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Accelerated net efflux of 3-*O*-methylglucose from rat adipocytes: a reevaluation

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Abstract

In a study of 3-*O*-methylglucose transport in insulin-stimulated rat adipocytes (catalyzed primarily by the GLUT4 isoform), it was reported that at 37°C the K_m and V_{max} were 2.8-fold higher for net efflux than for equilibrium exchange (Vinten, J. (1984) Biochim. Biophys. Acta 772, 244–250). Because of its implications for the relative sizes of steps in the transport cycle, we reinvestigated this phenomenon. Accelerated net efflux was apparent when the extracellular methylglucose was diluted 26-fold but not when it was diluted 11-fold. When analyzed according to the one-site alternating conformation model, the data indicate about a 1.7-fold higher V_{max} for efflux than for exchange, only about 40% of the difference reported previously. Together with other results in the literature, the accelerated net flux indicates that the conformational change of the loaded transporter from its outward-facing to its inward-facing form is likely the slowest step in the transport cycle, in contrast to the case for GLUT1. Experiments at 25°C indicate a lower degree of accelerated net flux than at 37°C. This is consistent with the above conformational change being the step with the lowest activation energy, as for GLUT1.

Key words: Glucose transport; Kinetics; GLUT1; GLUT4; Transport model; (Rat adipocyte)

1. Introduction

In a study of 3-*O*-methylglucose transport in rat adipocytes, Vinten [1] reported that the K_m and V_{max} were about 2.8-fold higher for net (zero-*trans*) efflux than for equilibrium exchange. As will be discussed, this has interesting implications for the relative sizes of various rate constants in the transport cycle, and how they compare to those for glucose transport in human erythrocytes. It is now known that transport in these two types of cells is catalyzed by two distinct isoforms, GLUT1 and GLUT4. Erythrocytes contain GLUT1 and adipocytes contain both types; however, in insulin-stimulated adipocytes, the transport activity is mostly from GLUT4 [2]. The amino acid sequences of GLUT1 and GLUT4 are known, and other information on the structures of these two proteins is being obtained from various lines of research. Therefore, it is of interest to compare their kinetic and regulatory properties, and eventually to try to account for any differences at the molecular level.

To understand the implications of the observations of Ref. 1, it is necessary to review some other studies of methylglucose transport in adipocytes. Several of these have found similar kinetic parameters for net (zero-*trans*) uptake and for equilibrium exchange. Whitesell and Gliemann [3] observed similar K_m values (about 3.5 mM) for net and exchange uptake in insulin-stimulated cells at 22°C, and the time courses of uptake at 20 mM methylglucose were consistent with similar parameters for the two processes. Taylor and Holman [4] observed K_m values of 6.1 mM for uptake and 4.4 mM for exchange in the case of insulin-stimulated cells at 37°C. May and Mikulecky [5] determined K_m values of about 10 mM for both net uptake and exchange in the case of basal cells at room temperature, while insulin-stimulated cells had similar initial rates at 20 mM methylglucose, also indicating similar parameters. Toyoda et al. [6], in experiments at 37°C, observed a decrease in K_m values from about 10 mM

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to about 5 mM upon insulin treatment. However, for both basal and insulin-stimulated cells the K_m values were similar for net uptake and exchange. Thus, in general these studies support the idea that net uptake has kinetic parameters that are roughly equal to those for exchange, both at room temperature and at 37°C. For insulin-stimulated cells, the reported K_m values are about 4–6 mM for both temperatures.

In contrast, glucose transport in erythrocytes shows faster exchange than net uptake. This accelerated exchange is, however, strongly temperature-dependent. Exchange has about 80-fold higher K_m and V_{max} values than net uptake at 0°C and about 8-fold higher values at 20°C (data summarized in Ref. 7). At 37°C, the K_m and V_{max} values for exchange were reported to be 30–100% higher than those for net uptake [8].

For a passive transporter showing Michaelis-Menten kinetics, the ratio of K_m values for the two directions of flux must be the same as the ratio of V_{max} values [10]. This ratio will be referred to as the *degree of asymmetry*. (Conversely, a symmetrical transporter will have the same parameters for the two directions of flux.) The erythrocyte transporter displays asymmetry of zero-*trans* flux, with efflux having K_m and V_{max} values that are higher than those for uptake but lower than those for exchange (except at high temperatures). The asymmetry is temperature-dependent, being about 20-fold at 0°C and 4-fold at 20°C [7]. At 38°C, efflux has slightly (7–22%) higher parameters than exchange [9]. Taken together, the uptake, efflux, and exchange results of Refs. 8 and 9 indicate an asymmetry of less than 2-fold at 37–38°C.

With respect to possible asymmetry in adipocyte glucose transport, Taylor and Holman [4] reported a higher K_m value for net uptake of methylglucose (6.1 mM) than for efflux (2.7 mM) in the case of insulin-stimulated cells at 37°C. However, the reported V_{max} values were nearly identical, indicating that there was some type of experimental error in these measurements. For basal cells, the same study also reported a higher K_m for uptake (5.4 mM) than for efflux (4.1 mM), but here there was an even greater discrepancy between the ratio of K_m values and the ratio of V_{max} values.

Vinten [1], on the other hand, reported that for insulin-stimulated adipocytes at 37°C, net efflux had about 2.8-fold higher K_m (14.8 mM) and V_{max} values than equilibrium exchange ($K_m = 5.2$ mM). Taken together with the similarity of parameters for uptake and exchange reported in Refs. 4 and 6, these results indicated that methylglucose transport in insulin-stimulated adipocytes at 37°C is asymmetric, with nearly 3-fold higher kinetic parameters for efflux than for uptake and exchange. While the higher parameters for efflux than for uptake would be similar to glucose transport in erythrocytes at low temperatures, the magnitude of the asymmetry would be greater than that found in erythrocytes at about 37°C. In addition, the higher parameters for efflux than for exchange would be a feature that occurs slightly, if at all, in erythrocytes near 37°C, and which is opposite of the case for erythrocytes at lower temperatures.

As part of the present investigation, the results of Vinten [1], together with other results from adipocytes and erythrocytes, were analyzed according to the one-site alternating conformation model (carrier model) (see Appendix). This model can account for most of the kinetic results obtained for glucose transport in erythrocytes [7], although it remains controversial (e.g., Ref. 11). The analysis revealed that different steps in the transport cycle are rate-determining for glucose uptake in erythrocytes (GLUT1) and in insulin-stimulated adipocytes (mostly GLUT4). Because of the interesting apparent differences in the kinetic features of the two transporters, we attempted to confirm the larger kinetic parameters for net efflux than for exchange reported in Ref. 1. Our results also indicate an accelerated net efflux at 37°C, although of lower magnitude than reported previously. Since glucose transport in erythrocytes shows large changes in asymmetry and exchange acceleration with temperature, we also compared net and exchange efflux at 25°C.

2. Materials and methods

2.1. Preparation of cells

Sprague-Dawley rats of 170 to 350 g were employed; similar results were obtained with rats of various weights in this range. The rats were decapitated following sedation with 40% CO_2/60% O_2. Adipocytes were prepared from the epididymal fat pads as described by Weber et al. [12], using Type I collagenase from Cooper Biomedical. The buffer for digestion and cell incubations was Krebs Ringer bicarbonate Hepes buffer [12] with 1% bovine serum albumin (Fraction V, Armour or Sigma) and 2 mM pyruvate [6]. Cells were suspended to 30% (v/v) in buffer containing 9 nM insulin (gift of Dr. R. Chance, Eli Lilly).

2.2. Assay procedures

Efflux assays were performed using modifications of the procedures used in previous studies [3,5–6]. Aliquots (39 μl) of cell suspension were added to 12×55 mm polystyrene tubes and incubated in a water bath at the assay temperature for 20 min, after which 9 μl of sugar solution was added. This solution contained 3-*O*-[*methyl*-^{14}C]-methyl-D-glucose and L-[1-^{3}H]glucose (DuPont NEN), and nonradioactive methylglucose (Sigma) at four times the final concentration, in albu-

min-free buffer. The cells were allowed to equilibrate with this solution for 30 min before the assays were performed.

To initiate efflux of methylglucose, tubes were held on a vortex mixer set at 600 rpm and 0.36 or 0.9 ml of efflux solution was added; this resulted in a 10.9- or 25.8-fold dilution of the extracellular water. The efflux solution was either albumin-free buffer (net efflux) or buffer containing nonradioactive methylglucose at the same final concentration as in the 30 min incubation (exchange efflux). To stop the efflux, 2 ml of albumin-free buffer containing 0.3 mM phloretin was added; this stopping solution was kept at room temperature. For zero time points, stopping solution was added before efflux solution. For time points of 5 s or longer, tubes were returned to the water bath between the initiation and the stopping of efflux.

Assays were done in groups of four. After completion of a group of four assays, 0.5 ml dinonyl phthalate (ICN) was added to each tube. The tubes were then spun for 30 s in a clinical centrifuge. Since the cells are less dense than the oil, which in turn is less dense than water, the centrifugation results in clumps of cells floating above the oil layer. These were removed with transfer pipets and transferred to counting vials. Scintillation fluid (6 ml) was added to each vial and the radioactivity determined.

The L-glucose counts were used to correct the methylglucose counts for variations in the extracellular water carried along with the cells [5], giving the radioactive methylglucose contained within the cells. Efflux was defined as the decrease in the intracellular content from the content at time zero. Complete equilibration of the methylglucose was estimated from the efflux in 20 min. Efflux values at earlier time points are expressed as fractions of complete equilibration.

2.3. Model values of initial rates and time courses

The one-site alternating conformation model (see Appendix) was used to calculate the transport rates and time courses for a given set of kinetic parameters under various experimental conditions. Three types of simulations were performed. For each, it was assumed that net uptake and exchange have the same kinetic parameters, as indicated by previous studies [3,5–6]. In the first type of simulation, it was assumed that there was no asymmetry (i.e., efflux also had the same parameters). In the second, a 2.78-fold asymmetry (the ratio of the V_{max} for efflux compared to that for exchange reported by Vinten [1]) was used; this gave the ratio of parameters for efflux to those for uptake and exchange. In the third, the asymmetry was varied to optimize the fits to the data.

For equilibrium exchange, the extracellular radioactive methylglucose concentration was 9.2% or 3.9% of the initial intracellular methylglucose concentration for the 10.9- or 25.8-fold dilutions, respectively. This means that similar fractions of the exchange efflux would not be experimentally detectable. Therefore, for initial rate calculations, the rates obtained from the Michaelis-Menten equation were multiplied by 0.908 or 0.961, respectively, to calculate the expected rates of loss of intracellular radioactive methylglucose. For time course simulations of exchange, a first-order approach to the equilibrium situation was assumed, using the Michaelis-Menten equation to calculate the rate constant for equilibration and setting the final intracellular concentration equal to 9.2% or 3.9% of its initial value.

For net efflux, the following equation (Eqn. 16 of Ref. 13) was used to calculate the unidirectional flux from outside (side 1) to inside (side 2):

$$u_{12} = \frac{KS_1 + S_1S_2}{K^2R_{00} + KR_{12}S_1 + KR_{21}S_2 + R_{ee}S_1S_2}$$

In this equation, S_1 and S_2 are the sugar concentrations on side 1 and side 2, respectively (S_1 is 9.2% or 3.9% of the initial S_2 value). The parameters R_{00}, R_{12}, R_{21}, and R_{ee} are defined in the Appendix, while K is the affinity of the transporter for sugar at very low sugar concentrations [13]. A similar equation, obtained by substituting 'S_2' for 'S_1' in the first term in the numerator, gives the unidirectional flux from inside to outside. The net flux is the difference between the two unidirectional fluxes. Time courses were simulated by applying the equation iteratively, using intervals of 0.05 s and adjusting the internal concentrations with each iteration. Use of smaller intervals did not affect the results significantly.

2.4. Fitting of model parameters to experimental data

Two types of fits were made. In the first, the initial rates of efflux were estimated. For 5 and 20 mM methylglucose, the earliest measured time points were used directly. For 50 mM methylglucose, the time courses were assumed to be approximated by first-order approaches to equilibrium, and the initial rates were estimated from the 10 and 20 s points. The mean initial rates of net and exchange efflux were determined for each concentration and extent of dilution (11- or 26-fold), and the ratios (net/exchange) calculated from these means. The model parameters (asymmetry and/or exchange K_m) were then adjusted to optimize the fit of the predicted to the observed ratios.

The second type of fit was to the time courses of uptake. For the experiments at 37°C, the 50 mM methylglucose data were employed; for the experiments at 25°C, all of the time points were used. The exchange V_{max} values were adjusted, in addition to the asymmetry and exchange K_m values, in optimizing the fits to the data.

For each type of fitting, a simple computer program in the BASIC language was employed. Each program accepted the exchange K_m and asymmetry (both types of fit), as well as the exchange V_{max} (fits of time course data), as inputs, and then calculated the model value (ratio of initial rates or extent of efflux) for each experimental condition as described above. Each calculated value was then compared to the corresponding experimental value, and the difference squared and weighted according to the number of experimental determinations. The calculated model values and errors, as well as the sum of the weighted squares of the errors, were reported as outputs of the program. The input parameters were adjusted manually to minimize the sum of the weighted squares of the errors.

3. Results

3.1. Net and exchange efflux at 37°C, 10.9-fold dilutions

Net efflux and equilibrium exchange efflux were measured for insulin-stimulated rat adipocytes at 37°C, the conditions for most of the experiments reported by Vinten [1]. We initially used a procedure that resulted in a 10.9-fold dilution of the extracellular medium. Three concentrations of 3-*O*-methylglucose (5, 20, and 50 mM) were tested. Results from these experiments are listed in the first section of Table 1. For 5 and 20 mM methylglucose, two time points (at which about 20% and 40% of the intracellular sugar had left the cells) were examined. For both concentrations, very similar extents of efflux were seen for net and exchange efflux. The ratios of net to exchange efflux did not differ greatly from 1.0. For 50 mM methylglucose, the time courses were examined for 40 s, during which about 60% of the intracellular methylglucose exited. Again, the ratios of net to exchange efflux were very close to 1.0 for the three time points tested.

The parameters reported by Vinten [1] predict that the initial rates of net efflux should be about 1.4-, 2.0-, and 2.4-fold faster than those for exchange at 5, 20, and 50 mM methylglucose, respectively. Therefore, it would appear that the data conflict with these predictions. However, when we analyzed the experimental situations using the one-site alternating conformation model (see Appendix), it became apparent that the residual extracellular methylglucose should significantly retard the rate of net efflux, compared to zero-*trans* conditions. This reduces the predicted net/exchange ratios to about 1.3, 1.5, and 1.3 for 5, 20, and 50 mM methylglucose, respectively. The experimental values still conflict with these predictions.

3.2. Net and exchange efflux of 50 mM methylglucose at 37°C, 25.8-fold dilution

To reduce the complicating effect of the extracellular methylglucose in the interpretation of the results, we carried out further experiments at 50 mM methyl-

Table 1
Net and exchange efflux of 3-*O*-methylglucose from insulin-stimulated rat adipocytes

[3OMG] (mM)	Time (s)	Equilibration		Net/exchange	Model fit	
		net	exchange		net	exchange
37°C, 10.9-fold dilution						
5	1	0.19 ± 0.05	0.26 ± 0.06	0.73 ± 0.26		
	3	0.42 ± 0.15	0.40 ± 0.04	1.05 ± 0.39		
20	5	0.25 ± 0.13	0.28 ± 0.13	0.89 ± 0.62		
	10	0.54 ± 0.08	0.46 ± 0.10	1.17 ± 0.31		
50	10	0.21 ± 0.07	0.25 ± 0.05	0.84 ± 0.33	0.19	0.23
	20	0.37 ± 0.10	0.38 ± 0.13	0.97 ± 0.42	0.37	0.40
	40	0.66 ± 0.07	0.66 ± 0.03	1.00 ± 0.12	0.68	0.64
37°C, 25.8-fold dilution						
50	10	0.32 ± 0.07	0.21 ± 0.05	1.52 ± 0.49	0.27	0.23
	20	0.51 ± 0.13	0.40 ± 0.11	1.28 ± 0.48	0.52	0.40
25°C, 10.9-fold dilution						
5	3	0.25 ± 0.05	0.26 ± 0.03	0.96 ± 0.22		
	5	0.41 ± 0.02	0.38 ± 0.06	1.08 ± 0.18		
20	10	0.24 ± 0.10	0.29 ± 0.07	0.83 ± 0.40		
	15	0.56 ± 0.08	0.47 ± 0.05	1.19 ± 0.21		

Adipocytes were loaded with the indicated concentration of 3-*O*-[^{14}C]methylglucose (3OMG). The efflux of the radioactive methylglucose was measured into medium containing no methylglucose (net efflux) or nonradioactive methylglucose at the same concentration (exchange efflux). The addition of the cell suspension to this medium resulted in either a 10.9- or 25.8-fold dilution of the extracellular loading medium, as indicated. Results in the first two columns are expressed as the fraction of complete equilibration (determined from the extent of efflux in 20 min), while the third column gives the ratio of net to exchange efflux. Results are means ± S.D. of 3 to 5 determinations. The last two columns give the best fit values for the 50 mM time course data, using the one-site alternating conformation model and the an exchange K_m of 4.06 mM, an exchange V_{max} of 1.40 mM/s, and an asymmetry of 1.66-fold.

glucose using a 25.8-fold dilution of the extracellular medium. Results are listed in the second section of Table 1. In contrast to the data obtained at 50 mM methylglucose and a 10.9-fold dilution, net efflux in these experiments exceeded exchange efflux by 1.52-fold at 10 s and 1.28-fold at 20 s. These results indicate that net efflux is accelerated with respect to exchange. However, the size of the effect was smaller than the 1.7-fold difference in initial rates predicted by the parameters of Vinten [1] when the residual extracellular methylglucose is taken into account.

We compared these results to the time course of efflux of 50 mM methylglucose reported in Fig. 5 of Ref. 1. While the two sets of exchange values were in good agreement, the net efflux that we observed was much lower than that previously reported. Some, but not all, of this difference can be accounted for by the difference in extracellular methylglucose (which was about 2 mM in our experiments and less than 1 mM in Ref. 1).

3.3. Fits of initial rate data to model

The data from the experiments listed in Table 1 were compared to the predictions of the one-site alternating conformation model to determine what degree of accelerated net flux could best account for the results (assuming that net uptake and exchange have similar kinetic parameters, this acceleration is also equal to the asymmetry of net flux). Two types of fits were made, as described in Materials and methods. In the first, the predicted ratios of net to exchange efflux under initial rate conditions were employed.

Results of this type of fit, with various constraints on the model parameters, are listed in the first section of Table 2. Fig. 1 compares the predictions of the fits to the observed ratios of initial rates (center bar of each group). For the case of symmetric transport (no accelerated net efflux), the fitting procedure did not converge; the goodness of fit continued to improve as the exchange K_m was increased beyond reasonable levels. Therefore, Fig. 1 plots the predicted values using the previously reported exchange K_m of 5.15 mM [1] (first bar in each group). While these were consistent with the 5 and 20 mM data, they were clearly much lower than the observed ratios at 50 mM, especially at the 26-fold dilution. The ratios calculated from the parameters of Ref. 1 are shown as the fifth bar of each group; only in the last experimental situation did these parameters provide a good fit to the data. When the 2.78-fold asymmetry of these parameters was retained but the exchange K_m was allowed to vary, the best fit was obtained with a K_m of 2.45 mM (fourth bar of each group); the calculated ratios fit the experimental data well in two of the four situations.

Setting the K_m at 5.15 mM and varying the asymmetry gave an optimal fit at a value of 1.98 for the latter (not shown). When both the asymmetry and K_m were varied, a slightly better fit was achieved for an asymmetry of 1.79-fold and a K_m of 8.21 mM (second bar of each group). This provided a calculated ratio within the standard deviation of the observations in three of the four situations. The sum of the weighted squares of the errors (Table 2, last column) was, for this fit, 1.8-fold lower than for the 2.78-fold asymmetry and K_m of 2.45 mM, 2.8-fold lower than for the parameters of Ref. 1, and 4.8-fold lower than for the symmetric parameters with $K_m = 5.15$ mM. Thus, the initial rate data support accelerated net exchange and asymmetry of transport, but with a lower degree of acceleration (1.8-fold) than reported previously (2.8-fold, Ref. 1).

Table 2
Fits of model parameters to experimental data

Parameter			Relative error
K_{ec} (mM)	V^{ec} (mM/s)	Asymmetry	
37°C, ratios of initial rates			
8.21		1.79	1.00
(5.15)		1.98	1.07
2.45		2.78	1.75
(5.15)		(2.78)	2.85
(5.15)		(1.00)	4.84
*		(1.00)	–
37°C, time courses			
4.06	1.40	1.66	1.00
(5.15)	1.41	1.54	1.12
1.64	1.30	(2.78)	1.87
15.9	1.80	(1.00)	3.20
(5.15)	1.03	(2.78)	9.62
25°C, ratios of initial rates			
6.60		1.08	1.00
9.19		(1.00)	1.91
*		(2.78)	–
25°C, time courses			
5.75	1.01	1.24	1.00
6.68	1.13	(1.00)	1.13
4.10	0.70	(2.78)	2.62

Parameters of the one-site alternating conformation model were fit to the data as described in Materials and methods. K_{ec} and V^{ec} are the K_m and V_{max}, respectively, for equilibrium exchange. In most cases one or two of the parameters were fixed; these values are indicated in parentheses. The sum of the weighted squares of the errors is listed in the last column, normalized to that of the best fit when all parameters were adjustable. For ratios of initial rates, the ratio is independent of V^{ec}, so this was not an adjustable parameter.
* Fit did not converge.

3.4. Fits of time course data to model

The second type of fit was to the 50 mM time course data. Here, the V_{max} for exchange was varied, in addition to the K_m and the asymmetry (for the calculations described above, the V_{max} does not affect the ratio of initial rates at a given methylglucose concentration).

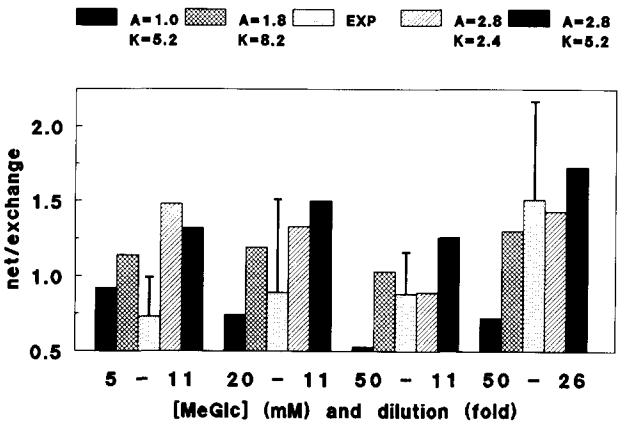


Fig. 1. Comparison of initial rate data to model predictions. Ratios of initial rates of net efflux to those for exchange observed in the experiments listed in the first two sections of Table 1 are compared to those calculated for the one-site alternating conformation model, using various values for the asymmetry of net flux and the exchange K_m. The four experimental conditions (methylglucose concentration and degree of dilution of extracellular methylglucose) are displayed on the x-axis, while the net/exchange ratios are plotted against the y-axis. For each experimental condition, the five bars represent, from left to right, symmetrical net flux, $K_m = 5.15$ mM; the best fit when both asymmetry (1.79) and K_m (8.21 mM) were varied; the experimental results, with standard deviations shown; the best fit when the asymmetry was set to 2.78 and K_m (2.45 mM) was varied; and ratios predicted by the parameters of Ref. 1 (asymmetry = 2.78 and K_m = 5.15 mM).

Results are listed in the second section of Table 2. As with the initial rate data, the best fit was obtained when both the exchange K_m and asymmetry were varied. The optimum value of the asymmetry (1.66-fold) was in good agreement with that determined from the initial rates (1.79-fold); the K_m and V_{max} were 4.06 mM and 1.40 mM/s. The values listed in the last two columns of Table 1 are the time courses calculated using these parameters; these give a good fit to the experimental data. The sum of the weighted squares of the errors was, for this fit, 12% lower than when the K_m was fixed at 5.15 mM and the asymmetry varied (which had optimal parameters of 1.56-fold asymmetry and 1.41 mM/s), about 1.9-fold lower than when the asymmetry was fixed at 2.78-fold (optimum parameters, 1.64 mM and 1.30 mM/s), and 3.2-fold lower than for a symmetric fit (optimum parameters, 15.9 mM and 1.80 mM/s). Moreover, the K_m value of the best fit (4 mM) was in much better agreement with the 5 mM value determined in Ref. 1 than the fits to asymmetries of 1.0- or 2.78-fold. Thus, as with the initial rate data, the fit of the time course data supports accelerated net efflux, but to a lower degree than reported previously.

In analyzing time course data, Vinten [1] found that the cells appeared to be inhomogeneous. Assuming that the population of cells had a log normal distribution of maximum velocities gave a much better fit to the data of Ref. 1 than was given by a uniform population. Accordingly, we modeled the time courses for cells with a similar distribution of velocities. Again, the best fit was obtained when the asymmetry was varied; the optimum value of the asymmetry was 1.59, similar to that obtained assuming a homogeneous population. However, the sum of the weighted squares of the errors was 1.5-fold lower for the homogeneous model than for the log normal distribution.

3.5. Net and exchange efflux at 25°C

As noted in the Introduction, glucose transport in erythrocytes (catalyzed by GLUT1) shows much greater asymmetry and exchange acceleration as the temperature is decreased below 37°C. Therefore, it was of interest to compare net and exchange efflux in insulin-stimulated adipocytes (catalyzed primarily by GLUT4) at 25°C in addition to 37°C. Net and exchange efflux of 5 and 20 mM methylglucose were studied using a 10.9-fold dilution of the extracellular medium. Results are listed in the third section of Table 1. As for the corresponding experiments at 37°C (Table 1, first section), the ratios of net to exchange efflux were very close to 1.0 at all time points examined. These results suggest that the relationships among the kinetic parameters are less temperature sensitive in rat adipocytes than in human erythrocytes.

A series of fits to the one-site alternating conformation model was made, similar to those done for the experiments at 37°C. Again it was assumed that uptake and exchange had similar parameters, as indicated by previous studies [3,5]. In the case of initial rates, the best fit was obtained with an asymmetry of 1.08 and an exchange K_m of 6.6 mM (Table 2, third section). When the asymmetry was fixed at 2.78, the fit was poor and did not converge at a plausible value for the K_m. In the case of the time course data, the best fit was obtained for an asymmetry of 1.24 and a K_m of 5.8 mM (Table 2, fourth section). This was only slightly better than for the best fit with no asymmetry, but had a 2.6-fold lower sum of the weighted squares of the errors than when the asymmetry was set at 2.78. While the 25°C data were less extensive than those at 37°C, these results indicate that the asymmetry is, if anything, smaller at the lower temperature. This is in contrast to the situation in erythrocytes, where the asymmetry is larger at lower temperatures.

4. Discussion

The results presented here confirm qualitatively the observation by Vinten [1] that for insulin-stimulated adipocytes at 37°C, net efflux of methylglucose is faster than exchange efflux. However, the degree of net flux acceleration that we observed (about 1.7-fold) is much

smaller than previously reported (about 2.8-fold). The reason for this difference is unknown; possibly it could be due to differences in the strains or sizes of the rats used, or to the different experimental procedures employed. It should be noted, however, that the time course of exchange efflux at 50 mM methylglucose which we observed was in very good agreement with that reported in Ref. 1 despite the much different techniques used in the two studies.

The procedure used in Ref. 1 had the advantage of being able to dilute the extracellular methylglucose more than 50-fold. In our experiments, it was necessary to consider the extracellular methylglucose, and to interpret the results using a model for the transport kinetics. However, in the case of the higher dilution employed, the accelerated net flux was apparent even without considering the model (second section of Table 1). Moreover, the use of two different dilution conditions, with resulting differences in extracellular methylglucose, provided additional information that could be used to evaluate the kinetics of the system.

Two different procedures were used to evaluate the degree of net flux acceleration indicated by our data. In one, model parameters were adjusted to optimize the fit to initial rates observed under different conditions. In the other, the parameters were fit to the time courses of efflux of 50 mM methylglucose for the two types of dilutions. The resulting optimal values of the acceleration (1.79-and 1.66-fold, respectively, for the two procedures) were in good agreement. Moreover, the exchange K_m values giving the optimal fits for these two procedures, 8.2 and 4.1 mM, respectively, were in reasonable agreement with values reported by others (about 5 mM [1,4,6]). Even if the exchange K_m were fixed at the value of 5.15 mM [1] rather than being an adjustable parameter, the best fit values for the net efflux acceleration (1.98- and 1.56-fold, respectively) were not greatly different, and were much lower than the previously reported value of 2.78-fold [1].

We examined the implications of these results for the kinetics of sugar transport in adipocytes and how they compare to the kinetics for human erythrocytes (Appendix). Using the apparent equality of net uptake and exchange reported previously [4,6], and assuming rapid binding and dissociation of the methylglucose, various limits can be placed on the rate constants for the conformational changes of the transporter. Table 3 lists the possible ranges of the rate constants for the parameters of Ref. 1 (third section of the table), as well as those deduced from our experiments (fourth section of the table). The possible ranges of rate constants for human erythrocyte glucose transport at 20°C (first section) and 37–38°C (second section) are also listed.

This analysis reveals different patterns for sugar transport in erythrocytes (catalyzed by GLUT1) and in insulin-stimulated adipocytes (catalyzed primarily by GLUT4). For glucose transport in erythrocytes, the conformational change of the unloaded transporter from inward- to outward-facing (whose rate constant is k_2) is clearly the slowest step at 20°C. This step is rate-determining for zero-*trans* uptake. The conformational change of the loaded transporter from outward- to inward-facing (whose rate constant is g_1) is at least 7.6-times as large. At 37–38°C, k_2 is also probably the smallest rate constant, although it is possible that it could be slightly greater than those for the inward movement of the loaded (g_1) and unloaded (k_1) transporter forms. However, g_1 would become rate-limiting for zero-*trans* uptake only if g_2 exceeded the other three rate constants by at least 4-fold.

Table 3
Possible ranges of rate constants for one-site alternating conformation model

Cell and temperature	Rate constant			
	g_1	g_2	k_1	k_2
Erythrocytes 20°C	8.6	∞	4.1	1.1
	∞	8.6	7.9	1.0
Erythrocytes 37–38°C	1.6	∞	1.9	2.5
	2.0	9.4	2.4	2.0
	13.0	1.9	∞	1.1
Adipocytes 37°C	1.0	∞	2.8	∞
Asymmetry = 2.78	1.6	2.8	∞	2.8
Adipocytes 37°C	1.0	∞	1.7	∞
Asymmetry = 1.72	2.0	2.0	10.5	2.0
	2.4	1.7	∞	1.7

The relationships between the rate constants, and their possible ranges, are derived in the Appendix. Each line of the table represents a possible combination of the four rate constants. These give the range of possible values of the constants as g_1 is varied from its minimum to its maximum value. In addition, for those cases in which it is possible for g_1 to be either greater or less than k_2, the conditions at which these two rate constants are equal are also listed. Each set of rate constants has been normalized to a value of 1.0 for the V_{max} for zero-*trans* uptake. Values for human erythrocytes are based on literature results. Values for insulin-stimulated rat adipocytes are based on the results of Ref. 1 (asymmetry = 2.78) or on the data of Table 1 (asymmetry = 1.72).

Compared to glucose transport in erythrocytes, the rate constants for methylglucose transport in adipocytes at 37°C show an increase in k_2 relative to g_2 and an increase in k_1 relative to g_1. The analysis based on the parameters of Ref. 1 shows that g_1 is the smallest rate constant; it, rather than k_2, is rate-determining for the physiologically relevant process of net uptake. The other rate constants are at least 1.8-fold larger. In addition, since $g_2 > g_1$, the loaded transporter will be predominantly in the outward-facing conformation.

The analysis based on our results yields a similar pattern. While it remains likely that g_1 is the smallest rate constant, it is possible that it is slightly larger than k_2 and g_2, but only if k_1 is much larger than the other rate constants. Similarly, the loaded transporter is likely

to be predominantly outward facing, unless k_1 is relatively large.

Thus, our results and those reported previously [1] indicate that the rate-determining steps for glucose uptake in human erythrocytes and insulin-stimulated rat adipocytes are likely to be different. However, this conclusion is based on data using different sugar substrates for the two types of transporters. It might be argued that conformational changes for transporters binding methylglucose, the nonphysiological substrate, could be slower than when glucose is bound. This could lower the magnitudes of g_1 and g_2 relative to k_1 and k_2, such that g_1 becomes rate-determining for uptake rather than k_2.

This possibility appears to be ruled out on the basis of literature results employing glucose with adipocytes and methylglucose with erythrocytes. For the former situation, Okuno and Gliemann [14] observed nearly identical V_{max} values for zero-*trans* uptake of glucose and methylglucose in the case of insulin-stimulated adipocytes at 37°C. While there might be concern that phosphorylation rather than transport of glucose could be rate-determining, the experiments were performed under initial rate conditions (with uptake of glucose less than 25% of the distribution space for methylglucose). Moreover, the slightly lower K_m for methylglucose (3.3 mM) compared to glucose (4.2 mM) is in agreement with the relative affinities of the transporter for the two sugars as assessed by inhibition of tracer glucose flux [3]. Since the V_{max} for zero-trans uptake is determined by g_1 and k_2, and k_2 is independent of sugar, these results imply that g_1 is similar for the two sugars.

For the latter situation, Baker and Widdas [15] described the results of various types of transport experiments using methylglucose with human erythrocytes at 16°C. The V_{max} for equilibrium exchange (determined by g_1 and g_2) was reported to be 12–38% lower for methylglucose than for glucose. This suggests that the conformational change of the transporter may be somewhat slower when methylglucose rather than glucose is bound. However, an analysis of the type described in the Appendix indicates that the relative sizes of the rate constants are similar to those for transport of glucose by erythrocytes at 20°C: the smallest rate constant is k_2, and both g_1 and g_2 are at least 10-to 20-fold greater than k_2. Taken together with the adipocyte results [14], these studies indicate that the conclusion that GLUT1 and GLUT4 show different rate-determining steps for net uptake is independent of whether glucose or methylglucose is assayed.

Possibly the different kinetic patterns of the two transporters result from differences in their carboxyl-terminal domains, which show the greatest divergence in the various glucose transporter forms. A recent study showed that replacing the carboxyl terminus of GLUT1 with that of GLUT2 gave a chimeric transporter with kinetic properties similar to GLUT2 [16].

In experiments using insulin-stimulated adipocytes near 22°C, Whitesell and Gliemann [3] found that both zero-*trans* and exchange uptake had similar K_m values (about 3.5 mM), while May and Mikulecky [5] observed similar initial rates of net and exchange uptake at 20 mM methylglucose. These data suggest that just as at 37°C, net uptake and exchange have similar kinetic properties near 22°C. We compared the net and exchange efflux of 5 and 20 mM methylglucose at 25°C, using a 10.9-fold dilution of the extracellular medium (Table 1, third section). The ratios of net to exchange efflux observed in these experiments were similar to those in the corresponding experiments at 37°C. Moreover, the best fits of the data to the model (Table 2, third and fourth sections) gave lower values of the asymmetry (which is equivalent to the net flux acceleration) than obtained at 37°C. Since the experiments at 25°C were not as extensive as those at 37°C, these conclusions are more tentative, but they are consistent with a similar or even lower degree of asymmetry at 25°C than at 37°C, the opposite of the situation for erythrocyte glucose transport.

In two previous studies [3,5], the time courses of zero-*trans* and equilibrium exchange uptake of 20 mM methylglucose were compared, using insulin-stimulated adipocytes at room temperature. Assuming equal kinetic parameters for these two processes, we could not obtain a good fit to the time course data of Ref. 5, Fig. 5, despite testing various values of the asymmetry and K_m. However, for the data of Ref. 3, Fig. 14, good fits to the data were obtained using a K_m of 3.5 mM [3] and asymmetry values of 1.0 or 1.72, but not using an asymmetry of 2.78. This supports the above conclusions from our results (Table 1) that the asymmetry at 20–25°C is similar to or less than that at 37°C.

Lowe and Walmsley [8] analyzed the temperature dependence of glucose transport in human erythrocytes, and concluded that step g_1 had the smallest activation energy, followed (in order) by g_2, k_1, and k_2. The increase in k_2 relative to k_1 decreases the asymmetry at higher temperatures, and the increase in k_1 relative to g_1 and g_2 increases the rate of net efflux compared to exchange. However, in adipocytes, the observation that net uptake and exchange have kinetic parameters that are similar to each other at both room temperature and at 37°C indicates that k_2 and g_2 are similar in magnitude at both temperatures, and thus have similar activation energies. In erythrocytes, k_2 was estimated to have an activation energy twice that of g_2.

Our results indicate that the accelerated net efflux may be slightly lower at 25°C than at 37°C. A lower accelerated net efflux with lower temperature implies that k_1 (required for net efflux) has a higher activation

energy than g_1 (required for exchange; both processes require the step with rate constant g_2). This is the same relative order of activation energies as deduced for erythrocytes [8].

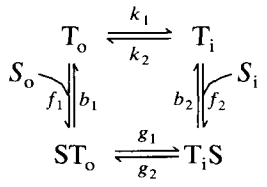
A lower accelerated net efflux at 25°C than at 37°C also implies a reduced directional asymmetry at the former temperature. Since g_1 is likely rate-determining for net uptake (Table 3), while k_1 and/or g_2 is rate-determining for efflux, this would mean that k_1 and/or g_2 has a greater activation energy than g_1. This is also consistent with the relative activation energies in erythrocytes. This may seem contradictory, since directional asymmetry is *greater* in erythrocytes at lower temperatures. However, this difference arises because k_2 (which has the greatest activation energy in erythrocytes) is rate-determining for uptake in erythrocytes, whereas in adipocytes the uptake rate is probably determined by g_1 (which has the smallest activation energy in erythrocytes).

Thus, earlier studies indicate that the relative activation energies for the transport steps in GLUT1 and GLUT4 differ in that g_2 and k_2 appear to have similar activation energies in GLUT4. However, our results indicate that g_1 may have the lowest activation energy for both transporter types.

In summary, our results, combined with others in the literature, indicate different patterns of both the relative sizes of the rate constants of the transport cycle, and their relative activation energies, for GLUT1 and GLUT4. Further studies of the structures of these proteins may identify the sources of these differences at the molecular level.

5. Appendix: relationships between rate constants of one-site alternating conformation model

The one-site alternating conformation model for sugar transport is shown below:



In this scheme, T_o and T_i represent the outward- and inward-facing conformations of the transporter, respectively; S_o and S_i extracellular and intracellular sugar, respectively; and ST_o and T_iS the outward- and inward-facing conformations with sugar bound, respectively. The rate constants are given the same designations as in Eilam and Stein [13], if side 1 is taken to be the outside and side 2 the inside of the cell.

The maximum velocities for zero-*trans* uptake, zero-*trans* efflux, and equilibrium exchange are designated V_{12}^{zt}, V_{21}^{zt}, and V^{ee}, respectively; the reciprocals of these values are designated R_{12}, R_{21}, and R_{ee}, respectively.

The values of these parameters in terms of the rate constants are:

$$R_{12} = \left(\frac{1}{b_2} + \frac{1}{g_1} \frac{(b_2 + g_2)}{b_2} + \frac{1}{k_2} \right) / T \quad (1)$$

$$R_{21} = \left(\frac{1}{b_1} + \frac{1}{g_2} \frac{(b_1 + g_1)}{b_1} + \frac{1}{k_1} \right) / T \quad (2)$$

$$R_{ee} = \left(\frac{1}{b_1} + \frac{1}{b_2} + \frac{1}{g_1} \frac{(b_2 + g_2)}{b_2} + \frac{1}{g_2} \frac{(b_1 + g_1)}{b_1} \right) / T \quad (3)$$

In the discussion that follows, rates will be compared only within the same type of cell, and only for a given temperature. Therefore, the total concentration of transporter forms (T) can be set equal to 1. If the assumption of rapid dissociation of glucose or methylglucose is made, such that b_1 and b_2 are very large compared to the other rate constants, the above equations simplify to

$$R_{12} = \frac{1}{g_1} + \frac{1}{k_2} \quad (4)$$

$$R_{21} = \frac{1}{g_2} + \frac{1}{k_1} \quad (5)$$

$$R_{ee} = \frac{1}{g_1} + \frac{1}{g_2} \quad (6)$$

In the remaining calculations, V_{max} values will be normalized to the smallest value for each set of conditions, which is that for zero-*trans* uptake. Therefore, $V_{12}^{zt} = 1$ and $R_{12} = 1$. Four sets of conditions will be considered:

Case 1: human erythrocytes at 20°C. A fit of literature data to the model is presented in Table 3 of Ref. 7; more recent results for zero-*trans* efflux and equilibrium exchange [17] are in good agreement with this fit. The model parameters give $R_{21} = 0.243$ and $R_{ee} = 0.117$.

Case 2: human erythrocytes at 37–38°C. Lowe and Walmsley [8] reported about a 1.3-fold higher V_{max} and a 2.0-fold higher K_m for exchange than for zero-*trans* uptake. Theoretically these two ratios should be the same, so their average (1.65) will be used, giving $R_{ee} = 1 : 1.65 = 0.606$. Brahm [9] reported a 1.07-fold higher V_{max} and 1.22-fold higher K_m for efflux vs. exchange. Taking the average of these (1.145) as the ratio of efflux to exchange parameters and multiplying by 1.65 gives 1.89 as the ratio of efflux to uptake. Then $R_{12} = 1 : 1.89 = 0.529$.

Case 3: insulin-stimulated rat adipocytes at 37°C, 2.78-fold asymmetry. Because similar parameters for net uptake and exchange were observed by Taylor and Holman [4] and Toyoda et al. [6], R_{ee} will be set equal to R_{12}, which has been defined to be 1. Using the 2.78-fold higher value of the V_{max} for efflux compared to exchange reported by Vinten [1] gives $R_{21} = 1:2.78 = 0.360$.

Case 4: insulin-stimulated rat adipocytes at 37°C, 1.72-fold asymmetry. R_{ee} is set equal to 1 as above. Using the experimental results of this paper, the best fit values of the asymmetry were 1.79 (initial rate data) and 1.66 (time course data); taking the mean of these (1.725) gives $R_{21} = 1:1.725 = 0.580$.

For each of the four cases, there are four unknowns (g_1, g_2, k_1, and k_2) but only three equations (for R_{12}, R_{21}, and R_{ee}). Thus, unique solutions for the sets of rate constants cannot be found without additional information. However, specifying one of these rate constants determines the other three. As will now be shown, a minimum value can be established for each rate constant, and in some cases a maximum value can be determined as well.

Since all the rate constants are positive numbers, each reciprocal rate (or resistance) term in Eqns. 4–6 must be less than the corresponding reciprocal V_{max} (R) value. In other words, each rate constant must be at least as large as any V_{max} value that it partly determines. This yields the following limits:

Case 1: g_1 and $g_2 > 8.55$; $k_1 > 4.12$; $k_2 > 1$
Case 2: $g_1 > 1.65$; g_2 and $k_1 > 1.89$; $k_2 > 1$
Case 3: g_1 and $k_2 > 1$; g_2 and $k_1 > 2.78$
Case 4: g_1 and $k_2 > 1$; g_2 and $k_1 > 1.72$

Further limits can be deduced by comparing Eqn. 6 to either Eqn. 4 or 5. For cases 3 and 4, the condition that $R_{ee} \approx R_{12}$, combined with Eqns. 4 and 6, indicates that $k_2 \approx g_2$ (unless both are so large that they have negligible effect on determining the V_{max} values). Together with the above limits, this means that $k_2 > 2.78$ or 1.72 for cases 3 and 4, respectively.

For cases 1 and 2, where $R_{12} > R_{ee}$, subtracting Eqn. 6 from Eqn. 4 gives

$$R_{12} - R_{ee} = \frac{1}{k_2} - \frac{1}{g_2}$$

which equals 0.883 or 0.394, respectively. Since $1/g_2$ is a positive number, this means that $1/k_2 > 0.883$ or 0.394, and $k_2 < 1.13$ or 2.54 for cases 1 and 2, respectively.

Similarly, for case 1, where $R_{21} > R_{ee}$, subtracting Eqn. 6 from Eqn. 5 gives

$$R_{21} - R_{ee} = \frac{1}{k_1} - \frac{1}{g_1}$$

which means that $1/k_1 > 0.126$ and $k_1 < 7.94$

For cases 2–4, where $R_{ee} > R_{21}$, subtracting Eqn. 5 from Eqn. 6 gives

$$R_{ee} - R_{21} = \frac{1}{g_1} - \frac{1}{k_1}$$

which equals 0.077, 0.640, or 0.420, respectively. Therefore, $g_1 < 13.0$, 1.56, or 2.38 for cases 2, 3, and 4, respectively.

Finally, for case 2, the Eq. above yields $1/g_1 > 0.077$; substituting in Eqn. 4 gives $1/k_2 < 0.923$ and $k_2 > 1.08$.

The possible ranges of rate constants are listed in Table 3.

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