Biochimica et Biophysica Acta 812 (1985) 27–32
Elsevier

BBA 72413

Transport properties of photolabile sugar analogues

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(Received July 9th, 1984)

Key words: Carbohydrate transport; Diazirine; Photolabile analog; (Erythrocyte)

The transport properties of photolabile substituted sugars have been determined in human erythrocytes. Half-saturation constants (K_i values) for these compounds have been estimated by measuring the inhibition of the uptake of 100 μM D-galactose. 4-Azido-4-deoxy-D-galactose has a K_i of 7.9 mM. 4-(2′-Diazo-3′,3′,3′-trifluoropropionyl)-D-glucose has a K_i of 5.9 mM. 3,7-Anhydro-2,2-azo-1,2-dideoxy-D-glycerol-L-mannooctitol has negligible affinity. 1′-Methoxy-3′,3′-azobutyl β-D-glucopyranoside has low affinity outside the cell but shows a K_i of 2.3 mM when equilibrated with the cells. D-Galactose competitively reduces the inhibition produced by this glucoside. The 4,4-azo-4-deoxy and the 6,6-azo-6-deoxy derivatives of D-glucose both have good affinity. The K_i values are 31.6 mM and 7.3 mM, respectively. The 4,4-azo and 6,6-azo derivatives are particularly important sugars because they contain nonbulky photolabile substituents. All of the above analogues have been tested to determine whether they can induce counterflow of D-galactose. The 4,4-azo and the 6,6-azo derivatives induce large transient accumulations of D-galactose, indicating that these sugars are capable of forming a mobile sugar-carrier complex. The other sugars do not show counterflow. Thus the 4,4-azo and the 6,6-azo derivatives could be extremely useful analogues which may label parts of the transport system that side-specific nontransported sugars cannot reach.

Introduction

As part of our study on the synthesis followed by the testing of a large range of photolabile sugar analogues we now report some of their transport properties. The diazirine group was considered to be particularly useful because it is small and can be photoactivated at wavelengths in excess of 300 nm. We were particularly interested to see whether a photolabile sugar derivative could be prepared which retained the ability to be transported. To this end, the C-4 [1] and C-6 diazirines were synthesised. To measure transport directly a radiolabelled sugar could be used. However, introduction of a radiolabel into either of these sugars involves lengthy and complicated routes, so we decided to indirectly evaluate whether a transported carrier-substrate complex was formed by carrying out counterflow experiments. We know that transport system inhibitors which do not form such a transported complex do not show counterflow [2]. We have therefore used the counterflow test to distinguish between those of our sugar derivatives which are transported substrates from those which are nontransported inhibitors. In fact, we show that 4-azido-4-deoxy-D-galactose and 4-(2′-diazo-3′,3′,3′-trifluoropropionyl)-D-glucose are nontransported (probably external) inhibitors, while 1′-methoxy-3′,3′-azobutyl β-D-glucopyrano-

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side is a good inside specific inhibitor which shows nonmediated but no facilitative uptake into the cell. The C-4 and the C-6 diazirines do induce a large transient accumulation of D-galactose and are thus transported by the carrier.

Materials and Methods

D-Galactose was from Sigma. D-[1-^{14}C]Galactose was from Amersham International. Phloretin was from K&K laboratories.

Preparation of sugar derivatives

The sugar derivatives used in this study were prepared by Kurz, Lehmann and Thieme [1]. The preparations are rather complicated and difficult, but some indication of the synthetic routes can be given and may be of general interest. 4-Azido-4-deoxy-D-galactose was prepared from methyl 2,3,6-tri-*O*-acetyl-4-*O*-methanesulfonyl α-D-glucopyranoside using (i) sodium azide, (ii) sodium methoxide, (iii) acidic ion-exchanger. 4,4-Azo-4-deoxy-D-glucose was synthesised from methyl 2,3,6-tri-*O*-benzyl α-D-glucopyranoside using (i) dimethyl sulphoxide/dicyclohexyl carbodiimide/orthophosphoric acid, (ii) palladium/charcoal/hydrogen, (iii) hexamethyl disilazane/trimethylsilyl chloride, (iv) ammonia/hydroxylamine *o*-sulphonic acid, (v) iodine/triethylamine, (vi) hexamethyl disilazene/trimethylsilyl chloride, (vii) trimethylsilyl iodide/water. 3,7-Anhydro-2,2-azo-1,2-dideoxy-D-glycerol-L-mannooctitol was synthesised from 4,5,6,8-tetra-*O*-acetyl-3,7-anhydro-1-deoxy-D-glycero-L-mannooct-2-ulose using (i) ammonia/hydroxylamine *O*-sulfonic acid, (ii) iodine/triethylamine, (iii) acetic anhydride, (iv) sodium methoxide. 1′-Methoxy-3′,3′-azobutyl β-D-glucopyranoside was prepared from 2,3,4,6-tetra-*O*-acetyl-1-*O*-trimethylsilyl β-D-glucose using (i) 3,3-azobutyraldehyde dimethyl acetal/trimethylsilyl triflate, (ii) sodium methoxide. 4-(2′-Diazo-3′,3′,3′-trifluoropropionyl)-D-glucose was prepared from 2,3 : 5,6-di-*O*-isopropylidene-D-glucose-dimethylacetal using (i) 2-diazo-3′,3′,3′-trifluoropropionyl chloride, (ii) acidic ion-exchanger. 6,6-Azo-6-deoxy-D-glucose was synthesised from methyl 2,3,4-tri-*O*-benzyl α-D-glucopyranoside in a manner similar to that indicated for the preparation of the 4,4-azo derivative (Lehmann, J. and Thieme, R., unpublished results).

Preparation of Erythrocytes

Human transfusion blood (1–3 weeks old) was separated from plasma and acid-citrate-dextrose by careful centrifugation below 20°C. Care was taken to remove white cells and platelets. The red cells were then washed a further four times in phosphate saline buffer (154 mM sodium chloride/12.5 mM sodium phosphate (pH 7.2)) at room temperature.

Transport experiments

These were carried out at 20°C. The radiolabelled substrate used throughout was D-[1-^{14}C]galactose at 100 μM. For most of the inhibition experiments, cells at 10% cytocrit were incubated with inhibitor for up to 90 min. 100 μl of these suspensions were added to 5 μl of 100 μM D-[1-^{14}C]galactose and incubated for varying times (usually 10 s). For these simple experiments, the kinetic consequences of diluting the equilibrated inhibitor by the small volume of labelled sugar solution were ignored. For the competition experiment in which 160 mM and 100 μM D-galactose were used, care was taken to ensure true equilibration. 80-μl cell aliquots which had been equilibrated with substrate and inhibitor were added to 10-μl aliquots containing the radiolabelled substrate and inhibitor.

In the counterflow experiments cells were equilibrated with approx. 80 mM of the test sugar and 20 μl of these packed cell suspensions were diluted in 0.8 ml of a solution containing 100 μM D-[1-^{14}C]galactose. The accumulation at the indicated times was calculated as a fraction of the D-[1-^{14}C]galactose which equilibrated in the absence of the test sugar.

In the above experiments the transport was terminated by adding stopping solution (generally 2.7 or 3 ml). Stopping solution contained 0.3 mM phloretin and 10 μM mercuric chloride in phosphate saline buffer. Samples were then spun in a refrigerated bench centrifuge for 1 min. The cell pellets were resuspended in a further aliquot of stopping solution and respun. Finally, trichloroacetic-acid-extracted radioactivity was estimated by liquid scintillation counting. Samples were processed no more than two at a time and the total amount of time that each sample was left in stopping solution was kept to a minimum. Using this

technique, the variation in duplicate samples was approx. 2% of the mean, the background or zero time radioactivity was less than 1% of the radiolabel at equilibrium and the apparent volume occupied by D-galactose at equilibrium was approx. 65 μl per 100 μl of packed cells.

Estimations of rates of transport were carried out at either very low D-galactose concentrations or under conditions in which D-galactose and inhibitors are at equilibrium across the membrane. Incubation times giving 50% filling or less were used. Under all these conditions the usual logarithmic expression for evaluating the rate constant can be used [3,4]. In this equation, k (the rate constant) is equal to $-(\ln(1-f))/t$ where f is the fractional filling and t is the time. Plots of v_0/v vs. inhibitor concentration are used to calculate the K_i value, since $v_0/v = 1 + I/K_i$ [3]. Here v_0 and v are the rate constants for uninhibited and inhibited transport, while I is the inhibitor concentration. Results are expressed as mean $\pm$ S.E. In the case of a transported inhibitor which has been equilibrated with the cells, the K_i or half-saturation constant is equal to the equilibrium exchange K_m [3].

Results and Discussion

The data used for the estimation of the half-saturation concentrations for 4-azido-4-deoxy-D-galactose, 4-(2'-diazo-3',3',3'-trifluoropropionyl)-D-glucose and for 3,7-anhydro-2,2-azo-1,2-dideoxy-D-glycero-L-mannooctitol are shown in Fig. 1. Since the latter compound is a 'C-glycopyranoside' and has the unfavourable D-galactopyranosyl instead of D-glucopyranosyl configuration, it is perhaps not surprising that it shows no inhibition. The affinity for 4-azido-4-deoxy-D-galactose is surprisingly high. The $K_i = 7.99 \pm 0.41$ mM. The half-saturation constant for D-galactose is about 75–80 mM [7]. The much higher affinity for the 4-azido derivative of D-galactose may be partly related to the observation that this compound is not translocated by the carrier (see below). Thus this sugar derivative may interact just with a high-affinity external site. In addition, the azido substitution for the hydroxyl at C-4 of D-galactose may enhance the binding to the external site.

4-(2'-Diazo-3',3',3'-trifluoropropionyl)-D-glucose also has very high affinity. The $K_i = 5.91 \pm 0.12$ mM. This is consistent with the postulate [5,6] that introducing a bulky group into the C-4 position of D-glucose has very little effect on affinity, because this region of the sugar projects into the external solution when the sugar is bound at the external site. We show below that this compound is not translocated by the carrier and it thus resembles 4,6-*O*-ethylidene-D-glucose both in its affinity and in its inability to undergo translocation through the transport system.

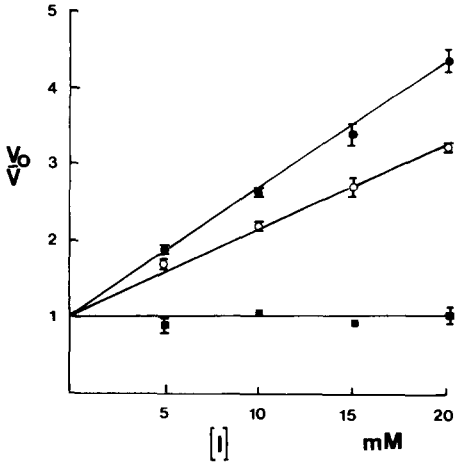
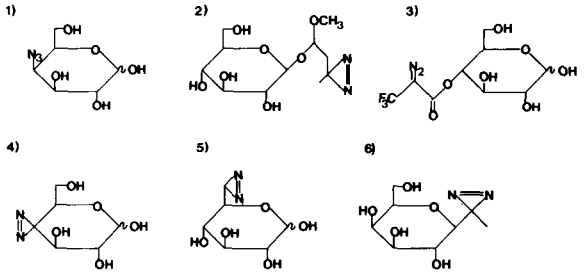


Fig. 1. Inhibition of 100 μM D-galactose uptake by 4-azido-4-deoxy-D-galactose (○) $K_i = 7.99 \pm 0.41$ mM ($n = 8$), by 4-(2'-diazo-3',3',3'-trifluoropropionyl)-D-glucose (●) $K_i = 5.91 \pm 0.12$ mM ($n = 8$) and by 3,7-anhydro-2,2-azo-1,2-dideoxy-D-glycero-L-mannooctitol (■). The average rate constant (v_0) for uninhibited D-galactose uptake was 0.072 s^{-1}. [I], inhibitor concentration.

The half-saturation constant for 4,4-azo-4-deoxy-D-glucose is very interesting. The K_i is 31.6 $\pm$ 2.67 mM (Fig. 2). From the structural formula shown in Scheme I it can be seen that the diazirine



Scheme I. Structural formulae of (1) 4-azido-4-deoxy-D-galactose, (2) 1'-methoxy-3',3'-azobutyl β-D-glucopyranoside, (3) 4-(2'-diazo-3',3',3'-trifluoropropionyl)-D-glucose, (4) 4,4-azo-4-deoxy-D-glucose, (5) 6,6-azo-6-deoxy-D-glucose, (6) 3,7-anhydro-2,2-azo-1,2-dideoxy-D-glycero-L-mannooctitol.

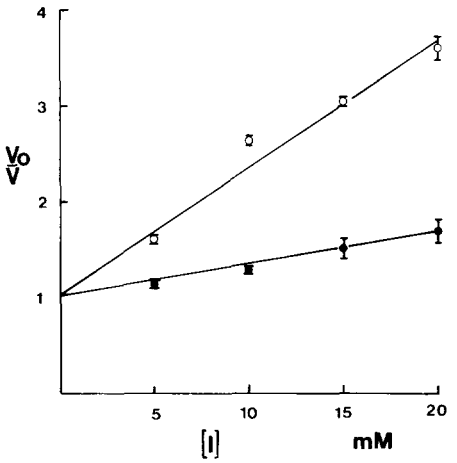


Fig. 2. Inhibition of 100 μM D-galactose uptake by equilibrated 4,4-azo-4-deoxy-D-glucose (●) $K_i = 31.46 \pm 2.67$ mM ($n = 8$) and by equilibrated 6,6-azo-6-deoxy-D-glucose (○) $K_i = 7.29 \pm 0.29$ mM ($n = 8$). The average rate constant (v_0) for uninhibited D-galactose uptake was 0.081 s^{-1}.

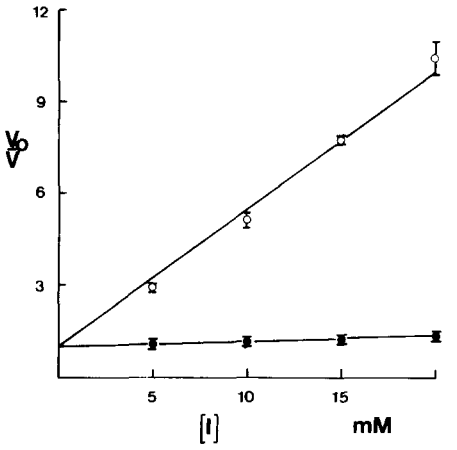


Fig. 3. Inhibition of 100 μM D-galactose uptake by equilibrated (○) and by external (●) 1'-methoxy-3',3'-azobutyl β-D-glucopyranoside. For the equilibrated inhibitor $K_i = 2.32 \pm 0.07$ mM ($n = 8$) and for external inhibitor $K_i = 78.0 \pm 15.88$ mM ($n = 8$). The average rate constant (v_0) for uninhibited D-galactose uptake was 0.089 s^{-1}.

group occupies a position normally occupied by the C-4 hydroxyls of D-glucose and of D-galactose. We show below that this is a transportable sugar, and its half-saturation constant can thus be compared with the half-saturation constants for equilibrated D-galactose and for D-glucose, which are approximately 80 mM and 12 mM respectively [7,9]. Thus the K_i for the 4,4-azo compound is about 3-fold greater than for D-glucose, but about 3-fold less than for D-galactose. In adipocytes, the affinity difference between D-glucose and D-galactose is much smaller [3] and there is a corresponding similarity between the K_i for D-glucose and for the 4,4-azo derivative. The adipocyte K_i values are 8 mM for D-glucose and 12 mM for the 4,4-azo derivative (unpublished results).

6,6-Azo-6-deoxy-D-glucose (Fig. 2) is an extremely good analogue and has affinity for the transport system that resembles, and may even be slightly better than, the parent compound, D-glucose. The K_i for the 6,6-azo derivative is 7.29 ± 0.29 mM. We show below that this analogue is transported by the carrier and is therefore an extremely useful and important sugar derivative.

Fig. 3 shows that 1'-methoxy-3',3'-azobutyl β-D-glucopyranoside is a very good inside inhibitor. When added with the substrate to just the outside of the cells the derivative shows very little inhibition. When equilibrated with the cells for 60–90 min, the K_i falls to 2.32 ± 0.07 mM. This inhibition is fully competitive with equilibrated D-galactose (Fig. 4). Raising the D-galactose concentration to 160 mM raises the $K_{i,app}$. The plots at 100 μM and at 160 mM D-galactose are virtually parallel, which is consistent with competition between substrate and the inhibitor.

The observation of inside specificity for this

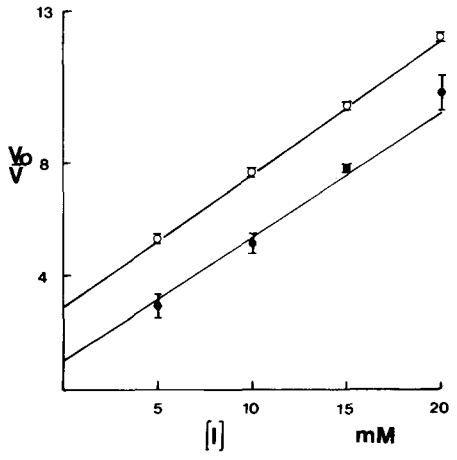


Fig. 4. Inhibition produced by 1'-methoxy-3',3'-azobutyl β-D-glucopyranoside at 100 μM (●) and at 160 mM (○) D-galactose. The rate constants for uninhibited D-galactose uptake are 0.084 s^{-1} at 100 μM D-galactose (v_0) and 0.029 s^{-1} at 160 mM D-galactose.

glucoside is consistent with our postulate [5,6] that the binding sites in the transport protein are organised in such a way that the orientation of the sugar at the external site is conserved at the internal site. Thus, at both of these sites C-1 faces inwards, while C-4 faces the external surface. In view of this side specificity and the photolability of this compound it may be possible to label domains of the transport system specifically associated with the inside site.

Fig. 5 shows the results from experiments in which each of the photolabile analogues was tested to determine whether they can induce counterflow of 100 μM D-galactose. The rationale for carrying out this test is that only the photolabile derivatives which are translocatable will show counterflow, while derivatives which simply bind but do not undergo the translocation step will not. The former compounds, when inside the cell, will produce a translocatable sugar-carrier complex which will only reduce the number of sites available for D-galactose backflux. This selective inhibition of backflux will give rise to a temporary D-galactose accumulation [10]. In comparison with this, the latter compounds will form dead-end complexes [11] with the transport system which will simply reduce the number of transport sites available for D-galactose influx and efflux.

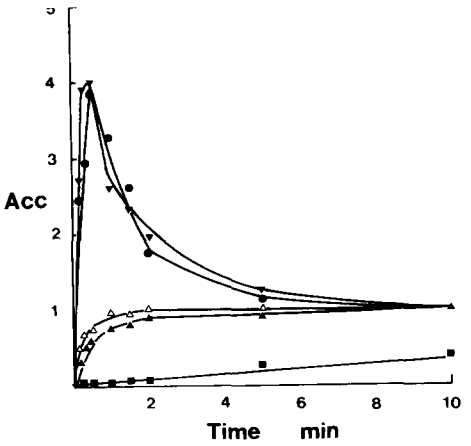


Fig. 5. The extent of counterflow induced by inhibitors which were preincubated with the cells for 1 h at 80 mM. The accumulation ratio (Acc) for 100 μM D-galactose in the presence of 4-azido-4-deoxy-D-galactose (△), 1′-methoxy-3′,3′-azobutyl β-D-glucopyranoside (■), 4-(2′-diazo-3′,3′,3′-trifluoropropionyl)-D-glucose (▲), 4,4-azo-4-deoxy-D-glucose (●), and 6,6-azo-6-deoxy-D-glucose (▼).

The results show that 4-azido-4-deoxy-D-galactose, 1′-methoxy-3′,3′-azobutyl β-D-glucopyranoside and 4-(2′-diazo-3′,3′,3′-trifluoropropionyl)-D-glucose do not cause counterflow. However, the 6,6-azo and 4,4-azo derivatives of D-glucose both induce a large transient accumulation of D-galactose, which indicates that both of these sugars are transported by the carrier.

In conclusion, it can be seen that the introduction of a photolabile group into the sugar can be accomplished without loss of affinity. In addition, we have found two photolabile analogues which form translocatable complexes with the transport system. This offers enormous advantages for transport system labelling, as these sugars may label parts of the transport system that side-specific nontranslocated sugars cannot reach. In addition, it is usually assumed that a ligand has to have high affinity to be a successful photoaffinity label. This is because the rate constant for dissociation is lower for a compound which has high affinity and therefore the time over which the protein is in contact with the photogenerated reactive species is greater [12]. However, this argument need not apply to a transported photoaffinity label, since the rate constants for translocation of the carrier complex with substrate may be lower than the dissociation rate constants for the complex. Thus transport will increase the time over which a photogenerated reactive species is in contact with the transport system protein. In the case of sugar transport, it has often been suggested that the transport mechanism exhibits rapid equilibrium and slow translocation kinetics [8].

Acknowledgements

P.J.W.M., B.A.P. and G.D.H. are grateful to the M.R.C. and to S.E.R.C. (U.K.) for financial support. R.T. and J.L. thank the Deutsche Forschungsgemeinscahft for financial support. R.T. thanks the Wissenschaftliche Gesellschaft Freiburg for a travel grant.

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