomes (Table II), indicating the external site is more sensitive.

The experiments presented here demonstrate that many of the kinetic properties of the erythrocyte glucose transporter can be observed after purification and reconstitution. The use of trypsin to generate a population of active transporters having the same orientation as in erythrocytes is a useful tool in further exploring the properties of this protein.

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REFERENCES