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SPATIAL REQUIREMENTS FOR INSULIN-SENSITIVE SUGAR TRANSPORT IN RAT ADIPOCYTES

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(1) Alkyl sugar inhibition of D-allose uptake into adipocytes has been used to explore the spatial requirements of the external sugar transport site in insulin-treated cells. α-methyl and β-methyl glucosides show low affinity indicating very little space around C-1. The high affinity of D-glucosamine (K_i = 9.05 ± 0.66 mM) is lost by *N*-acetylation. *N*-Acetyl-D-glucosamine shows no detectable affinity, indicating that a bulky group at C-2 is not accepted. Similarly 2,3-di-*O*-methyl-D-glucose (K_i = 42.1 ± 7.5 mM) has lower affinity than 3-*O*-methyl-D-glucose (K_i = 5.14 ± 0.32 mM) indicating very little space around C-2 but much more around C-3. A reduction in affinity does occur if a propyl group is introduced into the C-3 position. The K_i for 3-*O*-propyl-D-glucose is 11.26 ± 2.12 mM. 6-*O*-Methyl-D-galactose (K_i = 87.2 ± 17.9 mM) and 6-*O*-propyl-D-glucose (K_i = 78.07 ± 12.6 mM) show low affinity compared with D-galactose and D-glucose, indicating steric constraints around C-6. High affinity is restored in 6-*O*-pentyl-D-galactose (K_i = 4.66 ± 0.23 mM) possibly indicating a hydrophobic binding site around C-6). (2) In insulin treated cells 4,6-*O*-ethylidene-D-glucose (K_i = 6.11 ± 0.5 mM) and maltose (K_i = 23.5 ± 2.1 mM) are well accommodated by the site but trehalose shows no detectable inhibition. These results indicate that the site requires a specific orientation of the sugar as it approaches the transporter from the external solution. C-1 faces the inside while C-4 faces the external solution. (3) To determine the spatial and hydrogen bonding requirements for basal cells 40 μM 3-*O*-methyl-D-glucose was used as the substrate. Poor hydrogen bonding analogues and analogues with sterically hindering alkyl groups showed similar K_i values to those determined for insulin-treated cells. These results indicate that insulin does not change the specificity of the adipocyte transport system.

Introduction

In the investigation of human erythrocyte sugar transport alkyl derivatives of sugars have been used to explore the spatial requirements for binding to the transport active site. Baker and Widdas [1] have shown that 4,6-*O*-ethylidene-D-glucose is a good inhibitor of sugar transport with an affinity constant for the external face of the transporter that is comparable with that of D-glucose. Barnett et al. [2] showed similar behaviour with 6-*O*-alkyl derivatives of D-glucose and of D-galactose and concluded that since no loss of affinity occurred on the introduction of bulky alkyl groups into the C-4 and C-6 positions then there need not be a close approach of the transporter to these positions for strong binding. However, these substitutions resulted in loss of transport indicating that the spatial requirements for transport are different from those for external binding. Spatial requirements for external binding to the transporter were shown to be different from the spatial requirements for internal binding to the transporter since there is no glycoside binding site at the external surface but there is such a site at the internal surface [2,3].

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.

The present report examines whether the rat adipocyte sugar transport system is similar to the erythrocyte system and whether it also shows specific spatial requirements at the external site and whether the sugar shows a specific orientation in the adipocyte transporter.

In a previous report [4] we detailed information on the hydrogen bonding requirements for the transporter in insulin-treated cells. In this study we showed that the important hydrogen bonding groups were at the ring-oxygen, at C-1 and C-3. Also it was suggested that there was a weaker H-bond to C-6. The low affinity substrate D-allose was used in these experiments and in basal cells D-allose transport was very slow and at a rate comparable with that expected for non-mediated transport. Hence using D-allose we were unable to obtain information on the specificity of basal cells. In the present study we have used 3-*O*-methyl-D-glucose as the substrate to investigate hydrogen-bonding and spatial requirements for the transport site of basal cells. The results show that, for all the analogues tested, the affinity constants (K_i values) are very similar in insulin-treated and in basal cells indicating that insulin does not cause a major structural change in the transporter but instead increases the availability of sites of identical specificity.

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. Silicon oil was from Hopkins and Williams and phloretin was from K. & K. Laboratories. D-Allose, D-glucose, 5-thio-D-glucose, 1-deoxy-D-glucose, 2-deoxy-D-galactose, 3-*O*-methyl-D-glucose, 6-deoxy-D-glucose, D-glucosamine and *N*-acetyl-D-glucosamine were from Sigma. Trehalose, maltose and methyl-α-D-glucopyranoside were from B.D.H. Ltd., 2-deoxy-D-galactose, 2,3-di-*O*-methyl-D-glucose, 6-*O*-methyl-D-galactose, 4,6-*O*-ethylidene-D-glucose and methyl-β-D-glucopyranoside were from Koch-Light. 3-Deoxy-D-glucose was synthesised following the method of Anet [5]. D-[3-^{3}H]Allose was prepared following the method of Sowa and Thomas [6] as previously described [4].

Preparation of alkyl substituted sugars

3-*O*-[^{14}C]Methyl-D-glucose, 3-*O*-propyl-D-glucose, 6-*O*-propyl-D-glucose and 6-*O*-pentyl-D-galactose were prepared using similar methods. The general method has been previously described [3]. The method is described here for 3-*O*-[^{14}C]methyl-D-glucose. 250 mg of 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (Koch-Light) were dissolved in 1.5 ml of dry dioxan containing 300 mg of freshly powdered sodium hydroxide. This mixture was added to methyl iodide (1 mCi, >50 mCi/mmol supplied by the Radiochemical Centre, Amersham) in a break seal tube and after transfer to a round bottomed flask was heated at 40°C overnight. The reaction products were added to ice water/diethyl ether and the diethyl ether layer was separated and dried over anhydrous sodium sulphate. After evaporation to dryness 1 ml water plus 1 ml ethanol and 0.5 g Dowex 50 H^+ resin were added. After heating at 70°C for 4 h the resin was removed, the solution was washed with ether and after removal of solvents 3-*O*-[^{14}C]methyl-D-glucose was separated from impurities (mainly unlabelled D-glucose) by preparative paper chromatography. Yield 650 μCi.

For the preparation of 3-*O*-propyl-D-glucose, 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose was treated with excess *n*-propyl iodide. For 6-*O*-propyl-D-glucose 3,5-*O*-benzylidene-1,2-*O*-isopropylidene-α-D-glucofuranose was treated with *n*-propyl iodide, while 6-*O*-pentyl-D-galactose was prepared from 1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose and *n*-pentyl iodide.

Preparation of adipocytes

Isolated adipocytes were prepared from epididymal fat tissue from male Wister rats weighing 150–170 g. The method was essentially that of Foley et al [7]. Tissue, chopped with scissors was digested in Hepes buffer (pH = 7.4, 37°C) (140 mM Na^+, 4.7 mM K^+, 2.5 mM Ca^{2+}, 1.25 mM Mg^{2+}, 142 mM Cl^-, 2.5 $H_2PO_4^-/HPO_4^{2-}$, 1.25 mM SO_4^{2-}, 10 mM Hepes) containing 3.5% albumin, 0.5 mg/ml collagenase and 0.5 mM D-glucose. The digestion time was approx. 1 h. The digested tissue was filtered through a nylon mesh (mesh size 250 μm) and the isolated cells were carefully washed five times in Hepes/1% albumin buffer.

Transport measurements

These were carried out as previously described

[4,8]. 50 μl of cell suspension, in some cases preincubated with inhibitor and nonradiolabelled substrate for 30 min, was added to 15 μl of albumin free buffer containing radiolabelled substrate and an appropriate concentration of inhibitor at 37°C. 1.3 mM D-allose transport in insulin-treated cells was measured over 1, 2 and 3 min. Basal 40 μM 3-*O*-methyl-D-glucose transport was also measured over 1, 2 and 3 min. In all cases transport was terminated by the addition of 3 ml of 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:

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

where S_0 is the substrate concentration, which is low compared with the substrate K_m, f is the fractional filling and t is the time.

Inhibition constants (K_i) were calculated from the equation

$$v_0/v = 1 + I/K_i \quad (1)$$

where v is the inhibited rate and v_0 is the uninhibited rate and I is the inhibitor concentration.

In order to reveal spatial features of the external site the nontransported inhibitors were usually added with the substrate. Under these circumstances (for the monovalent carrier model and also for multiple site models [7,13]) it can be shown that the K_i determined using Eqn. 1 reflects the external dissociation constant for the inhibitor.

Results

Insulin-treated cells

We have previously shown that the K_i for D-glucose inhibition of D-allose transport is 8.62 ± 0.71 mM (n = 34). Fig. 1 shows the inhibition of allose uptake in insulin-treated cells. It is shown that the K_i value is greatly increased if a methyl group is introduced into either the α or into the β configuration. α-Methyl-D-glucoside has a K_i = 57.9 ± 13.6 mM (n = 19) while β-methyl-D-glucoside shows no detectable inhibition over the concentration range tested.

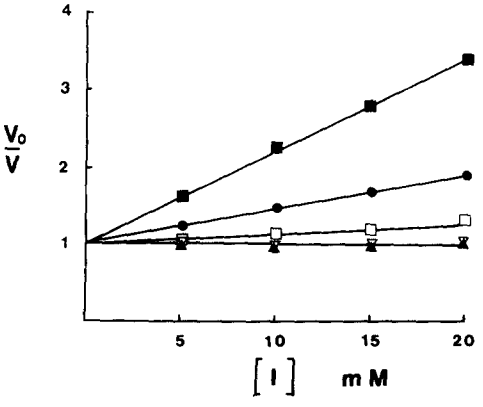


Fig. 1. Inhibition of the uptake of 1.3 mM D-allose in insulin-treated cells by (△) β-methyl-D-glucoside (30 min preincubation), (□) α-methyl-D-glucoside (30 min preincubation) and by (▲) trehalose, (●) maltose and (■) D-glucose.

Also shown in Fig. 1 is a comparison of the affinity of two disaccharides. Maltose has a K_i = 23.5 ± 2.1 mM (n = 12) while trehalose shows no detectable inhibition. This indicates that in trehalose (α-1,1-glucopyranosyl-glucopyranoside) hydrogen bonding to the glucose units is hindered because of the α-1,1-linkage and that the relatively unhindered C-4 and C-6 positions do not bind well. In maltose (α-1,4-glucopyranosyl-glucopyranose) the reducing glucose unit is accommodated by the site but the bulky non-reducing glucose unit must project from the site at C-4. These results indicate that the external site requires a specific orientation of the sugar and an unhindered C-1 position.

The results in Fig. 2 show that there is very little space available in the transport site around the C-2 position of the sugar. D-Glucosamine binds well (K_i = 9.05 ± 0.66 mM, n = 12) but introducing an acetate group to give *N*-acetyl-D-glucosamine reduces the affinity to undetectable levels. Carter-Su and Czech have also shown a high affinity of the transporter for D-glucosamine [9].

If 3-*O*-methyl-D-glucose (Fig. 3) is compared with 2,3-di-*O*-methyl-D-glucose (K_i = 42.09 ± 7.5 mM, n = 11) then it is seen that the additional methyl group at C-2 markedly reduces affinity. This again indicates that there is very little space available around C-2.

In contrast to the lack of accommodation of

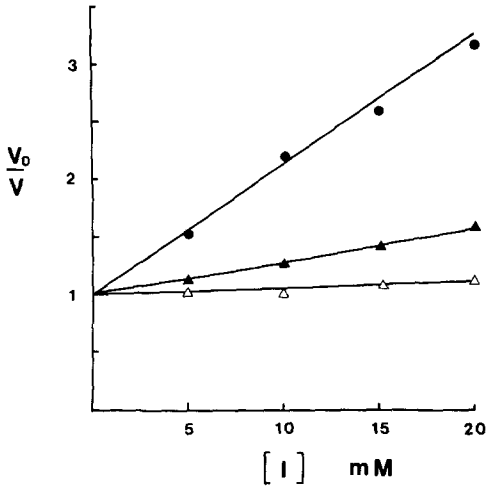


Fig. 2. Inhibition of the uptake of 1.3 mM D-allose in insulin-treated cells by (▲) 2,3-di-*O*-methyl-D-glucose, by (●) D-glucosamine and by (△) *N*-acetyl-D-glucosamine. After 30 min preincubation in each case.

methyl groups at C-1 and C-2 a C-3 methyl group is accepted well and 3-*O*-methyl-D-glucose has high affinity K_i = 5.41 ± 0.32 mM (n = 12). The affinity is higher than for D-glucose and this may be due to hydrophobic binding of the methyl group or alternatively the methyl group may confer increased electronegativity to the adjacent oxygen which is thought to accept a hydrogen bond from the membrane [4]. The K_i calculated for 3-*O*-methyl-D-glucose inhibition of D-allose is very similar to the equilibrium exchange K_m for 3-*O*-methyl-D-glucose [10,11]. Loten et al. [12] also measured 3-*O*-methyl-D-glucose inhibition of D-allose uptake in adipocytes and report a similar K_i value. The similarity of the K_m for 3-*O*-methyl-D-glucose transport to the K_i calculated for inhibition of D-allose transport by 3-*O*-methyl-D-glucose suggests that the two sugars are transported by the same transport system. Inhibition plots are linear using 3-*O*-methyl-D-glucose concentrations up to 40 mM providing an allowance is made for the small proportion of the flux (about 2%) that occurs through a route that is insensitive to 50 μM cytochalasin B (data not shown).

Increasing the size of the alkyl group at C-3 from a methyl to a propyl group results in a reduction in affinity. The K_i for 3-*O*-propyl-D-glucose is 11.26 ± 2.12 mM (n = 12). Thus although there is more space available at C-3 than at C-1 and C-2 the propyl group shows some steric hindrance.

4,6-*O*-Ethylidene-D-glucose is a good inhibitor of D-allose uptake with a K_i comparable with that of D-glucose (Fig. 4). The K_i for 4,6-*O*-ethylidene-D-glucose is 6.11 ± 0.50 mM (n = 12). This result indicates that a bulky group directed towards C-4 does

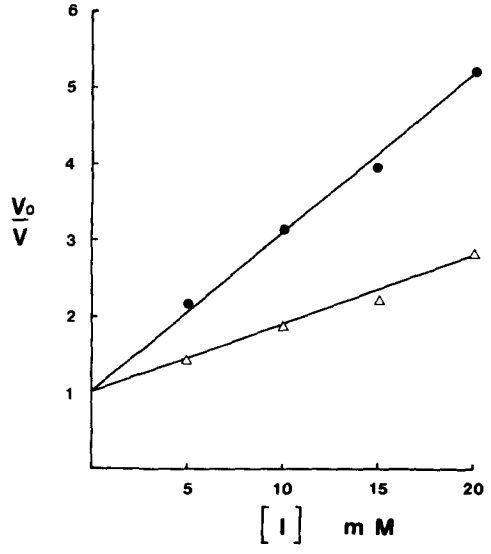


Fig. 3. Inhibition of the uptake of 1.3 mM D-allose in insulin-treated cells by (●) 3-*O*-methyl-D-glucose (30 min preincubation) and by (△) 3-*O*-propyl-D-glucose.

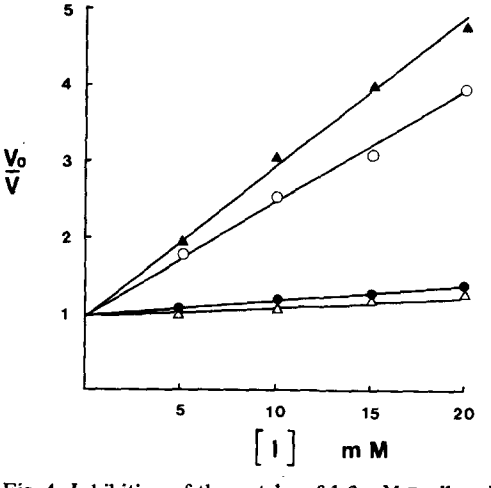


Fig. 4. Inhibition of the uptake of 1.3 mM D-allose in insulin-treated cells by (△) 6-*O*-methyl-D-galactose (30 min preincubation), by (○) 4,6-*O*-ethylidene-D-glucose, (▲) 6-*O*-pentyl-D-galactose and (●) 6-*O*-propyl-D-glucose.

not interfere with binding and supports the suggestion (based on the inhibition by maltose data) that the sugar orientation in the outer transport site is such that C-4 projects into the external solution. Using 4,6-*O*-ethylidene-D-[U-^{14}C]glucose we have shown that this compound is not transported by the D-glucose transporter (Holman, G.D. and Rees, W.D. unpublished data) hence this compound binds to the transport system but, because of interference by the ethylidene group, is not transported by it. This suggests that sugar translocation in adipocytes but not binding involves a conformational change in the transporter around the C-4 position of the sugar. In this respect the system is similar to the sugar transporter of the human erythrocyte.

The results with C-6 substituted alkyl groups are less easy to interpret. In the human erythrocyte introducing alkyl groups into the C-6 position resulted in an increase in affinity as the size of the alkyl group was increased from a methyl to a propyl group. This was thought to be due to interaction with a hydrophobic region that was adjacent to the hydrogen bonding group from the membrane to the C-6 position of the sugar. However, in the adipocyte both 6-*O*-methyl-D-galactose and 6-*O*-propyl-D-glucose have low affinity (compared with D-galactose and D-glucose, respectively) and both alkyl groups may be interfering with binding (The K_i for 6-*O*-methyl-D-galactose is 87.25 ± 17.9 mM, $n = 9$ and for 6-*O*-propyl-D-glucose the K_i is 78.07 ± 12.6 mM, $n = 23$.). Some hydrophobic interaction is probably occurring with 6-*O*-pentyl-D-galactose which is a good inhibitor (K_i = 4.7 ± 0.23 mM, $n = 12$). Some of this inhibition may be due to nonspecific or detergent like action although no increased lysis was observed. The difference between the high affinity for 4,6-*O*-ethylidene-D-glucose and the low affinity for 6-*O*-propyl-D-glucose (with both compounds having a bulky substitution at C-6) is probably due to the random orientation of the alkyl group that can occur in 6-*O*-propyl-D-glucose but not in 4,6-*O*-ethylidene-D-glucose. Thus the bulky group in 6-*O*-propyl-D-glucose could project towards, and interfere with binding to, the ring oxygen while in 4,6-*O*-ethylidene-D-glucose the bulky group must project towards C-4.

TABLE I

A COMPARISON OF THE SUGAR-TRANSPORT SPECIFICITY IN BASAL AND INSULIN-TREATED CELLS

In basal cells 40 μM 3-*O*-methyl-D-glucose was the substrate and K_i values were determined at 20 mM inhibitor. The mean basal uninhibited uptake rate is $t_{1/2}$ = 3.14 ± 0.5 min which is about 55-times slower than in insulin-treated cells. The K_i values are compared with the present and previously reported results on insulin-treated cells using D-allose as the substrate [a]. Results are mean ± S.E. NDI, no detectable inhibition.

Inhibitor	K_i (mM)			
	Basal	(*n*)	Insulin (10 nM)	(*n*)
D-Glucose	9.61 ± 2.24	12	8.62 ± 0.71	34
5-Thio-D-glucose	37.58 ± 6.6	5	42.1 ± 6.0	12
1-Deoxy-D-glucose	NDI	6	NDI	12
Methyl-β-D-glucoside	NDI	6	NDI	12
2-Deoxy-D-galactose	21.58 ± 2.04	6	20.75 ± 3.04	9
2,3-Di-*O*-methyl-D-glucose	38.73 ± 5.54	6	42.09 ± 7.48	11
3-*O*-Propyl-D-glucose	8.93 ± 0.8	6	11.26 ± 2.12	12
3-Deoxy-D-glucose	32.53 ± 1.58	3	40.31 ± 4.20	10
4,6-*O*-Ethylidene-D-glucose	8.00 ± 3.53	6	6.11 ± 0.50	12
D-Galactose	30.50 ± 7.22	6	24.49 ± 3.05	12
D-Fucose	35.34 ± 8.59	4	39.35 ± 5.03	15
6-Deoxy-D-glucose	14.17 ± 3.19	6	11.08 ± 0.63	11
6-*O*-Methyl-D-galactose	98.31 ± 30.18	6	87.25 ± 17.85	9
6-*O*-Pentyl-D-galactose	5.11 ± 1.51	5	4.66 ± 0.23	12

[a] The ratio of tracer permeability of 3-*O*-methyl-D-glucose/D-allose is approx. 45 for basal and insulin-treated cells.

Basal cells

Table I compares the hydrogen bonding and spatial requirements of insulin-treated and basal cells.

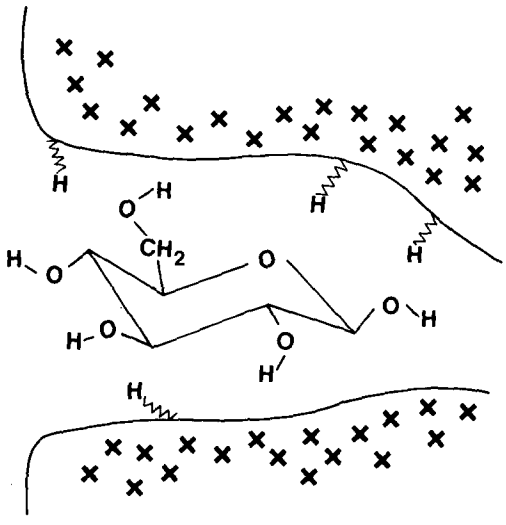
A different substrate (3-*O*-methyl-D-glucose) has been used for the determination of inhibition constants for basal cells. We have previously shown that the ratio of 3-*O*-methyl-D-glucose/D-allose permeability is approx. 45 for insulin-treated and basal cells. Also the similarity of the K_m for 3-*O*-methyl-D-glucose transport and the K_i for 3-*O*-methyl-D-glucose inhibition of D-allose transport suggests that the two sugars share the same system and therefore we feel that a comparison of the K_i values for basal and insulin-treated cells using two different substrates is reasonable.

In basal cells the poor hydrogen bonding analogues 5-thio-D-glucose, 1-deoxy-D-glucose, 3-deoxy-D-glucose, D-fucose and 6-deoxy-D-glucose indicate that, as in insulin-treated cells, the important hydrogen bonding positions are at the ring oxygen, C-1 and C-3 with a less important hydrogen bonding group at C-6. The low affinity for β-methyl-D-glucoside, 2,3-di-*O*-methyl-D-glucose, 3-*O*-propyl-D-glucose and for 6-*O*-methyl-D-galactose shown by basal cells show that lack of treatment with insulin has no effect on the spatial requirements of the site. Also shown in Table I is that D-glucose, D-galactose, 4,6-*O*-ethylidene-D-glucose and 6-*O*-pentyl-D-galactose have similar affinities in basal and in insulin-treated cells.

It can thus be concluded that insulin does not cause a major shape change in the site which might have been expected to affect the specificity requirements. Instead insulin probably leads to an increase in the number of sites of identical specificity.

Discussion

The basis of the experiments described in this report was to determine only the spatial requirements of the outer site of the transporter and the conclusions drawn are based mainly on the use of inhibitors added with the substrate to the external solution. The proposed features of the external site are shown in Scheme I. This scheme also shows the positions of the proposed hydrogen bonding sites at the ring oxygen at C-1 at C-3 and C-6. D-Glucose is shown in the $^4C_1$ pyranose ring form which we have previously shown is the preferred conformation. This conclusion is based on the demonstration that the fused pyranose ring in fluoro-β-D-glucoside has high affinity [4].



Scheme I. The scheme shows the proposed features of the active site at the external surface of the sugar transporter in adipocytes. The important hydrogen bonding sites are the ring oxygen, C-1 and C-3 and to a lesser extent C-6. A close approach of the transporter to the sugar at C-1 and C-2 and to a lesser extent at C-3 and C-6 but not at C-4 is suggested. The preferred conformation is $^4C_1$ glucopyranose.

The important spatial restrictions to binding externally to the transporter seem to be at C-1 and C-C-2 but not at C-4. This implies a strict orientation of the sugar in the site with C-1 of the sugar facing inward. In this sense the rat adipocyte and human erythrocyte transport systems for sugars seem very similar but differ only in small differences in affinity for various analogues. These differences may in part be due to the different temperatures at which inhibition constants were determined for the two systems. There may be a slightly looser fit at C-1 and C-3 in the adipocyte system compared with the human erythrocyte because α-methyl-D-glucoside and 3-*O*-propyl-D-glucose show more affinity as inhibitors in the adipocyte. This is a small difference compared with the very clear difference in spatial requirements between C-1 and C-4 shown by both systems.

Whether the spatial requirements at the inner face of the adipocyte sugar transport system are similar to those at the outer face will be the subject of further investigation.

Acknowledgement

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