Biochimica et Biophysica Acta, 1064 (1991) 37–48

ADONIS 000527369100168X

BBAMEM 75197

3-*O*-Methyl-D-glucose transport in rat red cells: effects of heavy water

R.J. Naftalin and R.J. Rist

Physiology Group, King's College London, London (U.K.)

(Received 7 August 1990)
(Revised manuscript received 8 January 1991)

Key words: Hexose transport; Transport kinetics; Carrier; Deuterium oxide; (Rat erythrocyte)

Transport of 3-*O*-methyl-D-glucose (3-OMG) in rat red blood cells (RBCs) has been examined at 24°C. The K_m and V_m of *zero-trans* net uptake are 2.3 ± 0.48 mM and 0.055 ± 0.003 μmol (ml cell water)$^{-1}$ min^{-1}, whereas the K_m and V_m for net exit are 2.1 ± 0.12 mM and 0.12 ± 0.01 μmol (ml cell water)$^{-1}$ min^{-1}. The K_m and V_m for *infinite-trans* exchange uptake are 2.24 ± 0.14 mM and 0.20 ± 0.04 μmol (ml cell water)$^{-1}$ min^{-1}. In agreement with Whitesell et al. (Abumrad, N.A., Briscoe, P., Beth, A.H. and Whitesell, R.R. (1988) Biochim. Biophys. Acta 938, 222–230), we find that there is no significant acceleration of the rate of exchange exit over net exit. Substitution of D_2O for water results in an increase in the V_m for *zero-trans* net uptake to 0.091 ± 0.004 μmol (ml cell water)$^{-1}$ min^{-1}. There is no change in the V_m or K_m for exchange uptake or net or exchange exit. Counterflow experiments indicate, in agreement with Helgerson and Carruthers (1989) Biochemistry 28, 4580–4594), that there is some compartmentalization of 3-OMG within the cells, perhaps resulting from slow complexation of the sugar with some intracellular component. The data can be simulated by assuming that transport across the membrane is mediated by either a fixed 2-site, or an alternating 1-site symmetrical transporter. With both models the observed asymmetries in net and exchange kinetics and in counterflow can be ascribed entirely to the complexation reaction of the sugar to an intracellular component. Also the D_2O effects can entirely be attributed to an increase in the rate of sugar movement between bound and free compartments.

Introduction

The rat RBC glucose transport system is a thousand times slower than that of human RBC. Consequently, it is a particularly convenient system for studying the kinetics of transport of a non-metabolizable sugar like 3-*O*-methyl-D-glucose (3-OMG). Whitesell et al. [1,2] consider that the transport system is asymmetrical, they observe acceleration of exchange uptake of labelled 3-OMG, but little or no acceleration of exchange exit, additionally they find that the K_m values for net and exchange exit over the temperature range 37–24°C are 2–3-times higher than the K_m for net uptake. On the other hand, Helgerson and Carruthers [3] report that the rat RBC has a symmetrical sugar transport system. They find a symmetrical *trans* stimulation of both labelled 3-OMG entry and exit by high concentrations of unlabelled sugar on the *trans* side at 24°C. They also find that the K_m values and V_m values for *zero-trans* net entry and exit are the same (0.9 mM and 0.065 μmol (ml cell water)$^{-1}$ min^{-1}). However, in counterflow experiments, where cells are loaded with 20 mM unlabelled 3-OMG and the time-course of uptake of 1 mM 3-OMG is also determined, they find evidence confirming earlier work [4,5] for compartmentalization of intracellular sugar. This compartmentalization could also explain the previously observed kinetic asymmetries [1,2].

There is a need to reexamine the kinetics of 3-OMG in rat cells to reconcile these differences. Additionally, we were interested to use the system to assess accurately the effect of heavy water substitution on the kinetics of sugar transport.

Materials and Methods

Red cells were obtained by exsanguination of 100–150 g Wistar rats. The cells were washed three times in isotonic NaCl-Hepes buffer (pH 7.3) by repeated centrifugation at room temperature at 4000 × *g* for 15 min

Correspondence: R.J. Naftalin, Physiology Group, King's College London, Strand, London WC2R 2LS, U.K.

to remove plasma, white cells and intracellular sugar. The cells were preincubated with loading solutions containing varying concentrations of 3-OMG at 37° C for 2 h.

Hepes buffer. The Hepes buffer contains 140 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 5 mM Hepes (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (Sigma) sodium salt (pH 7.3).

Stopping solution. The stopping solution was formulated to give a final concentration of phloretin 0.1 mM and $HgCl_2 = 1$ μM in the final mixture of cell suspension and stopping solution at 2–4° C.

Cell numbers were counted using a Coulter Counter model B and also an Elzone 280 PC cell counter was used to estimate cell volume. The intracellular concentration of sugar was estimated on the basis that $3 \cdot 10^{10}$ cells = 1 ml cell water. No correction was applied for the small changes in intracellular volume imposed by the initial difference in intracellular and extracellular tonicity imposed by asymmetrical distribution of loading concentrations of 3-OMG.

The following experimental protocols were observed:

Zero-trans net entry

Following preparation of sugar free cells, 0.05 ml of cells were added to 5 ml of isotonic Hepes buffer in plastic tubes containing varying concentrations of 3-OMG labelled with ^{3}H-label (Amersham) equilibrated in a shaking water bath for 30 min at 24° C.

Uptake was measured over a period of 5–15 min, during this time the concentration within the cells rose to approximately 5% of the external solution, assuming a single intracellular compartment. The uptake was stopped by addition of ice-cold stopping solution and the cells were pelleted by centrifugation at 4000 × *g* for 2 min, after two further washes the radioactivity was extracted from the dispersed cell pellets by extraction in 2 ml of 5% trichloroacetic acid. Aliquots of the deproteinized extracts were counted along with aliquots of extracellular fluid.

The radioactivity was counted in scintillation fluid containing 500 ml toluene, 500 ml Synperonic NX (Durham Chemical Distributors, Birtley, Chester-le-Street, Co. Durham, U.K.), 2.5 g of 2.5-diphenyloxazole (Sigma Chemical Co., Poole Dorset, U.K.).

Infinite-trans exchange uptake

Cells preloaded with 20 mM unlabelled 3-OMG at 37° C for 2 h were incubated as for *zero-trans* uptake at 24° C and the radioactivity extracted from the cells in the same way as for *zero-trans* uptake. This type of experiment is simplified in the rat red cell system and more accurate than with human RBCs, as the loss of unlabelled sugar from rat cells is minimal during the short incubation period.

Zero-trans net exit

Following an initial pre-loading period of 2 h in varying concentrations of ^{3}H-labelled 3-OMG at a haematocrit of 30%, the cells were pelleted and 0.05 ml of packed cells, 80% haematocrit was added to 10 ml of Hepes buffer at 24° C. The cells were dispersed and at intervals 1 ml aliquots of the cell suspension were added to ice-cold stopping solution and centrifuged. The cell pellets were extracted in 5% trichloroacetic acid as described above.

Infinite-cis net exit (Sen-Widdas)

Cells preloaded with 20 mM ^{3}H-labelled 3-OMG were added to Hepes buffer at 24° C containing varying concentrations of 3-OMG at the same specific activity of ^{3}H-label as in the loading solution. Diluted cell-free supernatants from the loading solution were used for this. The effect of varying external sugar concentrations on net exit from the cells on the decrease in radioactivity from the cell pellet was observed, as for *zero-trans* exit. The cell pellets were washed twice in Hepes buffer prior to extraction in 5% trichloroacetic acid.

Infinite-trans exchange exit

Exit of label from cells preloaded with 10 mM ^{3}H-labelled 3-OMG as above into solutions containing 20 mM unlabelled 3-OMG was determined over a period of 1 h.

Uphill counterflow

The uptake of 1 mM ^{3}H-labelled 3-OMG into cells preloaded with 20 mM unlabelled 3-OMG was examined by following the time course of label uptake and loss from aliquots of cell suspension. The time course of net exit of the total sugar from the cells into 1 mM 3-OMG was determined by measuring the decrease in label within the cells suspended in containing 1 mM ^{3}H-labelled buffer 3-OMG with the same specific activity as the preloading solution.

Substitution of D_2O for water in the cell suspensions

Heavy water (Flurochem 99.8%) replaced water in all buffers, the pD was adjusted with 1 M HCl and estimated with the usual pH meter. For uptake experiments cell water was replaced with D_2O by suspension and washing twice in heavy water solutions. In exit experiments water preloaded cells were used for both exit into water and heavy water.

Computer simulations

A three compartment model of sugar transport across a fixed 2-site transporter similar to that described previously [3,4] was written in GFA Basic for an Atari ST microcomputer. The time courses of fluxes were simulated by 4th order Runge-Kutta solutions of the differential equations for simultaneous fluxes of two sugar isotopes across either a symmetrical two site membrane transport with an 'unstirred layer' and a cytosolic compartment in series, or a one alternating symmetrical site with an unstirred layer and a cytosolic compartment in series [3] (Table I). It was decided not to implement any osmotic corrections for cell volume, since this introduces additional parameters and the corrections required are less than 7% at maximum. Additionally, the exchange component for the 2-site model was simplified from that described previously [4]. Three constants determine sugar flux across the membrane, (i) a single dissociation constant K of sugar for the binding sites at the exofacial and endofacial surface of the transporter, (ii) k_2, the unidirectional rate of net flux across the membrane in either direction and (iii) k_3, the homo-exchange flux rate. A single constant k_1 is required to describe the rate of sugar movement between the bound and free compartments and a compartmental volume v_1 = bound cell water volume, where v_2 = the free water volume (ml) $= 1 - v_1$ i.e., $v_1 + v_2 = 1$ ml.

This model was compared with an alternating 1-site model also with an endofacial unstirred layer. Conveniently the symmetrical alternating site model as used by Helgerson and Carruthers [3] has the same number of parameters (five) as the fixed 2-site model. This comprises (i) a symmetrical constant for free carrier movement across the membrane, (ii) k_2, a symmetrical constant for movement of the carrier sugar complex across the membrane, in addition to (iii) the symmetrical dissociation constant K, (iv) a single constant k_1 is required to describe the rate of sugar movement between the bound and free compartments and a compartmental volume v_1 = bound cell water volume, where v_2 = the free water volume (ml) $= 1 - v_1$ i.e., $v_1 + v_2 = 1$ ml as for the 2-site model. The results were matched with the counterflow data using a $\Sigma\chi^2$ test for goodness of fit and estimates of the standard deviations of the parameters were obtained from these fitting procedures.

Statistics. The Michaelis-Menten kinetic parameters for the observed data were obtained by non-linear least-squares best fit using the Enzfitter program and also Multifit 2.1 which employ the Marquardt [10] algorithms for fitting the parameters to the lines. Statistical significance of the data was obtained using Student's *t*-tests.

Results

Effects of substitution of heavy water for water on zero-trans net uptake of 3-OMG at 24°C

3-OMG uptake in rat RBCs is linear over the initial 10-min period. Net uptake of 3-OMG in the initial 10 min after exposure of the cells to labelled sugar is graphed as a function of external 3-OMG concentration (Fig. 1). There is a small linear component of uptake which is due to a shunt conductance [3]. A linear correction term = 0.002 (min^{-1} (ml cell water)$^{-1}$) × (3-OMG mM) is required to offset this conductance. This term was applied both to uptake and exit experiments in water and heavy water, but not to the counterflow experiments. With these corrections, the K_m for *zero-trans* uptake in water is 2.3 ± 0.48 mM and $V_m = 0.055 \pm 0.003$ μmol (ml cell water)$^{-1}$ min^{-1}. This K_m is approximately double that observed by Helgerson and Carruthers [3] but in good agreement with Whitesell et al. [2].

Substitution of heavy water for water increases the maximal initial rate of 3-OMG uptake significantly, 0.091 ± 0.004 μmol (ml cell water)$^{-1}$ min^{-1} (four experiments with duplicate determinations for each point, $P < 0.01$), but has no significant effect on the apparent affinity of the net uptake process. $K_m = 2.45 \pm 0.35$ mM.

Effects of substitution of heavy water for water on infinite-trans exchange uptake of 3-OMG at 24°C

Preloading the cells with 20 mM unlabelled 3-OMG increases the maximal rate of 3-OMG uptake into the cells by 4-fold above that observed with net uptake. The maximal rate of *infinite-trans* uptake of 3-OMG is 0.20 ± 0.01 μmol (ml cell water)$^{-1}$ min^{-1} and the K_m is 1.84 ± 0.3 mM. With D_2O present there is a small, but

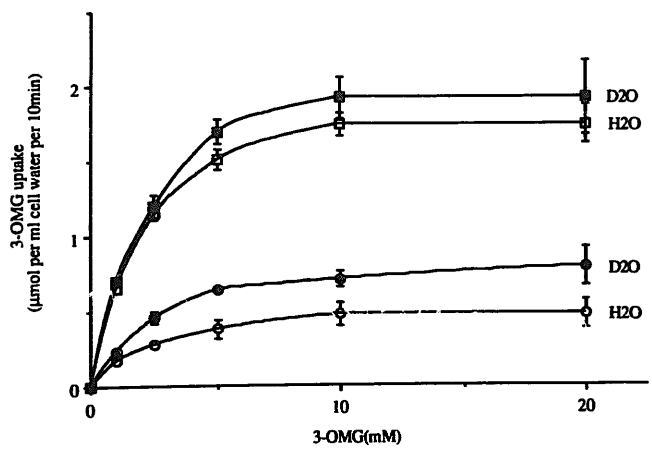

Fig. 1. Effects of pre-loading with 20 mM 3-OMG and D_2O on the concentration-dependent uptake of ^{3}H-3-OMG at 24°C. Sugar-free cells were pre-incubated with or without 20 mM unlabelled 3-OMG for 2 h at 37°C in Hepes-buffered saline. The uptake of varying concentrations of ^{3}H-3-OMG was observed, in the presence or absence of 99.8% D_2O at 24°C. For *zero-trans* net entry in water the $K_m = 2.3 \pm 0.48$ mM and $V_m = 0.055 \pm 0.003$ μmol (ml cell water)$^{-1}$ min^{-1} (circles). In D_2O the $K_m = 2.45 \pm 0.35$ mM and is not significantly different, however, the V_{max} is increased, 0.091 ± 0.004 μmol (ml cell water)$^{-1}$ min^{-1}, ($P < 0.01$). Pre-loading the cells with 20 mM unlabelled 3-OMG increases the maximal rate of uptake of 3-OMG 4-fold, when compared to net uptake. The V_{max} for *infinite-trans* uptake in water is 0.20 ± 0.01 μmol (ml cell water)$^{-1}$ min^{-1} and the K_m is 1.84 ± 0.3 mM (squares). D_2O substitution has small but insignificant effect on the K_m and V_{max} for *infinite-trans* uptake ($V_{max} = 0.22 \pm 0.014$ μmol (ml cell water)$^{-1}$ min^{-1}, $K_m = 2.00 \pm 0.46$ mM).

insignificant increase in the maximal velocity of *infinite-trans* uptake 0.22 ± 0.014 μmol (ml cell water)$^{-1}$ min^{-1} (Fig. 1). The K_m for *infinite-trans* uptake in D_2O is similar to that in water 2.00 ± 0.46 mM.

Effects of substitution of heavy water for water on zero-trans net exit and exchange exit of 3-OMG at 24°C

Zero-trans exit

The maximal rate of *zero-trans* net exit after correction for linear diffusion is 0.12 ± 0.013 μmol (ml cell water)$^{-1}$ min^{-1} and the K_m is 2.1 ± 0.4 mM (Fig. 3). Substitution of D_2O for water retards the initial rate of net exit of 3-OMG from cells preloaded with 10 mM sugar (Figs. 2 and 3). This is a transient effect and is not

TABLE I

Equations for fixed 2-site model symmetrical transporter

$xs_{in} = S_2/(K + S_2 + R_2)$; $xr_{in} = R_2/(K + S_2 + R_2)$

$xs_{out} = S_2/(K + S_3 + R_2)$; $xr_{out} = R_2/(K + S_3 + R_3)$

Prefex x refers to the fractional saturation of r or s on the inside or outside surfaces of the transporter, S, R refer to the concentration in mM of s and r in the external solution 3 or unstirred layer 2, respectively. K is the dissociation constant of R and S for the transporter at both inner and outer surfaces.

Net flux of s

$$k_2(xs_{out}(1 - xr_{in} - xs_{in}) - xs_{in}(1 - xr_{out} - xs_{out})) \quad (1)$$

Net flux of r

$$k_2(xr_{out}(1 - xr_{in} - xs_{in}) - xr_{in}(1 - xr_{out} - xs_{out})) \quad (2)$$

Exchange flux of s

$$k_2(xs_{out} \cdot xr_{in} - xs_{in} \cdot xr_{out}) \quad (3)$$

Exchange flux of r

$$k_3(xr_{out} \cdot xs_{in} - xr_{in} \cdot xs_{out}) \quad (4)$$

Diffusive flux of s and r between 1 and 2

$$k_2(S_1 - S_2); \quad k_1(R_1 - R_2) \quad (5,6)$$

k_3 is the symmetrical exchange constant, k_2 is the symmetrical net flux constant and k_1 is the rate constant of sugar movement between bound and free compartments with the cytosol.

Equations for symmetrical alternating 1-site model of sugar transport [3,5]

Numerator $S_{in} = S_3(1 + R_2/K + S_2/K)/K$; numerator $r_{in} = R_2(1 + R_2/K + S_2/K)/K$ (7,8)

Numerator $S_{out} = S_2(1 + R_2/K + S_2/K)K$; numerator $r_{out} = S_2(1 + R_3/K + S_3/K)/K$ (9,10)

Denominator =

$$2K^2/k_2 + K(k_2 + k_2)/(k_2 \cdot k_2)(S_2 + R_2 + S_2 + R_2 + 2)/(S_3 \cdot S_2 + R_2 \cdot R_2 + R_3 \cdot S_2 + S_3 \cdot R_2) \quad (11)$$

Influx of S = (numerator S_{out} − numerator S_{in})/denominator (12)

Influx of R = (numerator R_{out} − numerator R_{out})/denominator (13)

Prefix x refers to the fractional saturation of the carrier by r or s at the inner of outer surface; K refers to the symmetrical affinity of r and s for the carrier at both inner and outer surfaces. S, R refer to the concentration in mM of s and r in the external solution 3 or unstirred layer 2, respectively.

Diffusive flux of s and r between 1 and 2

$$k_1(S_1 - S_2); \quad k_1(R_1 - R_2) \quad (14,15)$$

In the alternating 1-site model k_3 is the symmetrical rate constant of carrier-sugar complex translation from side to side, k_2 is the symmetrical rate constant of empty carrier trnaslation from side to side and k_1 is the diffusion constant within the cytosol.

TABLE I (continued)

Comparison of observed and predicted parameters using the 1-site and fixed 2-site models

Parameters ± S.D. estimated from least-squares best fit of 1- and 2-site models to counterflow in water and heavy water. The only significant difference in the parameters fitting the lines to the data between water and heavy water is with k_1 for both 1- and 2-site models. The increase in v_1 is incorporated to improve the fits to the rates of uptake and exit.

	1-site		2-site	
	water	heavy water	water	heavy water
k_1 (min^{-1})	0.009 ± 0.001	0.020 ± 0.004^{x}	0.010 ± 0.0015	0.020 ± 0.004^{x}
k_2 (min^{-1})	0.06 ± 0.02	0.06 ± 0.025	0.200 ± 0.015	0.200 ± 0.015
k_3 (min^{-1})	0.24 ± 0.08	0.24 ± 0.05	0.49 ± 0.13	0.49 ± 013
K (mM)	0.50 ± 0.06	0.50 ± 0.06	1.55 ± 0.22	1.55 ± 0.6
v_1	0.91 ± 0.06	0.95 ± 0.07	0.85 ± 0.05	0.95 ± 0.08
X^2	2.004	2.007	0.377	1.06
P	< 0.005	< 0.005	< 0.001	< 0.005

observed after 60 min. However, when the cells are loaded with higher concentrations of 3-OMG, e.g. 20 mM, there is no observable D_2O-dependent retardation of the initial rate of exit (Fig. 3).

Exchange exit

Exit of 10 mM labelled 3-OMG into buffers containing unlabelled 20 mM 3-OMG is not increased significantly above the rate into sugar-free solutions (Fig. 2). These data indicate some form of asymmetry of the transport system. This result is in accord with Whitesell et al. [1,2] but not with Helgerson and Carruthers [3] who find a 400% acceleration of exchange exit over net exit of 3-OMG.

Infinite-cis net exit (Sen-Widdas procedure [6])

The time courses of net exit of 3-OMG loaded to 20 mM in either water or heavy water containing varying concentrations of 3-OMG are shown in Fig. 4. Substitution of heavy water for water has no significant effect on any of the observed time courses.

The *infinite-cis* exit K_m for 3-OMG obtained by non-linear least-squares regression is 1.8 ± 0.3 mM. This is not significantly different from the K_m for *zero-trans* uptake or *infinite-trans* uptake, but is higher than the value of 0.91 ± 0.08 mM reported by Helgerson and Carruthers [3].

Effect of D_2O on uphill counterflow of 3-OMG

The same conditions for *uphill counterflow* as those employed by Helgerson and Carruthers [3] are used. The time course of uptake of 1 mM ^{3}H-labelled 3-OMG into cells containing 20 mM unlabelled 3-OMG at 24°C was examined, along with the parallel net exit of 20 mM labelled 3-OMG into solution containing 1 mM 3-OMG

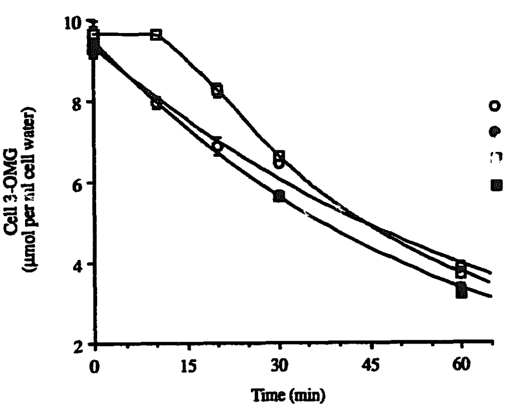

Fig. 2. Effects of external 20 mM unlabelled 3-OMG on the time course of exit of 10 mM ^{3}H-3-OMG, in the presence or absence of D_2O at 24°C. Cells pre-loaded for 2 h at 37°C with 10 mM ^{3}H-3-OMG were resuspended in sugar free or buffers containing 20 mM unlabelled 3-OMG in water or D_2O. The exit was followed for 60 min at 24°C. Initially D_2O retards the rate of *zero-trans* exit. With the rate of exit of 10 mM ^{3}H-3-OMG into 20 mM unlabelled 3-OMG in water is no greater than *zero-trans* exit. D_2O does not significantly alter exchange exit.

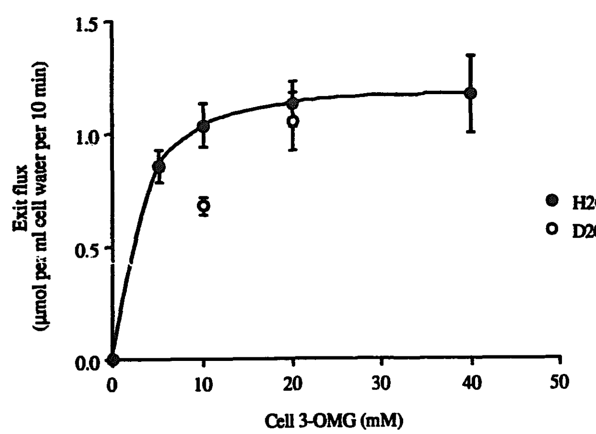

Fig. 3. Effect of varying internal [^{3}H-3-OMG] on initial exit rate into sugar-free buffers containing water or D_2O (*zero-trans* net exit). Cells were preloaded with varying concentrations of ^{3}H-3-OMG for 2 h at 37°C. The cells were resuspended in water or D_2O buffers at 24°C. Aliquots were removed at 0, 5 and 10 min and the initial rates of exit calculated. The V_m for *zero-trans* net exit in water is 0.12 ± 0.013 µmol (ml cell water)$^{-1}$ min^{-1} and the K_m is 2.1 ± 0.4 mM. D_2O retards the initial rate of *zero-trans* net exit at 10 mM internal 3-OMG ($P < 0.05$), but has no significant effect at 20 mM internal 3-OMG.

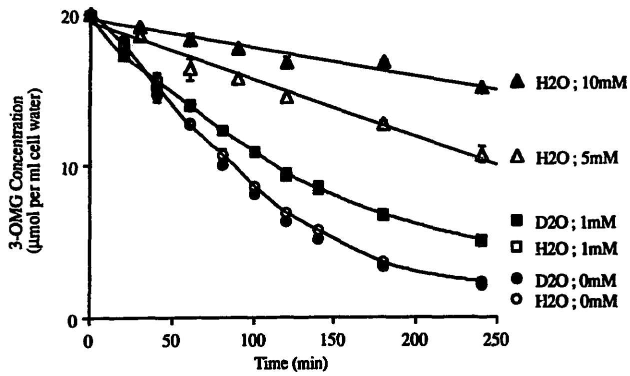

Fig. 4. Effect of D_2O on the time course of *infinite-cis* net exit from cells pre-loaded with 20 mM ^{3}H-3-OMG at 24°C. Cells pre-loaded with 20 mM ^{3}H-3-OMG for 3 h at 37°C were resuspended in water or D_2O buffers containing varying concentrations of 3-OMG with identical specific activities to the pre-loading solution. The *infinite-cis* net exit was followed over 4 h at 24°C. Increasing the external sugar concentration retards the exit of ^{3}H-3-OMG. Substitution with D_2O has no significant effect on exit of 3-OMG. The Sen-Widdas *infinite-cis* exit K_m for 3-OMG is 1.8 ± 0.3 mM estimated by non-linear least-squares fit to the data.

labelled with the same specific activity as the internal sugar. Fig. 5 shows these time courses. The uptake rate of 1 mM 3-OMG in water is the same as that of Helgerson and Carruthers [3], but the net exit rate of 3-OMG into 1 mM 3-OMG is about twice as fast as the rate they observed.

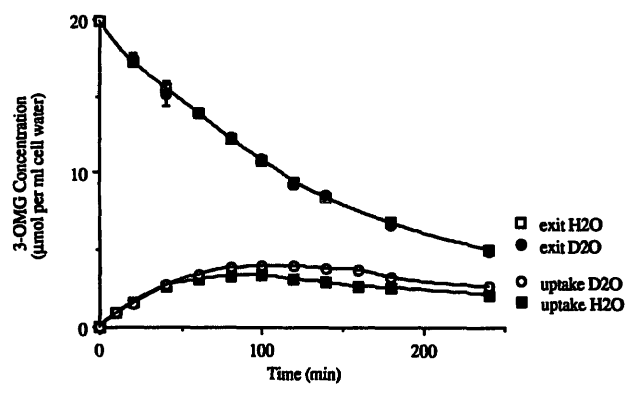

Fig. 5. Time course of uphill counterflow in the presence or absence of D_2O at 24°C. RBCs pre-incubated in 20 mM unlabelled 3-OMG for 2 h at 37°C. The uptake of 1 mM ^{3}H-3-OMG was followed over 4 h at 24°C, into the resuspended cells in water or D_2O-containing buffers. A parallel experiment in which the net exit of 20 mM ^{3}H-3-OMG into buffer containing 1 mM 3-OMG with the same specific activity (*infinite-cis* exit), was observed with or without D_2O at 24°C. This exit data are plotted with the uphill counterflow and shows that D_2O has no initial effect on the rate of uptake of 1 mM ^{3}H-3-OMG, but subsequently increases uptake. The maximal uptake with D_2O is at 100 min and is 4.2 ± 0.2 mM, whilst the peak for water is 3.1 ± 0.2 mM at 75 min. Net exit of 20 mM ^{3}H-3-OMG into buffer containing 1 mM 3-OMG is not significantly affected by D_2O substitution.

The rate of 3-OMG uptake in D_2O buffer is the same as for water over the initial 30 min period, but thereafter the uptake of labelled sugar into D_2O-treated cells is larger. The peak uptake of labelled sugar is 4.2 ± 0.2 mM after 100 min in D_2O, whereas in water the maximal uptake of label is observed at 75 min and only 3.1 ± 0.2 mM labelled 3-OMG is accumulated (four experiments). There is no difference in net exit of 3-OMG into D_2O or water containing buffer. The total intracellular 3-OMG at 75 min is 13.0 ± 0.3 mM and at 100 min is 10.3 ± 0.2 mM. These values permit an estimate of the sugar affinity for the carrier K in each condition according to formula [3–5] where:

$$K = (P_1 - (S_1/S_2)P_2)/((S_1/S_2) - 1)$$

P_1 and P_2 are the concentrations of 3-OMG inside and outside the cells at the counterflow peak, respectively, and S_1 and S_2 are the concentrations of labelled sugar inside and outside the cells at the counterflow peak, respectively.

Hence, in water $K = (13 - 3.1)/(3.1 - 1) = 4.7 \pm 0.25$ mM; whereas in D_2O $K = (10.3 - 4.2)/(4.2 - 1) = 1.9 \pm 0.25$ mM.

Discussion

The points of similarity with the data of Whitesell et al. [1,2] are:

(1) a 4-fold stimulation of *infinite-trans* exchange uptake over *zero-trans* net uptake but similar K_m values for *infinite-trans* exchange and *zero-trans* net uptake;

(2) a negligible increase of *exchange exit* over *zero-trans* exit;

(3) some apparent asymmetry of the rat sugar transport system. The $K = 4.7$ mM estimated by counterflow in water (assuming a uniform cytosolic compartment); whereas, the K_m value of *infinite-cis* exit is 1.8 mM. These findings suggest a lower apparent affinity for 3-OMG at the endofacial surface than at the external surface of the transporter although this is not substantiated by the K_m for *zero-trans* net exit $= 2.1 \pm 0.3$ mM.

The points of similarity between the data obtained in this study and those of Helgerson and Carruthers [3] are:

(1) the similar observed parameters for *infinite-trans* entry;

(2) the similar time courses of the uphill counterflow transient.

However, there are some differences:

(1) the K_m of *zero-trans* net uptake reported here and for exit are double those found in Ref. 3,

(2) the time course of net exit of 20 mM 3-OMG is approximately twice as fast as that reported in Ref. 3,

(3) no acceleration of exit of 3-OMG by external unlabelled 3-OMG is observed here.

Some of these differences may be due to the variability of cells and differences in the experimental methodologies adopted (see below).

Substitution of D_2O for water has asymmetric effects on transport; the V_m of *zero-trans* net uptake is increased by 40%, but *zero-trans* exit is unaffected, the

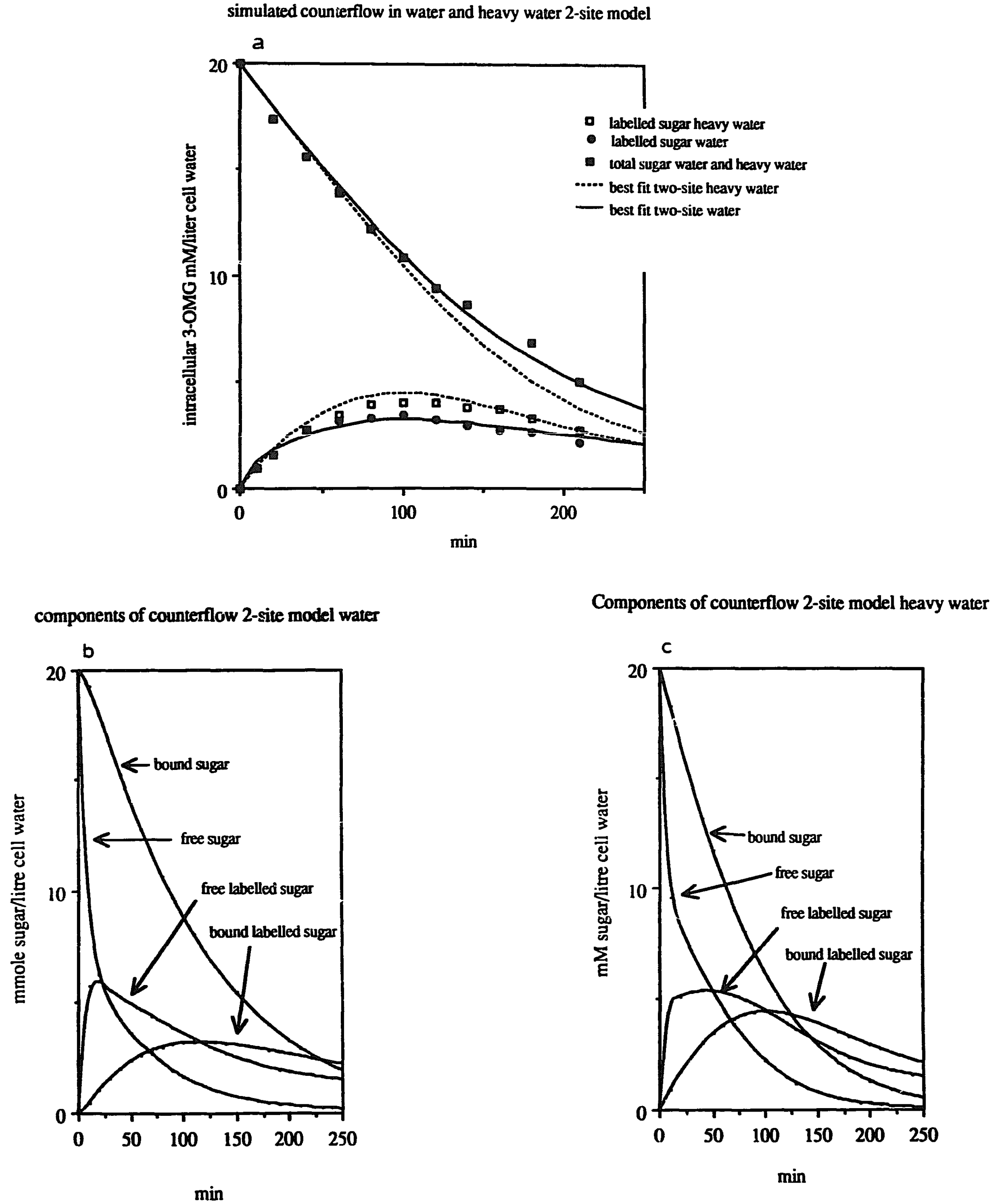

Fig. 6. Simulation of uphill counterflow in water and heavy water with a 2-site model. (a) Simulation of counterflow in water and heavy water based buffer. The filled squares are the observed data for net exit in both water and heavy water, the open squares are the observed data for uptake of 1 mM labelled 3-OMG into clels suspended in heavy water, the filled circles are the observed data for uptake 1 mM labelled 3-OMG in water. The broken lines are the simulated net exit and uptake in heavy water and the unbroken lines are the simulated net exit and labelled sugar uptake in water. (b) The simulated time-dependent changes in the concentration of bound and free components of intracellular total sugar and labelled sugar within their respective compartments in cells suspended in water using the 2-site model. (c) The simulated time-dependent changes in the concentration of bound and free components of intracellular total sugar and labelled sugar within their respective compartments in cells suspended in heavy water using the 2-site model.

distribution volume of labelled 3-OMG at the counterflow peak is increased by 25%. However, D_2O has no significant effect on exchange inflow or outflow (Figs. 1 and 2). These findings are consistent with a D_2O-dependent reduction in the endofacial unstirred layer effect (see below).

Simulation of 3-OMG flux and the effects of D_2O on 3-OMG flux

The rat RBC sugar transport system is three orders slower and much more symmetrical than the human RBC transport system. This slower rate can be simply ascribed to a lower number of glucose transporters/ unit

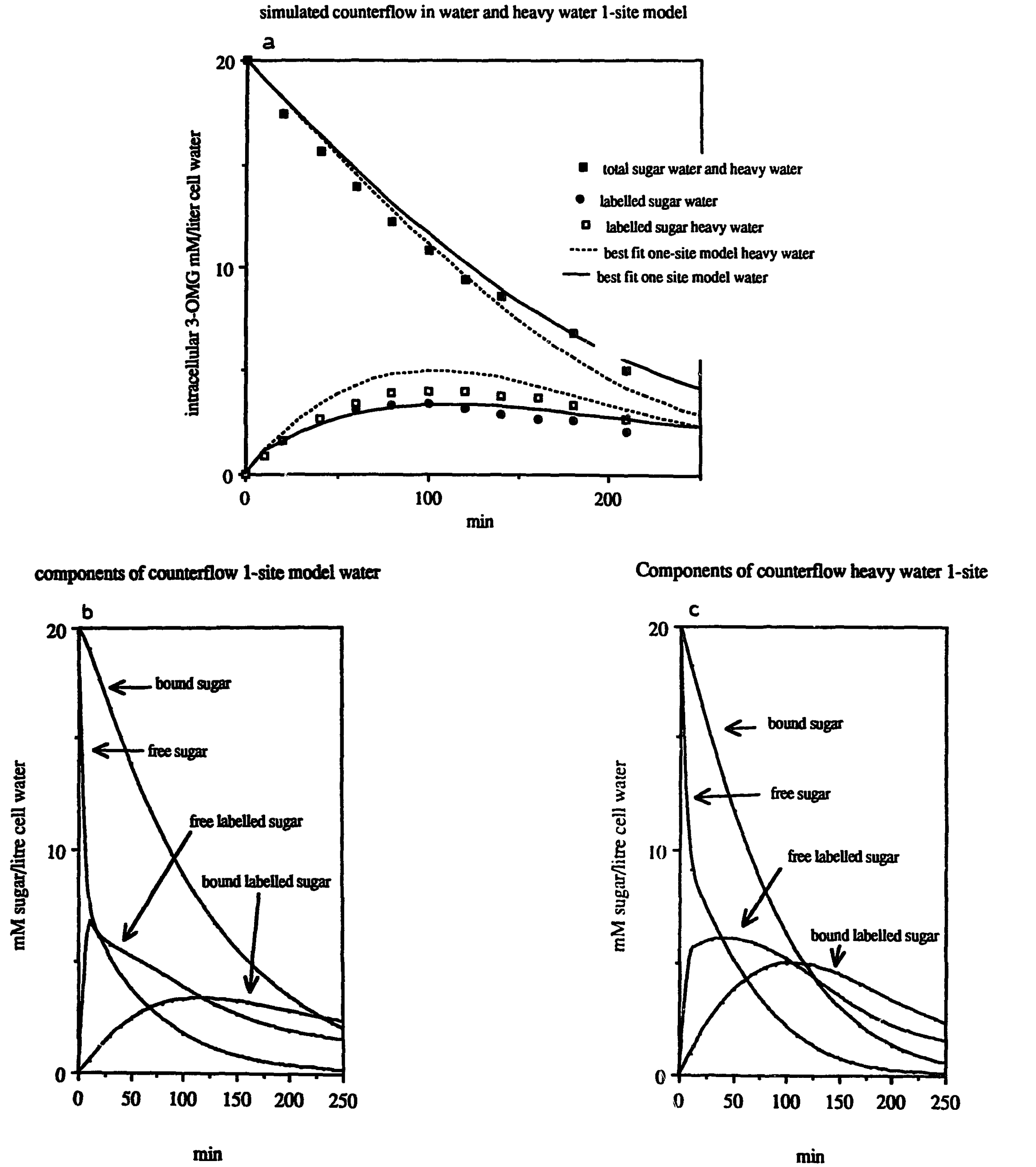

Fig. 7. Simulation of uphill counterflow in water and heavy water with a 1-site model. (a) Simulation of counterflow in water and heavy water based buffer using the 1-site model. The filled squares are the observed data for net exit in both water and heavy water; the open squares are the observed data for uptake of 1 mM labelled 3-OMG into cells suspended in heavy water, the filled circles are the observed data for uptake 1 mM labelled 3-OMG in water. The broken lines are the simulated net exit and uptake in heavy water and the unbroken lines are the simulated net exit and labelled sugar uptake in water. (b) The simulated time-dependent changes in the concentration of bound and free components of intracellular total sugar and labelled sugar within their respective compartments in cells suspended in water using the 1-site model. (c) The simulated time-dependent changes in the concentration of bound and free components of intracellular total sugar and labelled sugar within their respective compartments in cells suspended in heavy water using the 1-site model (see Table I).

area of rat RBC membrane than in human cells. A low rate of transport would, as Helgerson and Carruthers [3] suggested, account for the relatively small asymmetry of the rat sugar transport system in comparison with human, as the endofacial unstirred layer effect would be reduced.

Unstirred layer effect

The simplest explanation for (a) the small observed asymmetry in net entry and exit fluxes; (b) the smaller than ideal volume of intracellular distribution of 3-OMG during counterflow and (c) the large difference in exchange acceleration of entry and exit, is that labelled sugar does not equilibrate with the entire volume of cell water immediately following transport but is delayed, possibly due to a slow complexation reaction with an intracellular component. The size of this effect is related (i) to the relative volume of the free and bound sugar compartments, v_1/v_2 and (ii) to the rate of translation of sugar between the bound and free compartments, k_1 [3,4]. These effects can all be readily simulated with a three-compartment model as described previously [4]. Membrane transport is described by three symmetrical parameters, (see Methods and Table I).

Simulation of counterflows in water and heavy water with alternating 1-site or fixed 2-site models

Fig. 6 shows the 2-site model simulations of counterflow for water and heavy water. Fig. 7 shows the 1-site model simulations. All the simulations are the best fits to total label uptake from an external solution containing labelled 3-OMG (1 mM) into cells initially containing 20 mM unlabelled 3-OMG. The time-dependent decrease in the total amount of 3-OMG within the cells is also plotted and matched to the simulations.

With both models the optimal match for counterflow in D_2O is obtained by raising k_1 ($P < 0.01$). No significant change is required for any other parameter; although there is some improvement in fit if the free sugar compartment is reduced in size by D_2O substitu-

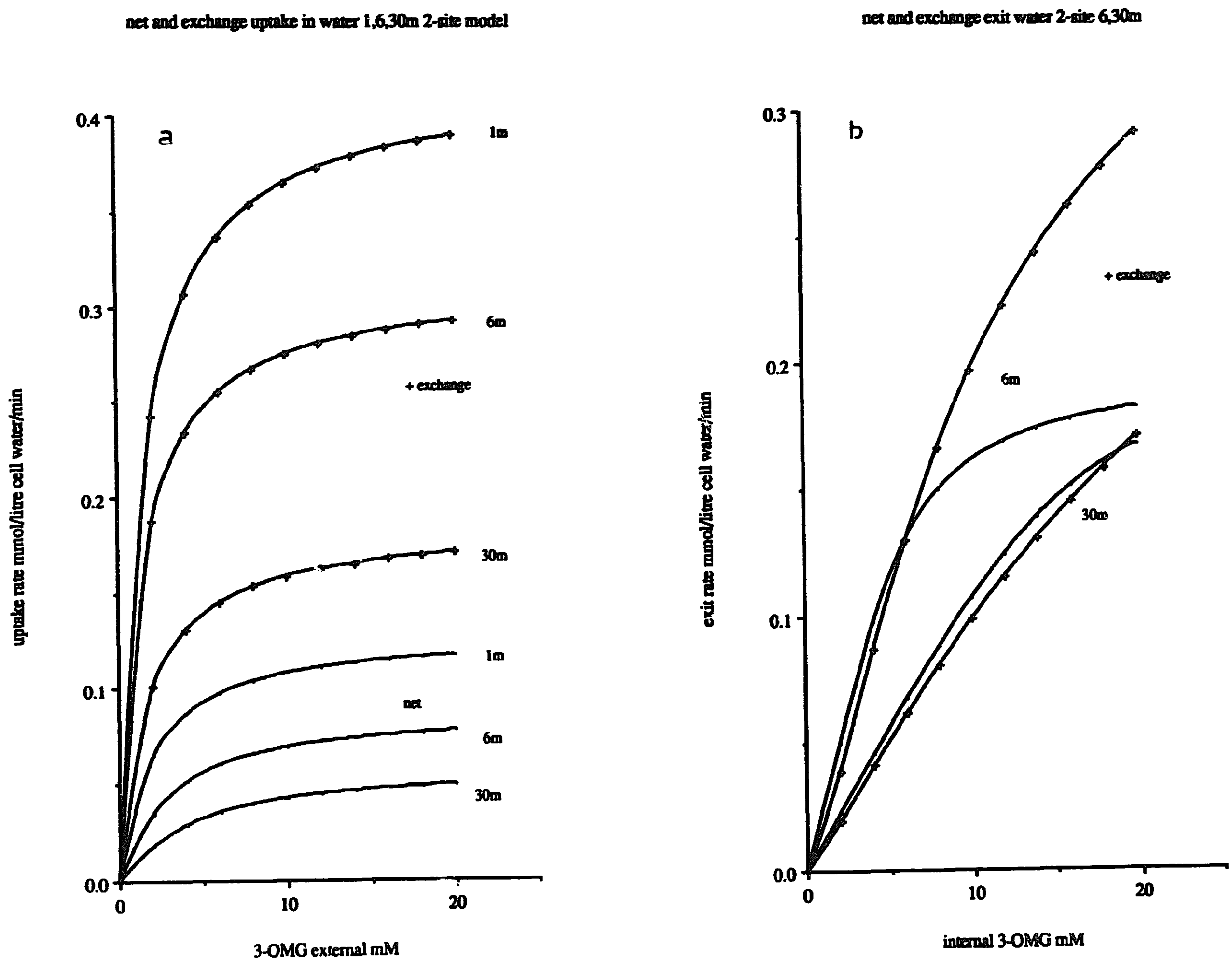

Fig. 8. Simulation of net and exchange uptake and net and exchange exit in water with the 2-site model. (a) Uptake fluxes as a function of external [3-OMG]. ———, net uptake was estimated from chord uptakes between 10 s and 1 min, 6 min and 30 min. + — +, exchange uptake into cells preloaded with 20 mM 3-OMG. (b) Exit fluxes as a function of total initial internal [3-OMG] as estimated by chord fluxes from 10 s to 6 min or 30 min. At 6 min it is possible to observe a stimulation of exchange exit over net exit, whereas at 30 min no acceleration of exchange exit is detectable.

tion for water. The counterflow simulations show that the D_2O-dependent increases in net uptake can be simulated with either the one or two site models in series with an unstirred layer. The D_2O-dependent acceleration of uptake requires approximately 30 min to develop. Initially because of the smaller free sugar pool in cells suspended in D_2O the uptake is retarded. The D_2O dependent reduction in volume of the free sugar pool may also account for the initial retardation of net efflux observed at low concentrations of sugar (Figs. 2 and 3).

The best match is obtained using the 2-site model with water where $\Sigma\chi^2 = 0.377$; the $\Sigma\chi^2$ for the 2-site model simulation in heavy water is 1.06; the best fits obtainable with the single-site models were $\Sigma\chi^2 = 2.0$ for both water and heavy water. Although the 1-site model is a poorer fit than for the 2-site model the goodness of fit of both models to the data is highly significant.

In Figs. 6b, 6c and 7b, 7c are shown the time courses of change in intracellular components of the sugar during counterflow: the concentrations of labelled sugar (bound and free) and the total concentration of sugar (labelled + unlabelled) in the bound and free compartments. A D_2O-dependent increase in k_1 increases the rate and amount of labelled sugar entering the bound compartment. This modification of both 1- or 2-site models predicts that the concentration of labelled sugar present within the free compartment remains higher for a longer duration in cells suspended in heavy water than in water based media. An increase in the rate of total net exit of sugar is also predicted with D_2O, however, this effect is too small and occurs too late in the time course to be detected.

Matching observed flux data to simulated fluxes with the symmetrical alternating 1-site and the fixed symmetrical 2-site model

A problem in matching models incorporating unstirred layers to experimental data is that the theoretical transport parameters predicted on the basis of the intrinsic constants of the transporter are modified by the unstirred layer and these apparent deviations from theoretical parameters become exaggerated as sugar flux advances beyond the initial perturbation. Thus although uptake and exit fluxes are quasi-linear, the rates of solute uptake or loss estimated by the differences in total solute or label within the cell between an early time datum, e.g., 10 s and 1, 6 or 30 min data (chord uptakes) generate large differences from the ideal transport parameters (see Figs. 8a and 8b). The model simulations were fitted to the data by first assuming that K, the dissociation constant = 1 mM and then minimizing $\Sigma\chi^2$ by small increments of k_1, k_2, k_3 and V_1; a second round of iterative fitting was done to optimize the fit of K.

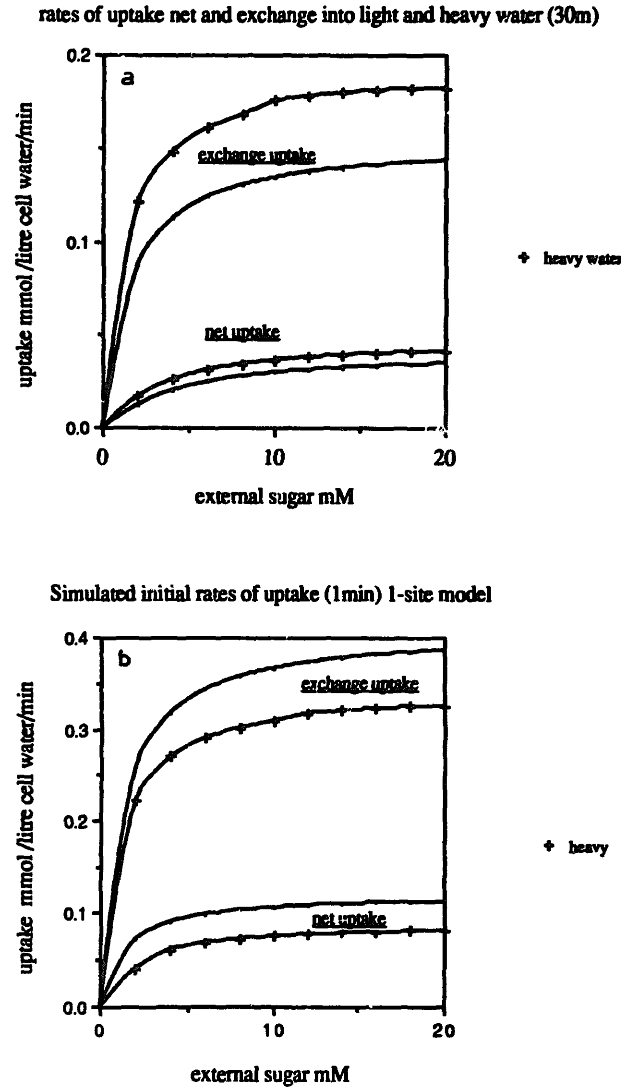

Fig. 9. Simulated rates of net uptake and exchange uptake into cells suspended in water or heavy water with the alternating 1-site model. (a) Simulated net uptake and exchange uptake into water (———) and heavy water (+—+) at 30 min. At this stage uptake of 3-OMG into cells suspended in heavy water is predicted to exceed that into cells suspended in water. (b) Simulated net and exchange uptake into water buffer (———) and heavy water buffer (+—+) at 1 min. At this stage uptake of 3-OMG is less into cells suspended in heavy water than into cells suspended in water.

Simulations of net fluxes

A selection of the simulated rates of net uptake and exit with both the 2-site models shown in Figs. 8a and 8b and the 1-site model Figs. 9a and 9b and the kinetic parameters derived form these simulations by non-linear regression [10] (Table II) indicate:

(a) that the unstirred layer effect accounts for the lower apparent affinity of 3-OMG for the endofacial than for the exofacial surface and the lower apparent maximal rate of net uptake than of net exit (Figs. 8a and 8b). Net entry of labelled sugar is retarded because the labelled sugar rapidly accumulates within the free compartment adjacent to endofacial surface of the transporter, this effect increases with time. The maximal rate of *zero-trans* net exit is not retarded by the unstirred layer effect, although in the low to intermediate concentration range exit where the endofacial sites are only partially saturated, sugar exit is rate limited by slow release from the bound compartment, this raises the apparent affinity for *zero-trans* net exit thus the apparent K_m for net exit is progressively raised as exit advances (Fig. 8b). The predicted parameters for net entry and exit obtained by matching the parameters from the counterflow experiments for both 1- and 2-site models are consistent with the observed parameters for net transport of 3-OMG in rat red cells bathed in either water or heavy water (Table II, Figs. 8 and 9).

Simulations of exchange

High concentrations of an unlabelled transported sugar within the cytosol compete with and reduce reflux of unbound labelled sugar and hence increase the rate of label uptake into the cells; thus *infinite-trans* entry is not retarded by the unstirred layer effect to the same extent as is *zero-trans* uptake (Figs. 8a and 8b). However, exchange exit of labelled sugar from cells into solutions containing unlabelled sugar is retarded as the unlabelled sugar accumulates within the unstirred layer and reduces the specific activity of the labelled sugar within the unstirred layer, hence after the initial 5 min of flux, exchange exit is not accelerated by unlabelled sugar in the *trans* solution to the same extent as exchange uptake. No acceleration of exchange exit over net exit is apparent with either the 1- or 2-site models on measuring the chord fluxes over the interval 10 s to 30 min (Fig. 8b).

These simulations provide an explanation both for the symmetrical acceleration of exchange exit and entry observed by Helgerson and Carruthers [3], who de-

TABLE II

Apparent Michaelis-Menten parameters K_m (mM) and V_m (mmol (litre cell water)$^{-1}$ min^{-1}) for alternating 1-site and fixed 2-site transporters as estimated by simulated chord uptakes in water and heavy water at 1 and 6 min after perturbation and observed parameters as described in Methods

Time	2-site		1-site		Observed parameters	
	K_m	V_m	K_m	V_m	K_m	V_m
Water						
Zero-trans uptake						
1 min	1.72±0.01	0.15±0.01	1.36±0.01	0.12±0.01	2.3 ±0.4	0.055±0.003
6 min	3.09±0.07	0.09±0.01	2.71±0.01	0.12±0.01		
Infinite-trans uptake						
1 min	1.46±0.01	0.43±0.001	1.08±0.01	0.34±0.01	1.84±0.30	0.20 ±0.01
6 min	1.32±0.01	0.31±0.01	1.10±0.01	0.24±0.01		
Heavy water						
Zero-trans uptake						
1 min	2.14±0.01	0.12±0.01	2.02±0.03	0.09±0.01	2.45±0.35	0.09 ±0.004
6 min	3.78±0.10	0.07±0.01	2.67±0.04	0.07±0.01		
Infinite-trans uptake						
1 min	1.96±0.04	0.21±0.01	1.08±0.01	0.34±0.01	1.84±0.30	0.20 ±0.01
6 min	1.39±0.01	0.25±0.01	1.10±0.01	0.24±0.01		
Water						
Zero-trans exit						
1 min	1.95±0.04	0.21±0.01	1.35±0.06	0.06±0.01	2.1 ±0.4	0.12 ±0.01
6 min	7.66±1.04	0.27±0.02	11.12±0.18	0.31±0.03		
Infinite-trans exit						
1 min	3.80±0.07	0.22±0.01	5.31±0.17	0.50±0.01	–	
6 min	40.6 ±9.3	0.94±0.17	30.11±1.78	0.66±0.03		
Heavy water						
Zero-trans exit						
1 min	2.14±0.01	0.12±0.01	3.59±0.46	0.23±0.01		
6 min	14.72±2.23	0.33±0.03	11.81±1.64	0.31±0.02		
Infinite-trans exit						
1 min	9.25±0.41	0.53±0.01	14.92±1.04	0.58±0.03		
6 min	40.60±9.33	0.94±0.17	27.32±1.02	0.55±0.95		
Infinite-cis exit (Sen-Widdas)						
Water						
6 min	1.01±0.23	0.16±0.03	1.03±0.59	0.14±0.14	1.8 ±0.3	0.18 ±0.03
Heavy water						
6 min	0.92±0.05	0.17±0.03	0.86±0.48	0.17±0.07		

termined exit over a shorter time interval than was done here, and also for the asymmetrical exchange process observed here and by Whitesell et al. [2].

Whitesell et al. [2] explain asymmetrical *trans*-acceleration of exchange on the basis that movement of the loaded carrier from inside to outside is the rate-limiting step of the net sugar exit, and exchange exit [1], i.e., the exchange rates of the loaded carrier are asymmetric, $k_{23} \gg k_{-21} \ll k_{-4}$, where k_{23} and k_{-21} are the unidirectional rates of inflow and outflow of the loaded carrier and k_{-4} is the rate of inflow of empty carrier.

However, the mobile carrier requires that exchange inflow and outflow must always be identical, even if k_{23} and k_{-21} are unequal, because a full cycle of the carrier is required to complete the exchange process. The exchange resistance $= R_{ee} = 1/V_{m(exchange)}$ is proportional to the sum of the reciprocals of both the forward and backward rates of the loaded carrier; $\propto (1/k_{23} + 1/k_{-21})$ [5]; hence the observed asymmetry of exchanging sugar fluxes in rat red cells must be due to some extrinsic factor, such as an asymmetric 'unstirred layer' at the endofacial surface [7–9].

Effects of heavy water substitution on sugar transport

Previous work [11,12] has indicated that substitution of heavy water for water inhibits net glucose transport in human red cells, but not exchange flux. A possible explanation for the difference between the effects we observed here and those with human cells could be that a heavy water-dependent reduction in free glucose space in human red cells would cause a more rapid and complete emptying of the intracellular free sugar pool during exit, or filling of the intracellular pool during uptake than in water. This would lead to a D_2O-dependent reduction in net transport. A similar inhibition of net flux is predicted by both 1- and 2-site models in the early stages of net exit or uptake with rat red cells and observed here (Figs. 2 and 3) but this effect is much attenuated because of the slow rate of membrane transport in these cells.

Discriminating between the single-alternating site or fixed double-site models

Helgerson and Carruthers [3] claim that the kinetics of the sugar transport by rat RBCs are not described well by an alternating carrier with unstirred layer effect. However, they only invoke the unstirred layer effect to explain their anomalous counterflow data and disregard it when explaining initial rates of net and exchange flux. This is justified by their assumption of a larger intracellular free pool (0.25 ml/ml cell water) than is required here. Here it is shown that the free water pool is much smaller, hence the unstirred layer effect has an immediate effect on the fluxes.

Thus, it is not possible on the basis of fitting model simulations to steady-state net fluxes or homoexchanges of 3-OMG fluxes in the rat red cell system to discriminate between 1-site and 2-site models. It may eventually be possible to use hetero-exchanges in rat cells as has been done before in human cells to reach a more firm conclusion [7].

References

1 Abumrad, N.A., Briscoe, P., Beth, A.H. and Whitesell, R.R. (1988) Biochim. Biophys. Acta 938, 222–230.
2 Whitesell, R.R., Regen, D.M., Beth, A.H., Pelletier, D.K. and Abumrad, N.A. (1989) Biochemistry 28, 5618–5625.
3 Helgerson, A.L. and Carruthers, A. (1989) Biochemistry 28, 4580–4594.
4 Naftalin, R.J., Smith, P.M., and Roselaar, S.E. (1985) Biochim. Biophys. Acta 820, 235–249.
5 Stein, W.D. (1986) in Transport and Diffusion across Cell Membranes, pp. 231–306, Academic Press, New York.
6 Sen, A.K. and Widdas, W.F., W.F. (1962) J. Physiol. (London) 160, 392–403.
7 Miller, D.M. (1968) Biophys. J. 8, 1329–1338.
8 Regen, D.M. and Tarpley, H.L. (1974) Biochim. Biophys. Acta 339, 218–233.
9 Naftalin, R.J. and Holman, G.D. (1977) in Membrane Transport in Red Cells (Ellory, J.C. and Lew, V.L., eds.), pp. 257–300, Academic Press, New York.
10 Marquardt, D.W. (1963) SIAM J. Appl. Math. 11, 431–441.
11 Faust, R.G. (1960) J. Cell Comp. Physiol. 56, 103–121.
12 Baker, G.F. and Naftalin, R.J. (1978) J. Physiol. (London) 280, 25P.