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HYDROGEN BONDING REQUIREMENTS FOR THE INSULIN-SENSITIVE SUGAR TRANSPORT SYSTEM OF RAT ADIPOCYTES

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(1) The $t_{1/2}$ for 1.3 mM D-allose uptake and efflux in insulin-stimulated adipocytes is 1.7 ± 0.1 min. In the absence of insulin mediated uptake of D-allose is virtually eliminated and the uptake rate ($t_{1/2}$ = 75.8 ± 4.99 min) is near that calculated for nonmediated transport. The kinetic parameters for D-allose zero-*trans* uptake in insulin-treated cells are K_{zt}^{oi} = 271.3 ± 34.2 mM, V_{zt}^{oi} = 1.15 ± 0.12 mM · s^{-1}. (2) A kinetic analysis of the single-gate transporter (carrier) model interacting with two substrates (or substrate plus inhibitor) is presented. The analysis shows that the heteroexchange rates for two substrates interacting with the transporter are not unique and can be calculated from the kinetic parameters for each sugar acting alone with the transporter. This means that the equations for substrate analogue inhibition of the transport of a low affinity substrate such as D-allose can be simplified. It is shown that for the single gate transporter the K_i for a substrate analogue inhibitor should equal the equilibrium exchange K_m for this analogue. (3) Analogues substituted at C-1 show a fused pyranose ring is accepted by the transporter. 1-Deoxy-D-glucose is transported but has low affinity for the transporter. High affinity can be restored by replacing a fluorine in the β-position at C-1. The K_i for D-glucose = 8.62 mM; the K_i for β-fluoro-D-glucose = 6.87 mM. Replacing the ring oxygen also results in a marked reduction in affinity. The K_i for 5-thio-D-glucose = 42.1 mM. (4) A hydroxyl in the *gluco* configuration at C-2 is not required as 2-deoxy-D-galactose (K_i = 20.75 mM) has a slightly higher affinity than D-galactose (K_i = 24.49 mM). A hydroxyl in the *manno* configuration at C-2 interferes with transport as D-talose (K_i = 35.4 mM) has a lower affinity than D-galactose. (5) D-Allose (K_m = 271.3 mM) and 3-deoxy-D-glucose (K_i = 40.31 mM) have low affinity but high affinity is restored by substituting a fluorine in the *gluco* configuration at C-3. The K_i for 3-fluoro-D-glucose = 7.97 mM. (6) Analogues modified at C-4 and C-6 do not show large losses in affinity. However, 6-deoxy-D-glucose (K_i = 11.08 mM) has lower affinity than D-glucose and 6-deoxy-D-galactose (K_i = 33.97 mM) has lower affinity than D-galactose. Fluorine substitution at C-6 of D-galactose restores high affinity. The K_i for 6-fluoro-D-galactose = 6.67 mM. Removal of the C-5 hydroxymethyl group results in a large affinity loss. The K_i for D-xylose = 45.5 mM. The K_i for L-arabinose = 49.69 mM. (7) These results indicate that the important hydrogen bonding positions involved in sugar interaction with the insulin-stimulated adipocytes transporter are the ring oxygen, C-1 and C-3. There may be a weaker hydrogen bond to C-6. Sugar hydroxyls in non-*gluco* configurations may sterically hinder transport.

Introduction

Sugar transport in rat adipocytes is increased by greater than 10-fold by insulin. This insulin stimulation has been studied in detail by Whitesell and Gliemann [1] who have developed techniques for measuring the transport of the non-metabolised D-glucose analogue, 3-*O*-methyl-D-glucose. Whitesell and Gliemann [1] and others (Vinten et al. [2], Siegel and Olefsky [3] and Czech [4]) have shown that insulin increases the V_{max} for 3-*O*-methyl-D-glucose uptake and exchange transport. We have recently extended

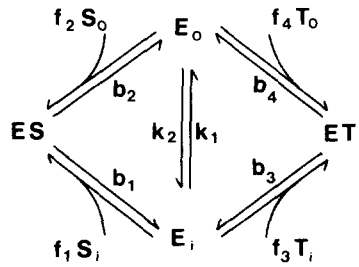
these studies on 3-*O*-methyl-D-glucose transport by showing symmetrical affinity constants in zero-*trans* entry and exit (transport into sugar-free solutions) and in infinite-*cis* entry and exit (transport into solutions of varying sugar concentrations) in basal and insulin-stimulated cells [5].

Despite extensive investigations on 3-*O*-methyl-D-glucose transport relatively little is known of the detailed specificity requirements of the adipocyte sugar transport system. One would like to know, for example, whether the insulin-dependent transport systems have similar specificity requirements to the well characterised non-insulin dependent systems such as that of the human erythrocyte. That adipocytes can transport 2-deoxy-D-glucose [6] makes the possibility of a similarity in specificity between this system and the Na-dependent systems unlikely since Na-dependent systems do not transport this analogue.

In the present investigation we develop the kinetics and a transport assay, for testing sugar analogues as inhibitors of transport of the low affinity sugar D-allose. D-Allose uptake is considerably slower than for 3-*O*-methyl-D-glucose and uptake can be studied over a time course of several minutes rather than seconds. Thus technical difficulties associated with measuring very rapid transport [5] are reduced. By measuring the relative inhibition by a series of D-glucose epimers, by deoxy-D-glucoses and by fluoro-substituted D-glucoses those sugar hydroxyls most important for hydrogen bonding have been determined. As far as possible we have used only those analogues which are considered to be poor substrates for hexokinase [7–9] so that inhibition is due to the analogue alone interacting with the transporter rather than a metabolite.

Determination of inhibition constants

As a starting point we consider the carrier model. The most readily acceptable molecular interpretation of the carrier model for sugar transport is that of a single reciprocating transport gate. Other models for sugar transport have been proposed with inner- and outer-membrane gates (for example the two-gate pore model of Foley et al. [10] or the allosteric pore model, Holman [11]) and these models will be of value if the single-gate (carrier) model can be shown to be invalid for each system under study.



Scheme I. The interaction of two substrates S and T with the carrier inside (E_i) and the carrier outside (E_o) to form the complexes ES and ET. f_1 and f_2 are the rate constants for formation of ES from inside and outside, respectively. f_3 and f_4 are the rate constants for formation of ET from inside and outside, respectively. b_1 and b_2 are the rate constants for breakdown of ES inside and outside while b_3 and b_4 are the rate constants for breakdown of ET inside and outside, respectively. k_1 is the rate constant for carrier movement from inside to outside while k_2 is the rate constant for carrier movement from outside to inside.

Stein and Lieb [12] and Geck [27] have given an important analysis of, and established rejection criteria for, the single-gate (carrier) model interacting with a single substrate. In order to extend this analysis to the two-substrate case we follow the approach of Stein and Lieb and as far as possible use comparable symbols. The model is shown in Scheme I. Rate constants for the translocation of the complexes ES and ET are omitted as these do not effect the steady-state rate equation [12].

From microscopic reversibility

$$b_2 k_2 f_1 = b_1 k_1 f_2$$

$$b_4 k_2 f_3 = b_3 k_1 f_4 \qquad (1)$$

$$\text{Tot} = E_o + E_i + ES + ET$$

After constructing the appropriate eight King-Altman diagrams and writing expressions for the concentrations of the intermediates, Eqn. 1 can be used to derive undirectional flux equations for substrates S and T

$$U^S_{oi} = \left(\frac{S_o}{K_S} + \frac{S_o S_i}{K_S^2} + \frac{S_o T_i}{K_S K_T}\right) \Big/ \left(R_{ZZ} + \frac{R^S_{oi} S_o}{K_S} + \frac{R^S_{io} S_i}{K_S} + \frac{R^T_{oi} T_o}{K_T} + \frac{R^T_{io} T_i}{K_T} + \frac{R^S_{ee} S_o S_i}{K_S^2} + \frac{R^T_{ee} T_o T_i}{K_T^2} + \frac{R^{ST}_{ee} S_o T_i}{K_S K_T} + \frac{R^{TS}_{ee} T_o S_i}{K_S K_T}\right) \qquad (2a)$$

$$U^S_{io} = \left(\frac{S_i}{K_S} + \frac{S_o S_i}{K_S^2} + \frac{S_i T_o}{K_S K_T}\right) \Big/ D \qquad (2b)$$

where D is the denominator in Eqn. 2

$$U_{oi}^{T} = \left(\frac{T_o}{K_T} + \frac{T_o T_i}{K_T^2} + \frac{T_o S_i}{K_S K_T}\right) \Big/ D \qquad (2c)$$

$$U_{io}^{T} = \left(\frac{T_i}{K_T} + \frac{T_o T_i}{K_T^2} + \frac{T_i S_o}{K_S K_T}\right) \Big/ D \qquad (2d)$$

Net fluxes of S are then

$$\text{net}_{oi}^{S} = \left(\frac{S_o - S_i}{K_S} + \frac{S_o T_i - S_i T_o}{K_S K_T}\right) \Big/ D$$

$$\text{net}_{io}^{S} = \left(\frac{S_i - S_o}{K_S} + \frac{S_i T_o - S_o T_i}{K_S K_T}\right) \Big/ D$$

with similar expressions for the net flux of T.

In these expressions

$$K_s = \left(\frac{k_1}{f_1} + \frac{k_2}{f_2}\right)$$

$$K_T = \left(\frac{k_1}{f_3} + \frac{k_2}{f_4}\right)$$

$$R_{ZZ} = \left(\frac{1}{k_1} + \frac{1}{k_2}\right)\frac{1}{\text{Tot}}$$

$$R_{oi}^{S} = \left(\frac{1}{b_1} + \frac{1}{k_1}\right)\frac{1}{\text{Tot}} \qquad R_{oi}^{T} = \left(\frac{1}{b_3} + \frac{1}{k_1}\right)\frac{1}{\text{Tot}}$$

$$R_{io}^{S} = \left(\frac{1}{b_2} + \frac{1}{k_2}\right)\frac{1}{\text{Tot}} \qquad R_{io}^{T} = \left(\frac{1}{b_4} + \frac{1}{k_2}\right)\frac{1}{\text{Tot}}$$

$$R_{ee}^{S} = \left(\frac{1}{b_1} + \frac{1}{b_2}\right)\frac{1}{\text{Tot}} \qquad R_{ee}^{T} = \left(\frac{1}{b_3} + \frac{1}{b_4}\right)\frac{1}{\text{Tot}}$$

$$R_{ee}^{ST} = \left(\frac{1}{b_1} + \frac{1}{b_4}\right)\frac{1}{\text{Tot}} \qquad R_{ee}^{TS} = \left(\frac{1}{b_2} + \frac{1}{b_3}\right)\frac{1}{\text{Tot}}$$

Each of the resistance terms (R's) can be determined from the V_{max} values obtained in experiments designed to give limiting forms of Eqn. 2. R_{io} and R_{oi} values can be determined in zero-*trans* experiments. R_{ee}^{S} and R_{ee}^{T} can be determined in exchange or infinite-*trans* experiments. The parameters R_{ee}^{ST} and R_{ee}^{TS} can be determined in heteroexchange or mixed infinite-*trans* experiments. R_{ee}^{ST} is determined when the influx of S_0 into infinite T_i or the efflux of T_i into infinite S_0 is measured. R_{ee}^{TS} is determined when the influx of T_0 into infinite S_i or the efflux of S_i into infinite T_0 is measured.

Of the resistance terms only five are unique and the following relationships exist among these parameters

$$R_{ZZ} = R_{oi}^{S} + R_{io}^{S} - R_{ee}^{S} = R_{oi}^{T} + R_{io}^{T} - R_{ee}^{T} \qquad (3)$$

From this relationship if any five of the six parameters (R_{oi}^{S}, R_{io}^{S}, R_{ee}^{S}, R_{oi}^{T}, R_{io}^{T}, R_{ee}^{T}) are known then the 6th can be calculated and used to test the model.

Also,

$$R_{ee}^{S} + R_{ee}^{T} = R_{ee}^{ST} + R_{ee}^{TS} \qquad (4)$$

Eqn. 4 is an extremely important result and has not been previously noted in other analyses of carrier transport [13]. It means that the sum of the reciprocals of the V_{max} values for exchange of sugars S and T should equal the sum of the reciprocals of the heteroexchange V_{max} values. Thus this equation provides a test of the single-gate (carrier) model and currently the equation is being tested for the interaction of sugars with the human erythrocyte sugar transport system.

In addition

$$R_{ee}^{ST} = R_{oi}^{S} + R_{io}^{T} - R_{ZZ}$$

$$R_{ee}^{TS} = R_{io}^{S} + R_{oi}^{T} - R_{ZZ} \qquad (5)$$

From Eqn. 5 it can be seen that the heteroexchange parameters for S and T (R_{ee}^{ST} and R_{ee}^{TS}) are not unique and can be calculated if the kinetic parameters for transport of S and T alone are known. Thus all the transport interactions between S and T are predictable. The equations for these interactions contain no new parameters but only different arrangements of them. The heteroexchange and mixed infinite-*trans* K_m values are predictable from simple equations derived from Eqn. 2 using the interrelationships among the parameters that are listed above. Eqn. 4 is also important because it allows further simplification of the carrier equation which allows it to be used in the determination of sugar analogue inhibition constants.

If the substrate is used at a low concentration relative to its K_m, if $S_0 = S_i$ and $T_0 = T_i$ in Eqn. 2 then uptake if radiolabelled substrate (S) is given

by the following equation:

$$V = U_{oi}^{S} = \frac{S}{R_{ZZ}K_S} \Big/ \left(1 + \frac{T \cdot R_{ee}^{T}}{K_T R_{ZZ}}\right) \quad (6)$$

Hence plotting S/V vs. T (the inhibitor concentration) gives the inhibition constant from the intercept on the abscissa and K/V for the substrate ($1/R_{ZZ}K_S$) from the ordinate.

Alternatively V_0/V (where V_0 is the uninhibited uptake) vs. T also gives the K_i from the intercept on the abscissa. From this equation it can be seen that the inhibition constant should equal the exchange K_m for the inhibitor. Also the inhibition constant should be independent of the substrate. If the inhibition constant is not equal to the exchange K_m for the inhibitor or if the inhibition constant varies depending upon which sugar is used as substrate then the carrier model can be rejected and multivalency is likely (that is more than one molecule of sugar (substrate or inhibitor) may interact with the transport system at any instant). The observation of substrate independent K_i values does not exclude the two-gate models. These means of distinguishing between transport models may form the basis of further experiments but the present investigation uses Eqn. 6 only to calculate a relative K_i and to compare inhibitor analogues.

If the assumption of equal inhibitor distribution is not met, as may be the case for a metabolised sugar such as D-glucose which may be removed internally, or if a nontransported inhibitor is only added externally, then the apparent K_i will mainly reflect the affinity of the external site. Also, we anticipate that the carrier model may be erroneous but that the K_i values that we have determined in this study will reflect the specificity of the predominant site in multiple-site models.

Materials and Methods

Crude collagenase (Type 1) was from Worthington Enzymes, Bovine serum albumin (fraction V) was from Sigma. Porcine monocomponent insulin was a gift from Novo Laboratories. Silicone oil was from Hopkins and Williams and phloretin was from K and K Laboratories.

Sugars. D-Allose, 1-deoxy-D-glucose, 5-thio-D-glucose, D-galactose, D-talose, D-fructose, L-arabinose, D-xylose, 2-deoxy-D-galactose, 6-deoxy-D-glucose were from Sigma and Koch-Light.

Other sugars were synthesised using the indicated methods. 3-Deoxy-D-glucose (Anet [14]), 3-deoxy-3-fluoro-D-glucose (Foster et al. [15]) β-D-glucopyranosyl fluoride (Michael and Klemer [16]), 6-deoxy-6-fluoro-D-galactose (Taylor and Kent [17]), 1-deoxy-D-[6-^{3}H]glucose (Barnett et al. [18]).

Radiolabelled D-allose was prepared by a modification of the method of Sowa and Thomas [19]. 260 mg of 1,2 : 5,6-di-*O*-isopropylidene-α-D-glucofuranose (Koch-Light) was dissolved in a mixture of dimethyl sulphoxide (0.6 ml) and acetic anhydride (0.4 ml) and left for 24 h at room temperature. The oxidised product 1,2 : 5,6-di-*O*-isopropylidene-α-D-*ribo*-hexofuranos-3-ulose was obtained in approx. 20% yield but was not purified at this stage. After removal of the solvents under reduced pressure 7 ml of ethanol was added and the solution stirred on ice. 25 mCi of tritiated sodium borohydride (The Radiochemical Centre Amersham 50–500 mCi/mmol) in 3 ml of water followed by 260 mg of cold sodium borohydride were then added. The product was extracted into chloroform, evaporated to dryness and applied to a silica gel column (3 × 30 cm Kieselgel 40) and run in ethyl acetate/petroleum ether (b.p. 40–60°C) (3 : 1, v/v). Tritiated di-isopropylidene-D-allose was well separated from its impurities (mainly nonradiolabelled di-isopropylide-D-glucose). After removal of solvents pure di-isopropylidene-D-[3-^{3}H]-allose was dissolved in 1 ml ethanol and 1 ml water with 0.5 g Dowex H^+ resin and magnetically stirred at 70°C for 3 h. D-[3-^{3}H]-Allose was then separated from solvent and was 99% pure. Yield 4 mCi, 34 mg. This preparative procedure could probably have been improved either by preparing more 1,2 : 5,6-di-*O*-isopropylidene-α-D-*ribo*-hexofuranos-3-ulose or by using a higher specific activity sodium borotritide.

Preparation of adipocytes. Isolated adipocytes were prepared from rat epididymal fat tissue from Wistar rats weighing 150–160 g. The method was essentially that of Foley et al. [10]. Tissue, chopped with scissors, was digested in Hepes buffer (pH = 7.4, 37°) (Na^+ 140 mM; K^+ 4.7 mM; Ca^{2+} 2.5 mM; Mg^{2+} 1.25 mM; Cl^- 142 mM; $H_2PO_4^-/HPO_4^{2-}$ 2.5 mM; SO_4^{2-} 1.25 mM; 4-(2-hydroxyethyl)-1-piperazine

ethane sulphonic acid 10 mM) containing 3.5% albumin, 0.5 mg/ml collagenase and 0.5 mM D-glucose. The digestion time was approximately 1 h. The digested tissue was filtered through a nylon mesh (mesh size 250 μm) and the isolated cells were carefully washed 5 times in Hepes 1% albumin buffer. Cell suspensions were adjusted to 44% cytocrit in buffer with or without 10 nM insulin.

D-Allose transport measurements. Inhibition of D-allose transport was measured using the oil flotation technique of Whitesell and Gliemann [1] with slight modifications. A 44% cell suspension was incubated for 30 min with D-allose (final concentration 1.3 mM) and inhibitor (0–40 mM final concentration range). This reduced the cell suspension to 40% cytocrit. 50 μl of this suspension was added to 15 μl of inhibitor (0–40 mM) with D-[3-^{3}H]allose (1.3 mM) in albumin free buffer. Uptake was measured after 0, 1, 2, 3 min and at equilibrium, 45 min. At equilibrium the D-allose equilibrium space was equal to the 3-*O*-methyl-D-glucose equilibrium space and was 1.8 μl/100 μl of packed cells. In each case transport was terminated by the addition of 3 ml Hepes buffer containing 0.3 mM phloretin. The cells were then spun through a 1 ml layer of silicone oil in a bench centrifuge for 1 min at 2 500 × *g*. The separated cells were removed from the top of the oil with a pipe cleaner. The trapped radioactivity was estimated by liquid scintillation counting. Average uptake rates were calculated from the equation

$$V = (S_0/t)\ln(1/(1-f))$$

where S_0 is the external concentration, f is the fractional filling and t is the time.

Average inhibition constants (K_i values) were calculated from the equation

$$V_0/V = 1 + (I/K_i)$$

where V is the inhibited rate and V_0 is the uninhibited rate and I is the inhibitor concentration.

Results

Our initial investigations were of uninhibited transport of D-allose in insulin-treated cells. The uptake of D-allose is shown in Fig. 1. The uptake follows a simple exponential function of time and fractional fillings of up to 75% were used to give linear plots of $\ln(S_0/(S_0 - S_T))$ vs. time. Also shown in Fig. 1 is the efflux of D-allose from adipocytes preincubated with D-allose for 45 min. The influx and efflux rates are equal. The D-allose equilibrium volume is equal to the 3-*O*-methyl-D-glucose equilibrium volume and this is consistent with D-allose being a transported but nonmetabolised D-glucose analogue.

Loten et al. [20] first studied D-allose uptake in rat adipocytes and have reported that it is nonmetabolised and transported by the insulin-dependent monosaccharide transport system. We have confirmed the insulin sensitivity in the presence of insulin the D-allose uptake rate $t_{1/2} = 1.7 \pm 0.1$ min. In the absence of insulin D-allose transport is 44-times slower $t_{1/2} = 75.8 \pm 5.0$ min (Fig. 1). In basal cells the D-allose uptake is incompletely inhibited by 50 μM cytochalasin B and D-glucose analogues and the rate is close to that previously calculated for nonmediated diffusion [1,28]. However, when tracer uptake rates of D-allose and 3-*O*-methyl-D-glucose are corrected for cytochalasin B (50 μM) uninhibitable uptake then the ratio of the halftimes of tracer D-allose/tracer 3-*O*-methyl-D-

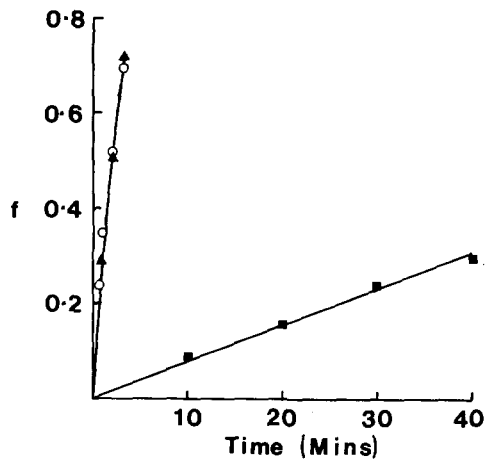


Fig. 1. Time courses for the fractional efflux (○) and fractional influx (▲) of 1.3 mM D-allose in cells treated with 10 nM insulin. In insulin-treated cells the influx $t_{1/2} = 1.77 \pm 0.13$ min (S.E., $n = 12$). In basal cells (■) $t_{1/2} = 75.88 \pm 4.99$ min (S.E., $n = 7$).

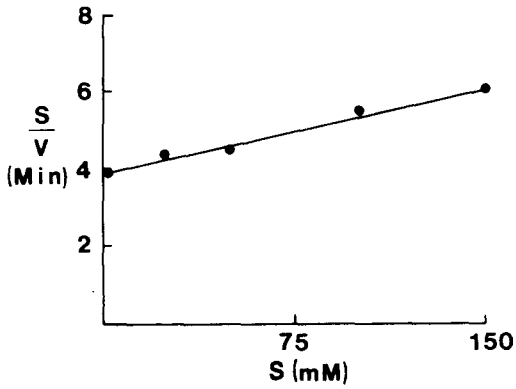


Fig. 2. Zero-*trans* influx of D-allose in cells treated with 10 nM insulin. $K_{zt}^{oi} = 271.3 \pm 34.2$ mM, $V_{zt}^{oi} = 1.15 \pm 0.12$ mM · s^{-1}. (Best fit estimates ±S.E. from weighted regression, $n = 5$).

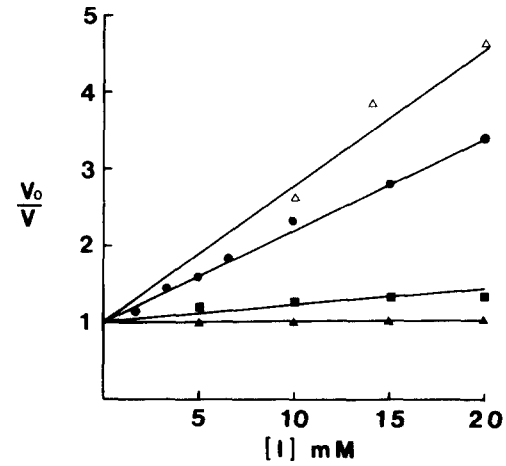


Fig. 3. Inhibition of D-allose transport (in cells treated with 10 nM insulin) by D-glucose analogues modified at C-1. ▲——▲, 1-Deoxy-D-glucose, $K_i > 150$ mM; ■——■, 5-thio-D-glucose, $K_i = 42.1 \pm 6.0$ mM (S.E., $n = 12$); ●——●, D-glucose, $K_i = 8.62 \pm 0.71$ mM (S.E., $n = 34$); △——△, β-D-glucopyranosyl fluoride, $K_i = 6.87 \pm 0.59$ mM (S.E., $n = 18$).

glucose uptake is approx. 45 in the presence and in the absence of insulin.

We have measured the kinetic parameters for zero-*trans* D-allose influx in insulin-treated cells (Fig. 2): $K_{zt}^{oi} = 271.3 \pm 34.2$ mM, $V_{zt}^{oi} = 68.7 \pm 7.25$ mM · min^{-1}. Hence the V_{max} for D-allose is similar to that previously measured for zero-*trans* 3-*O*-methyl-D-glucose influx (Taylor and Holman [5]). The K_m however is much higher and therefore at the concentration of D-allose used in the inhibition experiments the D-allose permeability (V/K) is very low.

C-1 analogues

Fig. 3 shows the inhibition by D-glucose analogues modified at C-1 or in the ring oxygen. Our results show that D-glucose has a $K_i = 8.62 \pm 0.71$ mM for inhibition of D-allose transport. Loten et al. [20] report a $K_i = 14.8$ mM for the same experiment. However, other groups have also reported a lower K_i value. Whitesell and Gliemann [1] have reported a D-glucose $K_i = 7$ mM using tracer 3-*O*-methyl-D-glucose as a substrate while Foley et al. [21] using L-arabinose as a substrate find a D-glucose $K_i = 8$ mM. Isolated plasma membranes behave differently. Cushman and Wardzala [22] report a $K_i = 30$ mM for D-glucose inhibition of cytochalasin B binding to plasma membranes while Ludvigsen and Jarrett [23], also working on adipocyte plasma membranes, report a D-glucose $K_m = 26$ mM. This anomaly may be due to the regulation of transport in intact adipocytes by sugar metabolites (Foley et al. [10]). The effect is being further investigated and the present investigation is confined as far as possible to a comparison of relative affinity of transported but poorly metabolised D-glucose analogues. We consider that providing the same substrate (D-allose) is used for other inhibitors then the relative K_i values give a measure of relative affinity.

Replacing the C-1 hydroxyl to produce 1-deoxy-D-glucose gave a large reduction in affinity with no detectable inhibition over the concentration range studied. A C-1 hydroxyl is not essential for transport however. Fig. 4 shows the uptake of 1-deoxy-D-glucose in the presence and in the absence of D-glucose. At the concentration tested uptake is slow but D-glucose clearly inhibits uptake confirming that 1-deoxy-D-glucose is transported by the D-glucose transport system. The initial uptake rate for 1-deoxy-D-glucose in the absence of D-glucose is 20 mM · min^{-1}. Assuming a V_{max} for 1-deoxy-D-glucose of 60–80 mM · min^{-1} (this is the V_{max} for both D-allose and for 3-*O*-methyl-D-glucose) then the approximate K_m for 1-deoxy-D-glucose is > 150 mM.

A high affinity can be restored by replacing a fluorine in the β-position at C-1. β-D-Glucopyra-

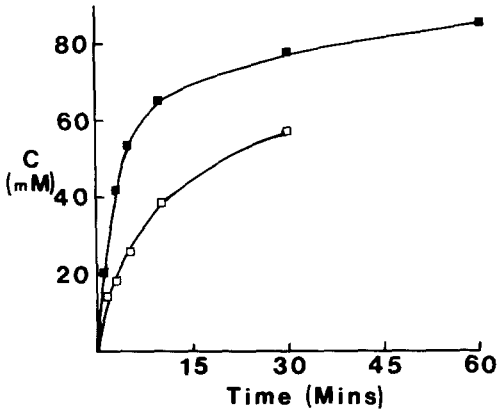


Fig. 4. A time course for the uptake of 85 mM 1-deoxy-D-[6-^{3}H]glucose in cells treated with 10 nM insulin in the presence (□) and in the absence (■) of 50 mM D-glucose. Results are the means of duplicate observations.

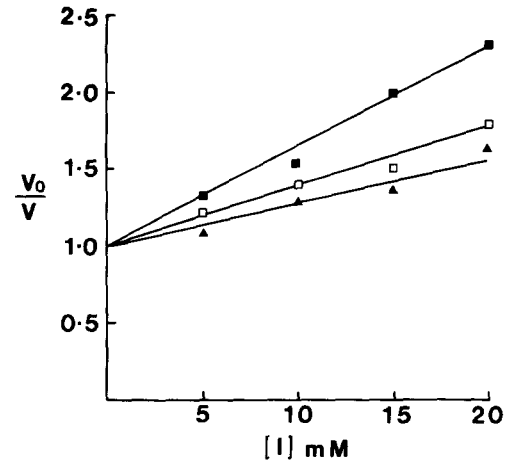


Fig. 5. Inhibition of D-allose transport (in cells treated with 10 nM insulin) by D-galactose analogues modified at C-2. ▲——▲, D-Talose, K_i = 35.39 ± 5.3 mM (S.E., n = 10); □——□, D-galactose, K_i = 24.49 ± 3.05 mM (S.E., n = 12); ■——■, 2-deoxy-D-galactose, K_i = 20.75 ± 3.04 mM (S.E., n = 9).

nosyl fluoride is a good inhibitor with a K_i = 6.87 ± 0.59 mM. We have previously used fluorine substitution for hydroxyl groups to indicate hydrogen bonding [18]. The electronegative fluorine appears to accept a H-group from the transport with no loss of affinity compared with the hydroxyl in D-glucose. This indicates that the electronegative O-group from the hydroxyl probably accepts a H-group from the transporter but that the H-group from the hydroxyl does not significantly bind to an electronegative group on the transporter. Replacing the ring-oxygen with a ring-sulphur results in a large loss of affinity (K_i = 42.1 ± 6.0 mM for 5-thio-D-glucose) indicating a H-bond from the transporter to the electronegative ring-oxygen.

Substitution at C-1 of D-glucose results in a fused pyranose ring form (ring opening cannot occur because of the absence of the anomeric hydroxyl). The results from C-1 substitution thus also indicate that the pyranose ring is accepted with high affinity by the site (β-fluoro-D-glucoside) and is transported (1-deoxy-D-glucose) and therefore that the open chain for sugars is not required.

C-2 analogues

The effect of modification in the C-2 position of D-glucose analogues is shown in Fig. 5. Because of the high affinity of 2-deoxy-D-glucose and of D-mannose for hexokinase, and a possible modification of transport by sugar metabolites [10], we confine our consideration of transport specificity to a comparison of D-galactose analogues modified at C-2. The inhibition produced by 2-deoxy-D-galactose K_i = 20.75 ± 3.04 mM is slightly greater than for D-galactose. K_i = 24.49 ± 3.05 mM. This indicates that there is no hydrogen bond to the C-2 position and that a hydroxyl in the *gluco* configuration at C-2 may hinder binding. However, a hydroxyl at C-2 in the *gluco* configuration interferes with binding less than a hydroxyl at C-2 in the *manno* configuration. Thus the D-galactose analogue with the *manno* configuration at C-2 (D-talose) has a K_i = 35.4 ± 5.3 mM.

C-3 analogues

D-Allose, the substrate used in this study is the C-3 epimer of D-glucose. It has a very low affinity K_m = 271.3 ± 34.2 mM when compared with D-glucose or D-galactose. 3-Deoxy-D-glucose also shows low affinity K_i = 40.31 ± 4.2 mM. This indicates that a hydrogen bond is probably directed to the *gluco* configuration at C-3 and that a hydroxyl in the *allo* configuration at C-3 interferes with transport. Transport inhibition can be greatly restored by replacing a fluorine in the *gluco* configuration. Thus the transporter has a high affinity for 3-fluoro-D-glucose K_i = 7.97 ± 0.44 mM indicating that a

hydrogen bond is probably directed towards the electronegative group (F or O) at C-3 rather than from the H-group of the hydroxyl at C-3.

C-4 and C-6 analogues

D-Galactose the C-4 epimer of D-glucose has a lower affinity than D-glucose. This reduction in affinity is much less than that observed when the D-glucose and D-galactose affinities for the human erythrocyte sugar transport system are compared. In the human erythrocyte the affinity for D-galactose is a tenth of the affinity for D-glucose [18]. Thus if a hydrogen bond to the *gluco* configuration at C-4 is involved in adipocyte transport it is relatively unimportant. Also, the possibility that the lowering of affinity is due to the hydroxyl in the *galacto* configuration at C-4 interfering with the binding at other positions cannot be excluded.

A C-6 hydroxyl or fluorine appears to slightly increase binding when the substitutions are compared with the relevant C-6 deoxy compound. Thus 6-deoxy-D-glucose (K_i = 11.08 ± 0.63 mM) has lower affinity than D-glucose while D-fucose (6-deoxy-D-galactose, K_i = 33.97 ± 5.03 mM) has lower affinity than D-galactose and 6-fluoro-D-galactose (K_i = 6.67 ± 0.37 mM). This indicates a hydrogen bond to C-6 but this bond is probably not as important as those bonds to the ring-oxygen, to C-1 and to C-3. Removal of the C-6 hydroxyl results in a greater affinity loss for the D-galactose derivative (D-fucose) than for the corresponding D-glucose derivative (6-deoxy-D-glucose). This may mean that the proposed H-bond to C-6 becomes more important when the bonding or spatial requirements at C-4 are not met. Removing the C-6 carbon (C-5 hydroxymethyl) group altogether also reduces affinity. Thus D-xylose, which can be regarded as a D-glucose analogue without a C-5 hydroxymethyl group, and L-arabinose, which can be regarded as a D-galactose analogue without the C-5 hydroxymethyl group, both have low affinity but there is little discrimination between the D-glucose and the D-galactose analogue. The K_i for D-xylose = 45.5 ± 3.25 mM while the K_i for L-arabinose is similar and equal to 49.69 ± 4.93 mM.

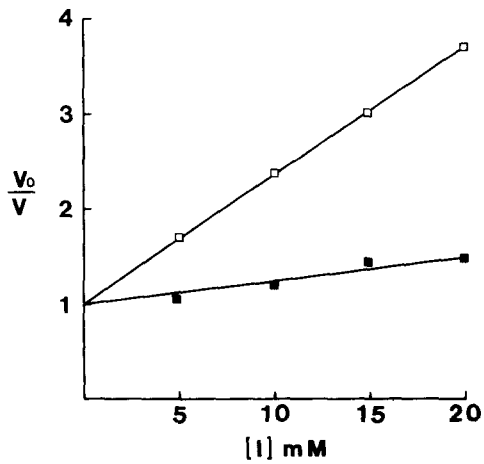


Fig. 6. Inhibition of D-allose transport (in cells treated with 10 nM insulin) by D-glucose analogues modified at C-3. ■———■, 3-Deoxy-D-glucose, K_i = 40.31 ± 4.2 mM (S.E., n = 10); □———□, 3-deoxy-3-fluoro-D-glucose, K_i = 7.97 ± 0.44 mM (S.E., n = 24).

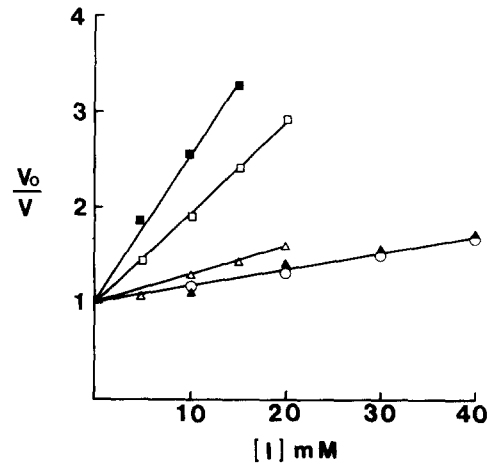
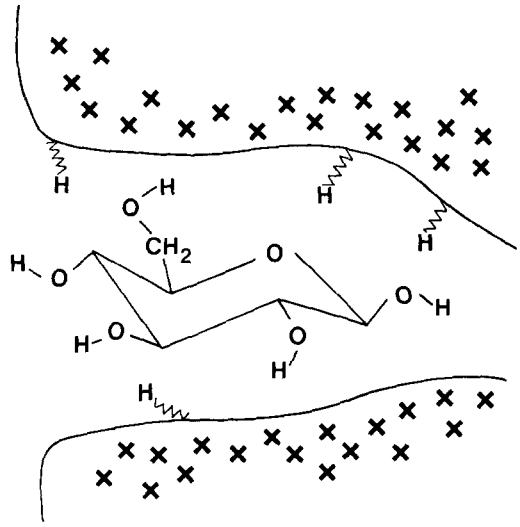


Fig. 7. Inhibition of D-allose transport (in cells treated with 10 nM insulin) by D-glucose analogues modified at C-4 and C-6. ▲———▲, L-Arabinose, K_i = 49.69 ± 4.93 mM (S.E., n = 21); ○———○, D-xylose, K_i = 45.56 ± 3.25 mM (S.E., n = 24); △———△, D-fucose, K_i = 33.97 ± 5.03 mM (S.E., n = 15); □———□, 6-deoxy-D-glucose, K_i = 11.08 ± 0.63 mM (S.E., n = 11); ■———■, 6-deoxy-6-fluoro-D-galactose, K_i = 6.67 ± 0.37 mM (S.E., n = 11).

Discussion

From the relative K_i values calculated for inhibition of D-allose transport (Results) it is possible to construct a model for sugar binding to the active site of the insulin sensitive adipocyte sugar trans-



Scheme II. A diagram of the proposed important H-bonding positions. These are to the ring-oxygen, C-1 and C-3 and to a lesser extent C-6. The preferred conformation is $^4C_1$ glucopyranose.

porter (Scheme II). The high affinity (β-fluoro-D-glucoside) and transport (1-deoxy-D-glucose) of the fused pyranose ring analogues indicate that the site accepts the pyranose ring. NMR investigations [24] have revealed that the α and particularly the β anomers of all the common monosaccharides exist predominately in the $^4C_1$ rather than the $^1C_4$ conformation. This will include all the analogues tested here and hence the possibility of selectivity based solely on the proportionate stability of the $^4C_1$ form [25] is unlikely. Thus relative affinities of the tested analogues will reflect binding to specific groups on the transporter.

The important hydrogen bonding positions on the sugar appear to be the ring-oxygen, the β-position at C-1, a hydroxyl in the *gluco* configuration at C-3 and to a lesser extent a hydroxyl at C-6. The C-2 and possibly the C-4 hydroxyls seem to be relatively unimportant. In many respects therefore the adipocyte sugar transport system resembles that of the human erythrocyte [18]. The most obvious differences, however, appear to indicate an apparent lack of specificity of the adipocytes system for the C-4 and C-6 positions. There are smaller differences between the affinities for D-glucose and D-galactose (and between other analogues of the D-glucose series when compared with the D-galactose series) in the rat adipocyte when compared with the human erythrocyte system. Also the loss of the C-6 hydrogen bond is less important in the adipocyte when compared with the human erythrocyte system.

Interpretation of the results in terms of specific hydrogen bonding groups may not be entirely straightforward and some further considerations need to be mentioned. Firstly, binding to the transport system and sugar translocation through the transporter are likely to be substrate induced phenomena. Thus although H-bonds are, at some stage in transport, involved at the ring-oxygen, C-1, C-3 and C-6 these positions need not necessarily all hydrogen bond together and some sharing of membrane bonding groups as the sugar moves is possible. Also, changes in the hydrogen bonding to water may be important at some stages of transport and this may also effect the apparent affinity constants.

Spatial requirements of the site may also affect the apparent affinity constants. Thus epimerisation at C-2 and particular C-3 result in hydroxyl configurations that confer a greater affinity loss than conversion to the deoxy compound. This may be because the inverted hydroxyls spatially interfere with the approach of hydroxyls and membrane groups at the nonepimerised positions. In human erythrocytes alkyl derivatives of sugars have been useful in the determination of the spatial requirements for the transport active site [26]. We have carried out a similar study on the adipocyte sugar transport system and the results (Holman, G.D. and Rees, W.D., unpublished results) generally show a looser fit and less exacting spatial requirements than for the human erythrocyte system.

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