A method is described which for the first time makes it possible to measure the initial velocity of uptake of the nonmetabolizable sugar analogue 3-O-methylglucose in adipocytes. The permeability of the rat adipocyte plasma membrane in the presence of very low concentrations of methylglucose (<<Km) was about 0.7 \times 10^{-6} \text{ cm s}^{-1} \text{ mm}^{-1} \text{ s}^{-1} both at 22°C and at 37°C. This corresponds to a half-time of uptake of about 18 s.

Insulin (1 \mu m) increased the permeability 3- to 6-fold at 22°C and 8- to 12-fold at 37°C. Insulin at 70 \mu m caused half of the maximal effect. The permeability due to nonmediated diffusion was approximately 2 \times 10^{-6} \text{ cm s}^{-1} \text{ mm}^{-1}. Thus, the permeability to 3-O-methylglucose is almost entirely accounted for by carrier-facilitated diffusion.

The following results were obtained at 22°C. The Km for 3-O-methylglucose equilibrium exchange (inside and outside sugar concentration being equal) was about 3.5 mM both in the absence and in the presence of insulin. Vmax was about 0.13 mmol s^{-1} \text{ l intracellular water}^{-1} and increased to about 0.8 mmol s^{-1} \text{ l intracellular water}^{-1} in the presence of insulin. The Km for net uptake (intracellular sugar concentration initially zero) in insulin-stimulated cells was 2.5 to 5 mM, suggesting the absence of any marked asymmetry of the transport system. The initial uptake of 3-O-methylglucose was inhibited by D-glucose with an inhibition constant of about 1 mM.

The hexose transport system in adipocytes has attracted much interest because it is markedly stimulated by insulin. Until now, it has not been possible to measure the transport of D-glucose directly due to the rapid metabolism of this sugar. Transport of D-glucose has been measured in adipocyte membrane vesicles, but this system is not stimulated by the addition of insulin to the vesicles (1, 2). However, several techniques are now available for the study of nonmetabolizable sugars or sugar analogues in adipocytes. Using modifications of the previously described oil technique (3) it has been possible to measure the rate of uptake of slowly transported sugars such as D-all0Se (4) or L-arabinose (5). However, these data were obtained using a metronome set at 120 beats/min and the incubation was terminated by the addition of 3 ml of albumin-free buffer containing (in mm) Na+, 140; K+, 4.7; \text{Ca}^{2+}, 2.5; \text{Mg}^{2+}, 1.25; \text{Cl}^{-}, 142; \text{H}_2\text{PO}_4^{-}/\text{H}_2\text{PO}_3^{-}, 2.5; \text{SO}_4^{2-}, 1.25; and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), 10. The concentration of bovine serum albumin was 10 mg/ml (unless indicated otherwise), and pH was 7.4. Collagenase (type I) was from Worthington, pig insulin (monocomponent) from NOVO and phloretin from K and K Laboratories. 3-O-[methyl-14C]Glucose (50 mCi/mmole) and 3-O-[methyl-14C]glucose (70 Ci/mmol) were from New England Nuclear, [U-14C]Glucose (300 mCi/mmol), L-[1-14C]glucose (4 Ci/mmol), hydroxy[14C]methoxyinulin (10 Ci/mmol), and [3H]2O were from The Radiochemical Centre, Amersham. Other chemicals were analytical grade.

Uptake of labeled 3-O-methylglucose was measured in round-bottom polypropylene culture tubes (11 \times 50 mm). Albumin free buffer, (12 \mu l), with 0.1 \mu Ci labeled 3-O-methylglucose was placed in the bottom of the tube. The adipocyte suspension was made 40% packed cell volume (approximately 4 \times 10^6 cells/ml) as described previously (3) and at time zero 40 \mu l of the suspension (16 \mu l of packed cells) was squirted on to the isotope with an automatic pipette. Low amounts of cells (less than about 10 \mu l of packed volume) led to incomplete cell recovery above the oil phase. The distance from the tip of the pipette to the isotope was about 10 mm. Timing was carried out using a metronome set at 120 beats/min and the incubation was terminated by the addition of 3 ml of albumin-free buffer containing 0.3 \mu l phloretin, 0.12% ethanol (v/v), and 0.05% dimethyl sulfoxide (v/v). Zero time was routinely determined by the addition of cells directly to 200 \mu l of stopping solution followed by addition of the remaining stopping solution. The phloretin buffer stopped sugar transport immediately (see below) and diluted the concentration of methylglucose in the aqueous phase of the incubation mixture (36 \mu l) by a factor of 85. Silicone oil (0.5 ml, density 0.99, viscosity 100 centistokes) was layered on top of the aqueous phase and the tubes very rapidly (6). The principle of the method is to follow the efflux of labeled methylene of preloaded cells, and the technique is not suited for uptake studies. The half-time of efflux was too short to be measured directly when the permeability of the adipocyte membrane to labeled methylglucose was maximal (i.e. 37°C, total methylglucose concentration <<Km, insulin present), but we were able to extrapolate a value of about 3 s. Czech (7, 8) has described a filter technique for measurement of the initial velocity of uptake of 3-O-methylglucose in adipocytes and reported a linear uptake for 20 to 30 s even when the permeability was maximal. The apparent transport rates reported by Czech were at least 20 times lower than those reported by us under comparable conditions (6, 7).

This suggested to us that initial velocities were underestimated when transport rates were measured with the technique described by Czech. In the present communication, we describe a new technique and we present results which further characterize the hexose transport system in adipocytes. Some preliminary results have been presented previously (9).

**MATERIALS AND METHODS**

Adipocytes were prepared from epididymal and perirenal fat from ad libitum fed male Wistar rats weighing 120 to 150 g and cell diameters were measured as previously described (10). The mean diameter was about 60 \mu m. The cells were suspended in buffer containing (in mm) Na+, 140; K+, 4.7; \text{Ca}^{2+}, 2.5; \text{Mg}^{2+}, 1.25; \text{Cl}^{-}, 142; \text{H}_2\text{PO}_4^{-}/\text{H}_2\text{PO}_3^{-}, 2.5; \text{SO}_4^{2-}, 1.25; and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), 10. The concentration of bovine serum albumin was 10 mg/ml (unless indicated otherwise), and pH was 7.4. Collagenase (type I) was from Worthington, pig insulin (monocomponent) from NOVO and phloretin from K and K Laboratories. 3-O-[methyl-14C]Glucose (50 mCi/mmole) and 3-O-[methyl-14C]glucose (70 Ci/mmol) were from New England Nuclear, [U-14C]Glucose (300 mCi/mmol), L-[1-14C]glucose (4 Ci/mmol), hydroxy[14C]methoxyinulin (10 Ci/mmol), and [3H]2O were from The Radiochemical Centre, Amersham. Other chemicals were analytical grade.

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were centrifuged within 2 min for 40 s at about 2000 × g in a Heraeus Christ MiniFuge 1. The 16 μl of cell sample formed one or two "islets" on top of the oil phase and were absorbed in one eighth of a pipette bent to form a brushlet. The 16 μl of packed cells (about 1.6 × 10^7 cells) in the oil phase trapped about 0.6 μl of the diluted extracellular medium. Thus, the amount of extracellular methylglucose in the cell pellet was reduced by a factor of approximately 5000 as compared to the amount in the initial incubation. The extracellular distribution space expressed in terms of the original 36 μl of extracellular medium was therefore 36/5000 or about 7 nI. Cells prepared as described exhibit an intracellular/methylglucose distribution space at equilibrium (equals the intracellular water space) of approximately 1.7% v/v or 0.28 μl/μl of packed cells. The ratio of intracellular/extracellular sugar at equilibrium was therefore around 40. The bent pipette with the cell pellet was added to a counting vial with 5 ml of scintillation fluid and radioactivity was measured. The method is outlined in Fig. 1 of the miniprint section. 1

RESULTS

Evaluation of Method—It is essential that the stopping solution work efficiently and almost immediately, even in cells with maximal permeability to methylglucose. Table I shows that the intracellular/methylglucose space calculated on non-diluted samples as total methylglucose space minus l-glucose space was not significantly different from the intracellular distribution spaces calculated after dilution of the extracellular medium with stopping solution. Fig. 2 shows that the half-time of efflux of methylglucose in insulin-stimulated cells was about 60 min in the presence of 0.3 mM phloretin. The combined results show that the time required for 0.3 mM phloretin to act is insignificant as compared to the half-time of efflux of labeled methylglucose in buffer, and that the loss of methylglucose from the intracellular pool of phloretin-treated cells is about 1%/min. It should be noted that efflux of 20 mM methylglucose in the presence of phloretin is even slower than that of "tracer" methylglucose alone. A rough estimate of the inhibition constant (K_i) of phloretin can be calculated from the curves assuming competitive inhibition. The half-time of efflux of tracer 3-O-methylglucose is about 3 s in the absence of phloretin (see below) and about 700 s in the presence of 0.3 μM phloretin (Fig. 2). Thus, K_i of phloretin is approximately 30 × 3/700 μM or 0.13 μM. It is concluded that the stopping procedure is adequate in the range of methylglucose concentrations used in the present experiments.

The coefficient of variation (n = 20) for methylglucose uptake at equilibrium (as well as for the "uptake" at time zero) was 7.1%. The coefficient of variation for uptake of methylglucose at 2 s in insulin-stimulated cells (intracellular methylglucose space about 30% of that at equilibrium) was 10.5% (n = 20). It appears, therefore, that the timing was sufficiently precise.

Some other aspects of the method are presented in the miniprint section 1 and the major points are summarized below:

1. The cell preparation is stable, i.e. the same initial uptake was measured in freshly prepared cells and in cells which had been standing for 2 h as 40% suspension at 22°C. It is preferable to use 3C-labeled methylglucose with the label in the methyl group (Fig. 3).

2. Ice cold buffer is not sufficient to stop efflux of labeled methylglucose and the stopping solution becomes increasingly less efficient with increasing albumin concentrations (Fig. 4).

3. The association of methylglucose with the cells appears to represent a distribution of methylglucose in aqueous phases. The contribution of a binding component to the total distribution space seems small (Table II).

4. Bulk mixing of the isotope with the aqueous phase of the cell suspension appears to be adequate (Table III).

5. The measured kinetic constants apply to the transport system of the plasma membrane and are not influenced to a significant degree by unstimulated layers in the extracellular aqueous medium (Fig. 5).

Time Course of Uptake of Methylglucose and the Effect of Insulin—Fig. 6 shows the uptake of tracer 3C-methylglucose (about 30 μM) at 22°C. It appears that the uptake follows an exponential course quite closely and that 1 μM insulin increases the rate of uptake about 5-fold. In this experiment, insulin was added 10 min before transport was measured and, as it appears from Fig. 7, about 4 min was required to obtain full expression

| Distribution space | Methylglucose (n = 5) | L-Glucose (n = 6) | Methylglucose minus L-glucose (n = 5) |
|--------------------|----------------------|------------------|-------------------------------------|
| A. Microfuge, no dilution | 3.05 ± 0.14 | 0.99 ± 0.06 | 2.06 ± 0.20 |
| B. Microfuge, dilution × 10 | 2.11 ± 0.15 | 0.17 ± 0.01 | 1.94 ± 0.16 |
| C. Present method | 1.88 ± 0.12 | 0.05 ± 0.01 | 1.83 ± 0.13 |

*Distribution spaces are calculated on the basis of undiluted medium.
lated from our previous experiments to "infinitely low" methylglucose concentrations (6). An estimate of the permeability to methylglucose in the presence of insulin at 22°C varied from 2.1 × 10⁻⁶ to 4.0 × 10⁻⁶ cm·s⁻¹.

Fig. 8 shows that an increase in temperature from 22°C to 37°C nearly doubled the maximal permeability of insulin-stimulated cells whereas the uptake declined slightly in the absence of insulin (cf. Fig. 7).

Fig. 9 shows that the steady state insulin dose-response relationship on methylglucose transport at 37°C is closely similar to that obtained on the rate of conversion of glucose to lipids (11). In this experiment, albumin (50 mg/ml) and bacitracin (0.5 mg/ml) were added to the medium in order to reduce protease mediated degradation of insulin (12). The transport rates (basal or maximally insulin-stimulated) obtained in this medium were not different from those obtained for albumin 10 mg/ml and in the absence of bacitracin (data not shown).

Permeability Due to Nonmediated Diffusion—Before studying the concentration dependence of methylglucose transport, it was important to investigate whether a significant fraction of the uptake of labeled methylglucose was due to nonmediated diffusion. Fig. 10 shows that the rate of uptake of L-[¹⁴C]glucose in the absence of insulin was less than 1% of that of [¹⁴C]methylglucose. Insulin enhanced the uptake suggesting that carrier-mediated transport is involved. Furthermore, 40 mM methylglucose caused a marked inhibition and the half-time of L-glucose uptake was, under these conditions, at least 150 min both in the absence and in the presence of insulin. This means that the component of the permeability to L-glucose, which may be due to nonmediated diffusion, is not more than about 0.5% of the permeability to methylglucose in "basal" cells and about 0.1% of the permeability in insulin-stimulated cells. These figures correspond well to the permeability to methylglucose obtained in the presence of 0.3 mM phloretin, cf. Fig. 2. However, methylglucose is more lipophilic than glucose and it may be argued that phloretin in high concentrations changes the general properties of the plasma membrane. Thus, the minimal permeability for L-glucose or for methylglucose in the presence of phloretin may not necessarily be relevant measures for the nonmediated diffusion permeability under normal incubation conditions.

We, therefore, measured uptake of 300 mM methylglucose (about 80 times \( K_m \), see below) under conditions approaching equilibrium exchange. The results shown in Fig. 11 indicate that the nonmediated diffusion permeability is not more than about 0.5% of the total permeability of insulin-stimulated cells to methylglucose present in a concentration \( < K_m \) and not more than 2 to 3% for basal cells. These figures must be considered as maximum estimates. The combined data indicated that carrier-mediated diffusion at least in the presence of insulin, accounts almost entirely for the total transport in the presence of total methylglucose concentrations up to about 5 times \( K_m \).

Countertransport—One of the characteristics of carrier-mediated diffusion is that addition of unlabeled sugar can induce a transient transport of the labeled sugar against its concentration gradient. Fig. 12 demonstrates that the addition of 25 mM methylglucose or glucose, but not sucrose, to the extracellular medium induces a transient decrease in the content of [¹⁴C]methylglucose in preloaded cells. Similar data have been shown with allose (4) and arabinose (5).

Substrate Dependence of Methylglucose Uptake—Fig. 13 shows the concentration dependence of the initial velocity of

Fig. 6. Time course of uptake of 50 μM methylglucose. The uptake was measured at 22°C as described under "Materials and Methods" in the absence of insulin (O—O) or in cells pretreated with 1 μM insulin (Δ—Δ). The lines represent exponential curves with half-times as indicated. The points represent measurements (duplicate values) from six independent experiments. The amount of radioactivity present in the cell pellet at time zero has been subtracted from each measurement. The methylglucose space at equilibrium was 1.8 μl/100 μl of packed cells (approximately 10⁶ cells).

Fig. 7. Time required for insulin to cause maximal activation. Uptake of methylglucose was measured after incubation for 2 s as described in the legend to Fig. 6. At time zero (arrow), 1 ml of cell suspension (40% v/v) was squirted on to 10 μl of buffer containing 1 μmol of insulin and the mixture was rapidly sucked back (in order to ensure mixing) and again ejected into the tube. Incubations with methylglucose (2 s) were carried out at the indicated time intervals (15 or 30 s) and the experiment was repeated three times on the same cell pool at each temperature; thus the points represent the mean of triplicate values. Some tubes were incubated for 5 min in order to obtain the distribution space at equilibrium. A—A, 37°C; Δ—Δ, 22°C. It should be noted that the half-time of association of 1 μM insulin to the insulin receptors is approximately 3 s (11).

of the effect of insulin at this high concentration at 22°C, whereas only about 60 s was required at 37°C. The half-time of uptake of tracer methylglucose was about 3 s in the presence of insulin and this agrees quite well with the values extrapolated from our previous experiments to "infinitely low" methylglucose concentrations (6). An estimate of the permeability of the plasma membrane to the labeled methylglucose can be derived from Fig. 6. The incubation contained 16 μl of packed cells with an intracellular distribution volume of 0.3 μl. It is seen from the curve that in insulin-treated cells about 23% of the equilibrium space is filled in 1 s. In other words, the amount of labeled methylglucose entering the cells per s corresponded to the amount present in 70 nl (7 × 10⁻⁵ cm³) of the extracellular medium. The mean surface area per cell was 1.1 × 10⁴ μm² (1.1 × 10⁴ cm²) and the total surface area was therefore about 18 cm². Thus, the permeability was about 3.9 × 10⁻⁶ cm·s⁻¹.

A moderate error is introduced in this calculation since the adipocyte diameters are in fact normally distributed with a coefficient of variation of 10% (10). In separate experiments we, therefore, measured the diameter distribution of 200 cells and calculated the total surface area of the 16 μl of packed cells. The maximal permeability to [¹⁴C]methylglucose in the presence of insulin at 22°C varied from 2.1 × 10⁻⁶ to 4.0 × 10⁻⁶ cm·s⁻¹.

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Substrate Dependence of Methylglucose Uptake—Fig. 13 shows the concentration dependence of the initial velocity of
because the time course of uptake deviates progressively from conditions (intracellular sugar concentration is initially zero) as indicated by the equation:

\[ \frac{S}{V} = \frac{K_m}{V_{max}} + \frac{S}{V_{max}} \]

The intercept with the ordinate represents \( K_m \) and the slope represents \( \frac{V_{max}}{V_{max} + S} \) for equilibrium exchange. Infranatant medium was then removed to make a 30% packed cell volume and the uptake of 50 \( \mu M \) \( ^{14}C \) methylglucose was measured after incubation for 2 s. 125I-labeled insulin was added to parallel incubations and 11% of the radioactivity was soluble in 12% trichloroacetic acid by the time transport was measured.

Fig. 10 (right). Uptake of L-glucose. Transport of 50 \( \mu M \) L-\( ^{14}C \)-methylglucose was measured as described for methylglucose. The triangles indicate incubation in the presence of 40 \( mM \) methylglucose. Open symbols, basal cells. Filled symbols, insulin-treated cells. An exponential curve with increasing sugar concentrations. If a transport system obeys Michaelis-Menten kinetics and is symmetrical (here defined as a system with the same \( K_m \) for equilibrium exchange and for net fluxes), the net influx of sugar \( (v_{net}) \) at any given infinitely small time interval during the influx is characterized by the equation:

\[ v_{net} = V_{max} \left( \frac{S}{K_m + S} - \frac{S}{K_m + S_0} \right) \]

where \( S_0 \) is the concentration of sugar in the extracellular aqueous phase and \( S \), the concentration in the intracellular aqueous phase in that small time interval. This flux equation has been integrated (13, 14) and in this form the equation expresses the intracellularly accumulated amount of sugar as a function of time.

Fig. 14 shows an experiment in which equilibrium exchange of methylglucose in insulin-stimulated cells was measured both with a very low methylglucose concentration (50 \( \mu M \)) and with 20 \( mM \). From these two curves, \( K_m \) (equilibrium exchange) was calculated as 3.7 \( mM \) and the curve for net uptake of 20 \( mM \) methylglucose was calculated assuming a symmetrical transport system. It is seen that the calculated curve for net uptake agrees well with the experimental points.

Inhibition Constants of Methylglucose and Glucose—The inhibition constant \( (K_i) \) of methylglucose on the uptake of \( ^{14}C \)-methylglucose is theoretically identical with \( K_m \) for net uptake of methylglucose provided that the initial velocity of uptake is measured. In practice, the nonexponential nature of the net uptake curve (cf. Fig. 14) will cause a small error in the estimate of the initial velocity in the presence of high concentrations of sugar. Fig. 15 shows that \( K_i \) for net uptake of methylglucose is of the same magnitude as \( K_m \) for equilibrium exchange. \( K_m \) ranged in four experiments from 2.5 \( mM \) to 5 \( mM \). This result, combined with the data shown in Fig. 13, suggests that the transport system in insulin-stimulated cells is symmetrical.

Fig. 15 also shows that \( K_i \) for D-glucose was about twice as high as for methylglucose. Furthermore, as shown in Fig. 16, the apparent initial velocity of influx of \( ^{14}C \)-glucose was about
The Maximal Permeability to Methylglucose—The half-time for equilibrium exchange and net influx when the substrate concentration is much lower than \( K_a \) for influx or exchange. The finding of similar half-times with the efflux method (6) and the present technique, therefore, supports the validity of the methods. Czech (7) reported average intracellular distribution spaces for methylglucose of 0.5 to 1.5 \( \mu \)l in cells from small rats, i.e. with mean diameters of about 60 \( \mu \)m. This is slightly smaller than the values reported by us and should, if anything, tend to decrease the half-time for equilibration. However, Czech (7) reported a linear uptake of tracer \([\text{H}]\text{methylglucose for at least 30 s at 37°C and in the presence of insulin, i.e. under conditions where we find a half-time of uptake of about 2 s (Fig. 9). These data indicate that the maximal permeability reported by us are at least 30-fold higher than those reported by Czech (7). It should be noted that Chandramouli et al. (2) found that 100 \( \mu \)M methylglucose was 80\% equilibrated in insulin-stimulated cells after incubation for 5 s at 24°C followed by centrifugation of the cells through oil for 15 s. This supports our results even though the incubation time was not well defined because no stopping solution was applied (2).

The transport rates in terms of pmol \( \times s^{-1} \) are difficult to compare directly since different authors have used different concentrations of methylglucose. However, it is reasonable to assume that the initial rate of uptake is proportional to the sugar concentration in a range up to 1 mM. Table IV shows the calculated initial rates of uptake of 100 \( \mu \)M methylglucose in insulin-stimulated cells. It appears that the rate of methylglucose uptake reported in the present paper are much higher than previously reported rates. There is about a 100-fold difference between our results and those reported by Czech (7, 15). It seems unlikely that this difference can be explained by differences in rats or collagenase preparations which, according to the authors' experience, may cause variations in the maximal permeability of not more than a factor of 3. Table IV also shows the rate of conversion of glucose to metabolic products. This figure must necessarily be smaller than the initial rate of glucose uptake (when measured on the same cells) which, again, is smaller than the initial rate of methylglucose uptake (Figs. 15 and 16). It appears that the rate of conversion of glucose to lipids plus \( \text{CO}_2 \) as reported by Czech (15) is 2 to 20 times higher than the initial rate of uptake of methylglucose. The data of Ref. 16 show that the apparent initial velocity of methylglucose uptake measured by the oil flotation method (3) after incubation for 20 s at 21°C is much lower than the rate of glucose conversion to products at 37°C. Czech (15) has reported that cells which responded at least 20-fold to insulin with respect to conversion of 0.2 \( \text{mm} \) [\( ^1^4\text{C} \)] glucose to \( \text{CO}_2 \) (or lipids, cf. Fig. 2 of Ref. 15) responded with a 2- to 3-fold increase of the initial velocity of uptake of tracer methylglucose at 37°C (7, 8). In our hands, the effect of insulin (fold increase) on methylglucose transport is considerably higher (Ref. 6, Figs. 7 and 8). Taken together, these data suggest that the initial velocity of tracer methylglucose in insulin-stimulated cells is markedly underestimated when measured as

\[
K_a = \frac{K_a \times (1 + S/K_a)}{H_{2\text{triglyceride}}}
\]

Here, \( K_a \) for equilibrium exchange was calculated as 3.6 \( \mu \)m. Curve 3 represents the net uptake of 20 \( \mu \)M methylglucose calculated for a symmetrical transport system with \( K_a = 3.6 \) \( \mu \)m (see text).

**Table IV**

| Reference | Calculated initial rate of uptake of 100 \( \mu \) M methylglucose | Calculated rate of conversion of 100 \( \mu \)M glucose to products (\( \text{pmol} \times s^{-1} \times 10^6 \text{cells}^{-1} \)) |
|-----------|---------------------------------------------------------------|--------------------------------------------------------------------------------|
| Present paper, Fig. 9 (2), Fig. 1 | 50 | 8/0.61 (\( \text{CO}_2 \) plus lipids) |
| (15), Fig. 3 and Fig. 1/ Fig. 2 | 0.35 | (21°C) 44 (total products) |
| (10), Table I | 20 | 22 (lipids) |

**Fig. 15.** Inhibition of \([\text{H}]\text{methylglucose uptake by methylglucose and glucose. Suspension (40 \mu l of 40\% (v/v) of insulin-stimulated cells (24 \mu l of aqueous phase) was added to 12 \mu l buffer containing isotope and methylglucose (O-O) or glucose (O-O) in concentrations three times those indicated on the abscissa. The incubation time was 1.5 s at 22°C. The points represent the mean of four replicates ± S.D.)**

**Fig. 14.** Net uptake of 20 mM methylglucose. The following procedures were carried out on cells from the same pool. Uptake of 50 \( \mu \)M \([\text{H}]\text{methylglucose (A------)}. Uptake of 20 mM methylglucose in cells equilibrated for 60 min with 20 mM methylglucose (O---O). Uptake of 20 mM methylglucose into sugar-free cells (O---O), i.e. the aqueous phase of the 40 \mu l of 40\% (v/v) cell sample did not contain methylglucose whereas the 12 \mu l of buffer with isotope contained 00 \( \mu \)M methylglucose. The graph represents data from three experiments. Curves 1 and 2 represent exponential curves with half-times of 2.9 s and 19 s, respectively.
described by Czech (7, 8). In our view, the main problems are as follows. 1) Addition of 10 μl of isotope solution to 100 μl of cell suspension does not give instantaneous mixing of the aqueous phases (Table III). 2) It is too late to take the first time point after 15 to 30 s. 3) Ice cold buffer does not stop efflux adequately (Fig. 4). 4) The use of [14C]methylglucose may have been a problem in some cases (Fig. 3). On the other hand, it should also be emphasized that it is clearly possible to demonstrate effects of insulin and other factors which increase the permeability of the adipocyte membrane to hexose with the technique described by Czech (8), and that it has provided the basis for important observations in relation to insulin’s mechanism of action.

The Kinetic Constants—The two major requirements for evaluation of kinetic constants are the ability to measure the initial velocity in the relevant concentration range and the absence of a large component of nonmediated diffusion. These requirements seem to be fulfilled in the present experiments. Czech reported (Fig. 3 of Ref. 7) that nearly half of the initial uptake of 90 mM methylglucose was not inhibited by 40 μM cytochalasin B which, as shown by Vinten (17), is a competitive inhibitor with an inhibition constant of 250 nM. The present results (Fig. 2) show that the half-time of efflux of 20 mM methylglucose in the presence of 0.3 mM phloretin is at least 60 to 190 min whereas the half-time for 90 mM methylglucose efflux (equilibrium exchange) in nonstimulated cells is not more than 4 min (6). We would therefore estimate nonmediated diffusion to be maximally 1% under these conditions. Czech (7) found that a component of the transport system has a high affinity to methylglucose with a Kᵣ of about 1.5 mM. Czech (7) and Olefsky (18), who used 10-s incubations followed by centrifugation through oil without stopping solution, have also reported that the initial rate of uptake was proportional to the methylglucose concentration in the range 5 to 20 mM after subtraction of the values obtained in the presence of cytochalasin B. This high Kᵣ component was large and accounted for about half of the total mediated transport of 10 mM methylglucose. It should be noted that the cited studies were carried out as net uptake experiments (intracellular methylglucose concentration initially zero). The true initial velocity is difficult to measure due to the nonexponential nature of the uptake curves (Fig. 14), and the bias will be different at different concentrations of methylglucose. This might be the reason for the apparent heterogeneity of the transport system (7, 18). It may be concluded from our studies that, if a heterogeneity exists, the present method is not precise enough to reveal it. This argument also applies to a possible asymmetry of the transport system (Fig. 14). The finding that the inhibition constant of glucose (about 7 mM at 22°C) on the initial uptake of tracer methylglucose is about twice as high as that of methylglucose (about 3.5 mM) is in agreement with the results of Loten et al. (4) who determined the inhibition constant of glucose on the initial uptake of allose in adipocytes as about 13 mM at 37°C and that of methylglucose as about 4 mM. The differences in the values may be due to differences in temperature. There is an apparent discrepancy between these results and Olefsky’s (18) finding that the inhibition constant of glucose on tracer deoxyglucose uptake is 1.0 to 2.3 mM and that of methylglucose is 4.5 to 9.0 mM. However, deoxyglucose is phosphorylated by hexokinase and trapped in the cell as deoxyglucose phosphate. Olefsky (18) incubated the cells for 3 min and by that time essentially all intracellular labeled deoxyglucose was in the phosphorylated form. D-Glucose is also phosphorylated and the apparent Kᵣ for glucose metabolism, particularly in the presence of insulin, is much lower than the apparent Kᵣ for glucose transport reported in the present paper (4, 6). Glucose transport cannot be considered rate-limiting for glucose metabolism under all conditions and it might, therefore, inhibit the phosphorylation of deoxyglucose at lower concentrations than those required to inhibit deoxyglucose transport. We have actually found that the inhibition constant of glucose on deoxyglucose transport (incubation 3 s) is considerably higher than the inhibition constant on deoxyglucose phosphorylation (incubation 3 min).1 The data suggest that the correct Kᵣ for transport of d-glucose at physiological temperature is of the order of 10 mM.

Is the Insulin-stimulated Transport System Different from the Nonstimulated?—The finding that insulin increases Vₘₓ without changing Kᵣ significantly can be interpreted in two ways. Insulin might either increase the number of carriers or it might increase the turnover on already functioning carriers (or both mechanisms might be involved). Vinten (17) found that Vₘₓ of stimulated and nonstimulated methylglucose transport showed the same temperature dependence (18 to 37°C), and that insulin did not affect Kᵣ significantly in this range of temperatures. This is compatible with the hypothesis that insulin increases the number of functioning carriers. This mechanism was also suggested by Olefsky (18) on the basis of studies on the temperature dependence of 2-deoxyglucose uptake. However, the present studies show that the uptake of tracer methylglucose (i.e. concentration << Kᵣ) in stimulated cells exhibits a temperature dependence different from that of nonstimulated cells (22 to 37°C, Fig. 8). This phenomenon was also noted by Czech (7). The data suggest that the effect of insulin is not exclusively brought about by an increase in the number of carriers available to the hexose. In further support of this hypothesis, we have noted that the stimulated and nonstimulated transport system show different dependences on changes in pH.2 These phenomena are at present under further investigation.

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1 J. Gliemann, unpublished observation.

2 J. Gliemann, unpublished observation.
**3-O-Methylglucose Uptake in Adipocytes**

**SUPPLEMENTAL MATERIAL**

**KINETIC PARAMETERS OF TRANSPORT OF 3-O-METHYLGLUCOSE AND GLUCOSE IN ADIPOCYTES**

BY RICHARD R. WHITESELL AND JØRGEN GLIEMANN

![Diagram of 3-O-Methylglucose Uptake in Adipocytes](image)

**Fig. 1.** The method for measuring uptake of methylglucose. The left panel shows the situation just before the incubation is started. Note that the buffer with isotope is a highly reacted reagent. The pipette was inserted carefully as described, and the 40 μl cell suspension mixed with the 12 μl drop to form a volume with a fairly horizontal surface (middle panel). The stopping solution was added at both test and the 3 μl was added in the pipetting when the incubation time was 1.5 min. The stopping was almost immediate, even though it takes 3 μl to pipette 3 μl. Because only a small volume is required to expose the surface of the large volume served to dilute the extracellular isotope with injection periods below 1.5 min, 0.5 ml of stopping solution was added to the large volume shortly after. The right panel shows the situation where the addition of oil and centrifugation.

![Graphs of Uptake and Efflux](image)

**Fig. 2.** Stability of the separation and quantitative efflux of 3-O-methylglucose. The right panel shows the methylglucose uptake at 240 μM of duplicate values in cells preincubated with 1 μCi/ml 22°C. The amount of radioactivity present in cells to which stopping solution was added at time zero is indicated by the dashed line. The right panel shows the efflux of 3-O-methylglucose from cells loaded with 3-O-methylglucose for 10 min and then stopped. The samples were centrifuged at the times indicated. Note that the amount of radioactivity in the cell pellet at "infinitesimal time" efflux is nearly identical with the amount present at zero time of uptake. The upper curve level represents efflux from cells preincubated with 1 μCi/ml 22°C. A large fraction of the radioactivity was retained in the cells. We obtained this pattern with 2 batches of trilaminated methylglucose, which apparently contained trace impurities. It is therefore recommended that 3-O-methylglucose be used even though some batches of unlabeled methylglucose have given results.

![Table of Data](image)

**Table II.** Evaluation of the distribution space obtained at time zero. The data for the distribution space is derived from the distribution of the 3-O-methylglucose in the intracellular and extracellular spaces. The distribution space used distribution space at time zero measured under standard conditions (stopping solution added first). It appears that the distribution spaces are the same for 3-O-methylglucose and 3-O-methylglucose. It is therefore recommended that 3-O-methylglucose be used even though some batches of unlabeled methylglucose have given results.

![Graph of Efflux of 14C-Methylglucose in Ice Cold Buffer and Albumin-Containing Stopping Solution](image)

**Fig. 4.** Efflux of 14C-methylglucose in ice cold buffer and albumin-containing stopping solution. Ice cold buffer was used in the technique described by Each (11) to remove extracellular space. Methylglucose was added to the stirred suspension of cells at time zero. The sample of methylglucose from cells treated with 1 μCi/ml 10 min followed by 14C-methylglucose for 10 min at 37°C. 49 μl aliquots were transferred to the 3 ml polystyrene tubes and 1 ml of ice cold buffer was added at time zero. 2 μl of stopping solution was added at the time indicated. By the addition of oil and centrifugation (which), it appears that the half-time of efflux is about 20 s.

Aliquots are subsequently measured in the incubation buffer and since albumin binds phosphatidyl it is of interest to study the effect of albumin in the stopping solution. The cells were loaded with the same amounts of 3-O-methylglucose and then for a stopping solution containing albumin as indicated. 2.5 μl albumin-free stopping solution was added at the times shown on the abscissa. It appears that stopping is impaired when the solution contained more than 0.25% albumin. The points represent the means of duplicate values.
3-O-Methylglucose Uptake in Adipocytes

Table III: Mixing of the bulk aqueous phase. To obtain reliable transport data the isotope-controlling buffer should contain the aqueous phase of the cell suspension. This aspect was controlled in the following way. The 3 ml incubation chamber was filled with 15 ml of buffer containing 11.2 nM 3-O-methylglucose in the aqueous phase. This was added to 10 ml of a 10 pmol methylglucose solution. The mixture was shaken vigorously for 5 minutes at 100 rpm. The 3-O-methylglucose uptake was determined by measuring the radioactivity in the incubation mixture. The results are given in Figure 1.

![Diagram](http://www.jbc.org/)

Fig. 1. Lack of importance of unstirred layers. The figure is a schematic illustration of the most probable diffusion situation with respect to assessing the bulk mixing of a complete, i.e., the diffusion distance of isotope to all cells is about the same. Consider a cell with a diameter of 50 nm and a bulk mixing of 50 nm. The cell is spherical and has a volume of 10 µm. Suppose that an unstirred layer were present, as represented by the shaded region. The cell will then be surrounded by an unstirred layer whose thickness is now assumed for simplicity and at a first approximation that is surrounding the cell. The volume of this shell is 10 µm and the thickness is 50 nm. In the model all cells are rounded off to the nearest 1 in 10 cells; therefore, the distance of methylglucose to diffuse is 50 nm. The diffusion coefficient in the cell is 30 mm²/s. It follows from Einstein's first relation for diffusion that

\[ Z = 2 \times 10^{-5} \]  

(1)

Since the medium is 10 mm²/s, the time is in seconds.

Fig. 2. Uptake of 3-O-methylglucose by hexokinase in removed cell suspensions. The cells were incubated in 3-O-methylglucose for 10 min at 20°C and the distribution phase at equilibrium was determined at 30°C. At the end of the incubation period, the cells were washed three times with 10 ml of buffer and the supernatant was removed. The cells were then suspended in 5 ml of buffer and the uptake was determined.

![Diagram](http://www.jbc.org/)

Fig. 3. Uptake of 3-O-methylglucose by hexokinase in removed cell suspensions. The cells were incubated in 3-O-methylglucose for 10 min at 20°C and the distribution phase at equilibrium was determined at 30°C. At the end of the incubation period, the cells were washed three times with 10 ml of buffer and the supernatant was removed. The cells were then suspended in 5 ml of buffer and the uptake was determined.

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![Diagram](http://www.jbc.org/)

Fig. 5. Uptake of 3-O-methylglucose by hexokinase in removed cell suspensions. The cells were incubated in 3-O-methylglucose for 10 min at 20°C and the distribution phase at equilibrium was determined at 30°C. At the end of the incubation period, the cells were washed three times with 10 ml of buffer and the supernatant was removed. The cells were then suspended in 5 ml of buffer and the uptake was determined.

![Diagram](http://www.jbc.org/)

Fig. 6. Uptake of 3-O-methylglucose by hexokinase in removed cell suspensions. The cells were incubated in 3-O-methylglucose for 10 min at 20°C and the distribution phase at equilibrium was determined at 30°C. At the end of the incubation period, the cells were washed three times with 10 ml of buffer and the supernatant was removed. The cells were then suspended in 5 ml of buffer and the uptake was determined.

![Diagram](http://www.jbc.org/)

Fig. 7. Uptake of 3-O-methylglucose by hexokinase in removed cell suspensions. The cells were incubated in 3-O-methylglucose for 10 min at 20°C and the distribution phase at equilibrium was determined at 30°C. At the end of the incubation period, the cells were washed three times with 10 ml of buffer and the supernatant was removed. The cells were then suspended in 5 ml of buffer and the uptake was determined.
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