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Kinetics of 3-*O*-Methyl-D-glucose Transport in Isolated Rat Hepatocytes

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3-*O*-Methyl-D-glucose transport across the plasma membrane of isolated rat hepatocytes was followed for net entry of the sugar into sugar-free cells (zero *trans* entry), net exit of sugar into sugar-free medium (zero *trans* exit) and for unidirectional entry and exit fluxes when cells had been equilibrated with sugar in the extracellular medium (equilibrium exchange entry and exit). These measurements were performed at 20°C and pH 7.4 by the use of simple manual methods. Initial rates of transport showed a Michaelis–Menten dependency on the sugar concentration at the *cis* side of the membrane over the range of concentrations tested (100 μM to 100 mM). Transport was found to be symmetrical with no evidence of substrate stimulation of transport from the *trans* side of the membrane. Parameters (mean values ± S.E.M.) of transport were estimated as $V_{max.}$ 86.2 ± 9.7 mmol/litre of cell water per min and K_m 18.1 ± 5.9 mM for exchange entry, $V_{max.}$ 78.8 ± 5.3 mmol/litre of cell water per min and K_m 17.6 ± 3.5 mM for exchange exit, $V_{max.}$ 81.9 ± 4.6 mmol/litre of cell water per min and K_m 20.2 ± 2.7 mM for zero *trans* entry and $V_{max.}$ 84.1 ± 8.4 mmol/litre of cell water per min and K_m 16.8 ± 4.6 mM for zero *trans* exit.

Liver-perfusion studies in rats (Williams *et al.*, 1968) and dogs (Goresky & Nadeau, 1974) have shown that D-glucose rapidly equilibrates between the liver and blood plasma. Although the intact liver presents great technical difficulties for the study of cellular-transport phenomena both groups of workers produced evidence for a sterospecific inhibitable equilibrative hexose-transport system in this tissue.

Suspensions of isolated hepatic parenchymal cells have been widely used in the study of hepatic metabolism (Krebs *et al.*, 1974) and provide a convenient system in which to observe membrane transport. Studies of hexose transport in isolated rat hepatocytes (Baur & Heldt, 1977) indicated that mediation was by an equilibrative system showing strong similarities to the glucose-transport system of the human erythrocyte, but giving substantially lower transport rates than anticipated from the liver-perfusion studies.

In the present report the transport of 3-*O*-methyl-D-glucose, which is not metabolized in isolated hepatocytes (Kletzien *et al.*, 1976), has been explored in detail to provide a basis for the study of transport of metabolically important sugars in the liver.

Materials and Methods

Male Sprague–Dawley rats (250–350 g) were deprived of food for 24 or 48 h before cell preparation.

3-*O*-Methyl-D-glucose, L-glucose, phlorrhizin, heparin and inulin were obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K. Collagenase was from Boehringer Corporation (London) Ltd., Lewes, East Sussex, U.K. Phloretin was from Phase Separations, Queensferry, Clwyd, U.K.

Sagatal veterinary anaesthetic was from May and Baker, Dagenham, Essex, U.K. PCS scintillation fluid was obtained through Hopkin and Williams, Chadwell Heath, Essex, U.K.

[U-^{14}C]-3-*O*-Methyl-D-glucose (sp. radioactivity 74.2 mCi/mmol), [^{3}H]inulin (sp. radioactivity 1.09 Ci/mmol), L-[1-^{14}C]glucose (sp. radioactivity 61.4 mCi/mmol) and D-[U-^{14}C]glucose (sp. radioactivity 248 mCi/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K.

All other chemicals were of the purest grade available from standard suppliers.

Centrifugation steps were performed with an MSE Super Minor bench centrifuge with swing-out head. All transport experiments were performed in 100 mm × 13 mm plastic or silicone-treated glass tubes.

Cell preparation

Isolated rat hepatocytes were prepared by the method of Berry & Friend (1969) as modified by Elliott *et al.* (1976). The hepatocytes were pelleted by centrifugation (50*g* for 2 min), washed by resuspension in approx. 10 vol. of fresh Krebs–Henseleit buffer, pH 7.4 (Krebs & Henseleit, 1932) and pelleted again. This washing procedure was repeated with phosphate–Ringer buffer (Krebs, 1933) and the cells suspended in fresh phosphate–Ringer buffer. The Ca^{2+} concentration of the phosphate–Ringer buffer

used in this work was decreased by half from that of Krebs (1933).

Transport measurements

The terminology used to describe transport experiments in the present paper is that of Eilam & Stein (1973). *Cis* refers to the side of the membrane from which transport is taking place, and *trans* refers to the opposite side of the membrane.

Zero-trans-entry measurements. The isolated cells were cooled on ice and endogenous sugars were removed by at least three washes with fresh phosphate–Ringer buffer over a period of up to 2h.

The hepatocyte suspension (approx. 50–75mg dry wt./ml) was equilibrated to 20°C and 0.2ml portions of this suspension were placed in experimental tubes pre-equilibrated to 20°C.

Transport was initiated by the rapid addition of 0.6ml of 3-*O*-methyl-D-glucose in phosphate–Ringer buffer at 20°C including radioactive tracer (approx. 0.1μCi/ml). Addition was from a Finnpipette (0.2–1.0ml) with a disposable tip slightly bevelled to allow rapid delivery of the solution while the experimental tube was held on a variable-speed vortex mixer (Rotamixer Deluxe, from Baird and Tatlock, Chadwell Heath, Essex, U.K.), mixing at 780–820 oscillations/min.

Transport was terminated at the appropriate time by the rapid addition of 1.5ml of ice-cold stopper solution [300μM-phloretin, 0.7% (v/v) ethanol, 80μM-$HgCl_2$, 50μM-KI in 1% (w/v) NaCl] from a 2ml disposable syringe.

The cells were rapidly separated from the medium by centrifugation to 300*g*, the supernatant removed by aspiration and the cell pellet resuspended with gentle shaking in 1.0ml of fresh ice-cold stopper solution. The cells were pelleted by centrifugation to 750*g* and the supernatant removed. Tubes were treated in pairs so that the cells were in the stopper solution for less than 4min in total.

Time-zero values were obtained by the addition of the labelled 3-*O*-methyl-D-glucose solution to hepatocytes previously well mixed with stopper solution and by the addition of the hepatocyte suspension to stopper solution ready mixed with labelled-sugar solution. Both were performed in duplicate and treated as described above.

Time-infinity values (20–80min, depending on the concentration used) were performed by incubation of duplicate tubes in a water bath at 20°C, being removed for gentle stirring at 5 to 10min intervals.

Cell pellets were osmotically shocked by the addition of 0.2ml of water and vigorously mixed on a vortex mixer. The suspension was deproteinized by the addition of 0.1ml of 20% $HClO_4$ with further vigorous mixing followed by centrifugation (2000*g* for 5min).

The supernatant fluid was removed and the radioactivity was counted in PCS scintillation fluid with a Packard 3385 scintillation counter to 10000 counts. Count rate was corrected for quenching by an external-standard method.

The radioactivity of the original methylglucose solution and external medium in the experimental tubes was similarly determined with 0.1ml samples.

Dry weights were measured by using 0.8 or 1.0ml of cell suspension, the cells being pelleted by centrifugation and supernatant fluid was removed before drying.

Zero-trans-exit measurements. After preparation, the cells were washed with the required solution of 3-*O*-methyl-D-glucose in phosphate–Ringer buffer and incubated at room temperature (16–22°C) for 5 to 30min, depending on concentration, before a second wash with the 3-*O*-methyl-D-glucose solution. The cells were further incubated on ice for up to 90min with at least two further washes with the 3-*O*-methyl-D-glucose solution.

The cell suspension of about 50% hepatocrit was equilibrated to 20°C with 2μCi of radioactively labelled 3-*O*-methyl-D-glucose for 10 to 30min, depending on concentration.

Portions (0.2ml) of the suspension were placed in the experimental tubes and the cells were pelleted by centrifugation at 350*g* for 10s. The supernatant was carefully removed by aspiration and the tubes equilibrated to 20°C.

Efflux was initiated by the rapid addition of 1.0ml of phosphate–Ringer buffer at 20°C while the tube was stirred on a vortex mixer. Transport was terminated by the rapid addition of 2.5ml of ice-cold stopper solution.

Washing, deproteinization and scintillation counting were performed as detailed for zero *trans* entry.

In net-flux experiments osmotic effects were minimized by changes in the NaCl concentration of phosphate–Ringer buffer when concentrations of over 30mM-3-*O*-methyl-D-glucose were used.

Equilibrium exchange entry and exit methods. The hepatocytes were preloaded with the required 3-*O*-methyl-D-glucose concentration as detailed for the zero-*trans*-exit method. For exchange-exit measurements the method given for zero *trans* exit was followed with an equilibrium concentration of unlabelled 3-*O*-methyl-D-glucose in the exit medium.

For exchange entry the radioactive tracer was omitted from the cells and influx of radioactive tracer from the external medium followed as outlined for zero *trans* entry.

In both net-flux and equilibrium-exchange measurements between 20 and 30 experimental time points were taken to define the progress curve from 0 to 50 (or 60)s, most points being taken between 2.5 and 20s of incubation.

Statistical treatment of data

In the absence of specific knowledge of the relative accuracy of experimental estimations of initial rates over a wide range of substrate concentrations the kinetic constants were estimated both by the method of Wilkinson (1961) on unweighted initial-rate data and by the method of Bannister *et al.* (1976) with weighting for constant percentage error in initial rates. Computation was performed by using programmes in FORTRAN and BASIC run on CDC7600 and Cyber 72 computers.

Control measurements

Use of phosphate–Ringer buffer in transport experiments. Baur & Heldt (1977) showed that hexose transport in isolated rat hepatocytes is not affected by the replacement of Krebs–Henseleit buffer with phosphate-based buffer. Measurements with phosphate–Ringer buffer and Krebs–Henseleit buffer in 3-*O*-methyl-D-glucose zero-*trans*-entry experiments confirmed this. The phosphate buffer was used routinely to avoid difficulties caused by CO_2 equilibration during the transport measurements.

Efficiency of the stopper solution. Preliminary measurements confirmed that phloretin is a stronger inhibitor of 3-*O*-methyl-D-glucose transport than phlorrhizin in this system (Baur & Heldt, 1977). It was found that $HgCl_2$ in the concentration range 0.25–2.0mM gave limited inhibition of 3-*O*-methyl-D-glucose efflux, so a stopper solution based on that used in human erythrocyte glucose-transport studies (Eilam & Stein, 1973) was adopted.

Control measurements with the stopper solution showed that isolated hepatocytes preloaded with 10mM-3-*O*-methyl-D-glucose retained 90% of their intracellular radioactivity for 20min in ice-cold stopper solution or 75% after five washes over 15min in ice-cold stopper solution.

The use of two types of time-zero measurement detailed in the zero-*trans*-entry methodology and performed for all entry measurements gave no consistent differences in radioactivity between the tubes indicating that the onset of inhibition was very rapid in comparison with entry fluxes under these conditions.

Stirring rates and unstirred layers. The effects of inadequate stirring and unstirred layers on membrane transport are well known (Winne, 1973). The stirring rate of the hepatocyte suspension was limited by the fragility of these cells and the rate adopted was an empirical compromise between stirring efficiency and cell damage.

Water spaces. Internal water spaces of 1.0–1.4μl/mg dry wt. were found by using the radioactively labelled 3-*O*-methyl-D-glucose and phloretin stopper solution. Preliminary experiments showed that the stopper solution adopted was an effective inhibitor of D-glucose transport, which equilibrated with a similar internal water space. L-Glucose also equilibrated with this space, but very much more slowly, the time for half equilibration at 10mM-L-glucose being about 20min at 20°C. Preliminary experiments with inulin (0.2μCi/mg) indicated that this space represented 50–60% of the total inulin impermeable space. These results are comparable with those obtained by Kletzien *et al.* (1975, 1976).

Gluconeogenesis and cell viability. Hepatocytes from both 24h- and 48h-starved rats showed negligible rates of gluconeogenesis (less than 10nmol/h per mg dry wt. at 37°C) in the absence of exogenous substrate.

Cell viability was routinely checked by the rate of glucose production from 5mM-L-lactate (see Elliott *et al.*, 1976) which was 192 ± 19nmol/h per mg dry wt. at 37°C (21 observations).

Results and Discussion

For all the experimental types initial rates of 3-*O*-methyl-D-glucose transport were estimated as tangents by eye to the progress curve at time zero (Figs. 1 and 2). In zero-*trans*-exit experiments the volume of extracellular 3-*O*-methyl-D-glucose solution carried over into the exit medium by the cell pellet was estimated in each experiment. Volumes of 15–30μl were found giving an external sugar con-

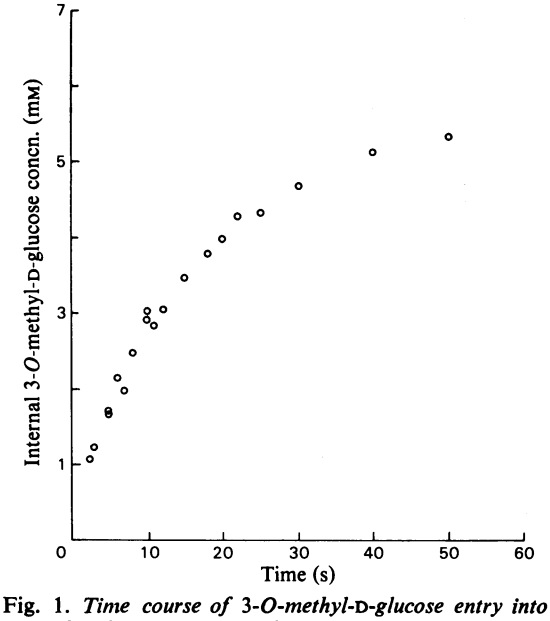

Fig. 1. *Time course of* 3-*O*-*methyl*-D-*glucose entry into sugar-free hepatocytes at* 20°C *and pH*7.4 *in phosphate–Ringer buffer*

Experimental conditions were those described for zero *trans* entry in the Materials and Methods section. External [3-*O*-methyl-D-glucose] at time zero was 5.85mM.

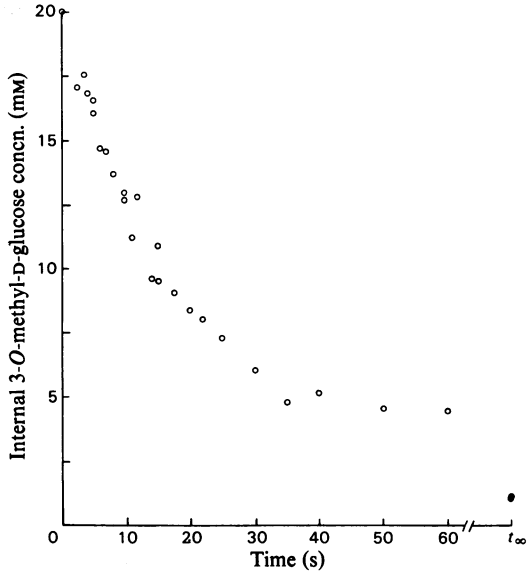

Fig. 2. *Time course of* 3-*O*-*methyl*-D-*glucose exit from preloaded hepatocytes into phosphate–Ringer buffer at* 20°*C and pH* 7.4
Experimental conditions were those described for zero *trans* exit in the Materials and Methods section.

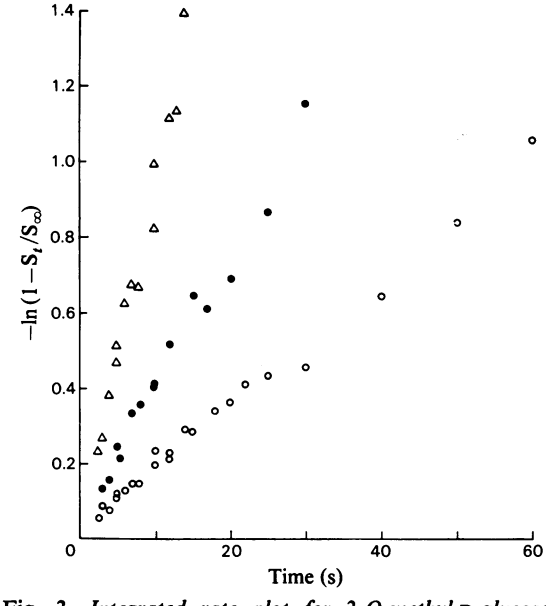

Fig. 3. *Integrated rate plot for* 3-*O*-*methyl*-D-*glucose equilibrium exchange entry measurements in phosphate–Ringer buffer at* 20°*C and pH* 7.4
S_t is the intracellular radioactivity at time t and S_∞ is the intracellular radioactivity at time infinity (25 min at the lowest 3-*O*-methyl-D-glucose concentration, 60 min at the highest). Experimental conditions were those described in the Materials and Methods section. The concentrations of 3-*O*-methyl-D-glucose were: △, 3 mM; ●, 15 mM; ○, 60 mM.

centration at time zero of less than 3% of the internal concentration. At sugar concentrations substantially below the apparent K_m of transport the half-equilibration times for both entry and exit dropped to a minimum of about 7 s.

For unidirectional measurements of exchange fluxes simple integrated rate-equation plots (Lacko *et al.*, 1972; Eilam & Stein, 1973) were performed (Figs. 3 and 4) in addition to the direct estimation of initial rates. Results shown in Tables 1 and 2 indicate that both methods of estimation of initial rates yield similar kinetic parameters.

A plot of initial rate of transport against substrate concentration for all the experimental types is given in Fig. 5. All show a Michaelis–Menten

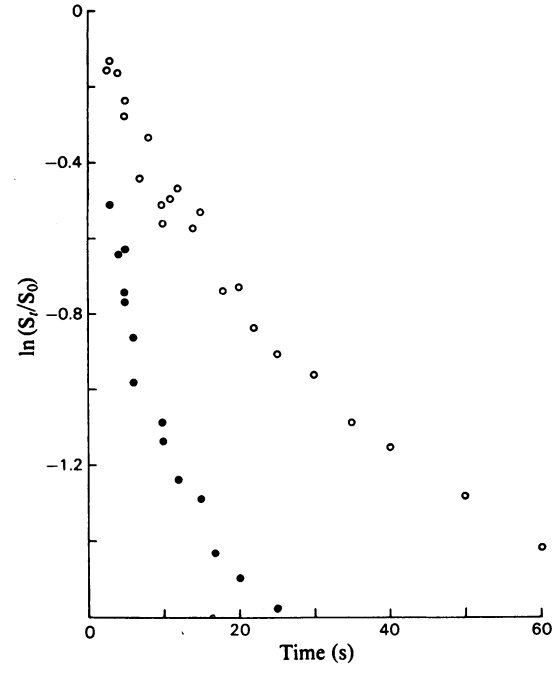

Fig. 4. *Integrated rate plot for* 3-*O*-*methyl*-D-*glucose equilibrium exchange exit measurements in phosphate–Ringer buffer at* 20°*C and pH* 7.4
S_t is the intracellular radioactivity remaining after time t and S_0 is the intracellular radioactivity at time zero. Experimental conditions were those described in the Materials and Methods section. The concentrations of 3-*O*-methyl-D-glucose were: ●, 0.3 mM; ○, 20 mM.

relationship between the initial velocity of transport and substrate concentration.

The kinetic constants estimated from the results are given in Tables 1 and 2. The agreement between weighted (Bannister *et al.*, 1976) and unweighted (Wilkinson 1961) estimates argues against systematic errors in the rate estimation over the range of substrate concentrations examined.

Plots of initial transport rate against substrate concentrations on logarithmic scales have been used to test for a variety of possible transport mechanisms (Hoare, 1972). Since both exchange and net fluxes appear to become identical and lie along a slope of gradient one at substrate concentrations substantially below the K_m of transport the possibility of multiple transport systems or polyvalency of the transport system towards 3-O-methyl-D-glucose may be rejected.

It is apparent that 3-O-methyl-D-glucose crosses

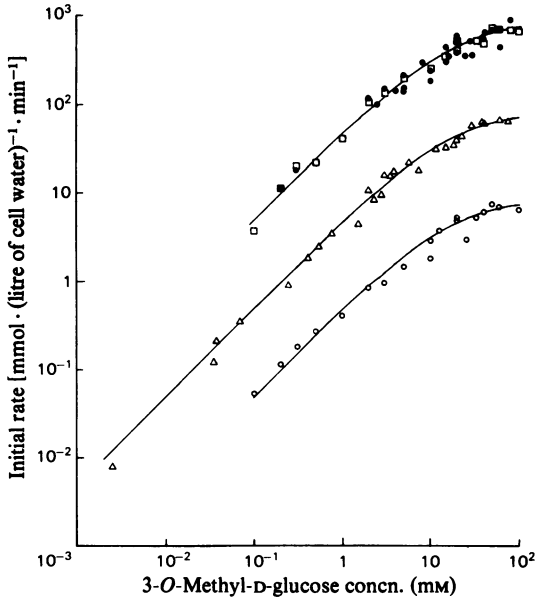

Fig. 5. *Transport of 3-O-methyl-D-glucose at 20°C and pH 7.4 in phosphate–Ringer buffer*

The solid line is the Michaelis–Menten curve. $V_{max.} = 86.2$ mmol/litre of cell water per min and $K_m = 18.1$ mM. Equilibrium-exchange initial rates have been multiplied by 10 and zero-*trans*-exit initial rates by 10^{-1} for clarity. Symbols: ○, zero *trans* exit; △, zero *trans* entry; □, equilibrium exchange exit; ●, equilibrium exchange entry.

Table 1. *Kinetic constants for the transport of 3-O-methyl-D-glucose in isolated rat hepatocytes at 20°C and pH 7.4 in phosphate–Ringer buffer*

Numbers in parentheses indicate the number of initial rates determined. W refers to parameters estimated by the method of Wilkinson (1961). B refers to estimates by the method of Bannister *et al.* (1976) with data points weighted for constant percentage error in initial velocity. The results by the method of Wilkinson (1961) are means ± S.E.M.

Experiment	$V_{max.}$ (mmol/litre of cell water per min)	K_m (mM)	Method of estimation
Zero *trans* entry (27)	81.9 ± 4.6	20.2 ± 2.7	W
	82.3	20.5	B
Zero *trans* exit (18)	84.1 ± 8.4	16.8 ± 4.6	W
	91.5	21.6	B
Equilibrium exchange entry (30)	86.2 ± 9.7	18.1 ± 4.9	W
	87.9	19.0	B
Equilibrium exchange exit (18)	78.8 ± 5.3	17.6 ± 3.5	W
	77.9	16.9	B

Table 2. *Kinetic constants for the exchange transport of 3-O-methyl-D-glucose in isolated rat hepatocytes at 20°C and pH 7.4 in phosphate–Ringer buffer*

Parameters were fitted by the method of Wilkinson (1961) (W) and Bannister *et al.* (1976) (B) to rates derived from integrated rate-equation plots. For the method of Bannister *et al.* (1976) data points were weighted for constant percentage error in velocity. The numbers in parentheses indicate the number of rates determined. The results by the method of Wilkinson (1961) are means ± S.E.M.

Experiment	$V_{max.}$ (mmol/litre of cell water per min)	K_m (mM)	Method of estimation
Equilibrium exchange entry (30)	86.5 ± 11.4	16.8 ± 6.4	W
	91.7	16.9	B
Equilibrium exchange exit (18)	75.9 ± 7.4	15.3 ± 4.3	W
	79.0	17.4	B

the plasma membrane of the isolated rat hepatocyte by a single univalent symmetrical facilitated-diffusion system. This system shows strong similarities to that described for 3-*O*-methyl-D-glucose transport in rabbit erythrocytes (Regen & Morgan, 1964) in that efflux was unaffected by external sugar and influx was unaffected by internal sugar. The lack of an acceleration of sugar movement by sugar on the opposite side of the membrane is in contrast with evidence from 3-*O*-methyl-D-glucose in monolayer cultures of hepatocytes (Kletzien *et al.*, 1975) and D-glucose transport in human erythrocytes (Mawe & Hempling, 1965; Levine & Stein, 1966), but the symmetry of transport is in agreement with the finding of Goresky & Nadeau (1974) for D-glucose in perfused dog liver.

The K_m for transport given in Table 1 is lower than the 30mM estimated for entry by Baur & Heldt (1977), who calculated initial transport rates from the difference in uptake between 10 and 20 (or 30)s. In view of half-equilibration times as low as 7s found in this report it is possible that systematic underestimates of transport rates by Baur & Heldt (1977) could account for the difference in K_m values. It is noteworthy that rat Novikoff hepatoma cells have been shown to have a very much lower K_m of 1.73mM for 3-*O*-methyl-D-glucose transport at 23°C (Graff *et al.*, 1978).

The results given in the present report show that simple manual methods similar to those used in the study of glucose transport in human erythrocytes may be used to observe sugar transport in isolated hepatic parenchymal cells. The kinetics of 3-*O*-methyl-D-glucose transport at 20°C in these cells are consistent with a simple carrier model in which movement of carrier and carrier–substrate complex are rate limiting and governed by the same rate constant.

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