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Interaction of Sugar Acetals with the Human Erythrocyte Glucose Transport System

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Summary. All of the eleven tested bi- and tricyclic sugar acetals were found to be competitive inhibitors of glucose exit from human red cells. The highest-affinity agents were those locked into a Cl conformation by fusion with a *m*-dioxane ring attached to a phenyl group (methyl-4,6-O-benzylidene-D-glucopyranoside, $K_i = 0.73$ mM; 4,6-O-benzylidene-D-glucopyranose, $K_i = 0.13$ mM at 23 °C). All of the isopropylidene acetals had less affinity than D-glucose (Sen-Widdas $K_m = 2.5$ mM) for the transport system.

Sugars in which two or more hydroxyl groups were cross-linked by isopropylidene groups apparently penetrated the red cell membrane by simple diffusion, as shown by a lack of (1) saturation in the entry process, (2) competition for entry with other acetals or D-glucose, and (3) inhibition by phloretin or Hg^{2+} on entry rates.

Kinetic analysis of sugar permeation of the erythrocyte membrane has led to the hypothesis that there exists a specific membrane receptor or carrier molecule with which the permeating sugar must interact during some phase of the translocation process (LeFevre, 1961; Stein, 1967). The carrier molecule is generally assumed to be a specific membrane protein or lipoprotein and although considerable effort has gone into the isolation of the carrier molecule, the results to date have been uniformly unsuccessful (LeFevre & Masiak, 1970; Masiak & LeFevre, 1972).

A technique which appears to hold great promise for the identification of the carrier molecule or sugar receptor is affinity labeling, which has been very useful in the identification of active sites of enzymes and antibodies (Singer, 1967). Affinity labeling involves the design and synthesis of an active-site-directed alkylating agent which is a stereochemical analogue of the normal substrate of the active site (receptor binding site) containing a

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protein-reactive group. Such an agent is then used to attach a covalently bound chemical tag to the active site and, hence, label the macromolecule containing the active site (Wofsy, Metzger & Singer, 1962).

Inherent to the design of the labeling reagent is a knowledge of the bulk tolerance of the active site; i.e., one must know what type of modifications can be made in the normal substrate while retaining affinity for the binding site. Since information is lacking on the bulk tolerance of the sugar receptor site of the carrier molecule, we undertook a study of the interaction of cyclic acetal monosaccharide derivatives with the erythrocyte glucose transport system. These derivatives have one or more pairs of hydroxyl groups cross-linked with alkylidene groups (ethylidene, benzylidene or isopropylidene) to form fused ring systems, and are commonly used as intermediates in carbohydrate synthesis. It was the purpose of this study to determine if any of these intermediates possessed the necessary characteristics for consideration as a potential precursor for an affinity-labeling reagent.

Ethylidene-glucose (4,6-O-ethylidene-D-glucopyranose) has been shown by Baker and Widdas (1973) to be a competitive inhibitor of glucose exit which penetrates the cell membrane by simple diffusion. Our results indicate that in fact all of the eleven sugar acetals tested are competitive inhibitors of glucose exit, with K_i correlated with the degree and the nature of the substitution. Isopropylidene acetals penetrated the erythrocyte membrane by the process of simple diffusion. The benzylidene acetals showed the highest affinity for the transport receptor (some 3 to 18 times that of D-glucose), while the isopropylidene acetals all had less affinity than D-glucose.

Materials and Methods

Sugars

Sugar acetals were obtained from the following sources: 1,2:3,5-di-O-, 1,2-O-, and 5-O-carbomethoxy-1,2-O-isopropylidene-D-xylofuranose, from Pfanstiehl Labs.; 1,2-O-, and 1,2:5,6-di-O-isopropylidene-D-glucofuranose, 4,6-O-ethylidene-D-glucopyranose, and 1,2:3,4-di-O-isopropylidene-D-galactopyranose, from Aldrich Chemical Co.; methyl-4,6-O-benzylidene-α-D-glucopyranoside and 1,2:4,5-di-O-isopropylidene-D-fructopyranose, from Pierce Chemical Co. 6-O-Acetyl-1,2-O-isopropylidene-D-glucofuranose was the gift of Dr. R. K. Crane. 4,6-O-benzylidene-D-glucopyranose was synthesized by the method of Fletcher (1963). Sugar acetals were checked for purity by thin-layer chromatography and repurified by recrystallization or vacuum distillation if necessary.

Methods

Blood was collected in heparin by venipuncture and used within 4 days of collection. Prior to use the red cells were washed three times with buffered saline (0.15 M NaCl,

pH 7.3, 10 mM sodium phosphate). Sugar transports were measured at 23 °C by photoelectric recording of changes in cell volume accompanying sugar migration (Ørskov, 1935).

Entry rates of the various sugar acetals were measured by the method of Widdas (1954) and permeability constants were derived using the method of Baker and Rogers (1972). Determination of the various transport constants was by the method of Sen and Widdas (1962). Briefly, this involves recording the net exit of the test penetrant from preloaded cells (60 min at 37 °C with 110 mM D-glucose; 10% cell suspension, v/v, in buffered saline) upon transfer to a lower glucose concentration, and estimation from these records of the extrapolated "exit time" (the time which would mark complete equilibration if the initial rate of exodus were maintained in each case). A plot of this exit time as a function of the external penetrant concentration then defines apparent kinetic constants expressing the saturation characteristics of the entry flux (as in Fig. 1). The alteration of such plots by fixed concentrations of other interactants (as in Fig. 2) allows also the calculation of apparent K_i's for such interaction.

The rate constants for inactivation of the transport system by 1-fluoro-2,4-dinitrobenzene (FDNB) in the presence and absence of various substrates and inhibitors were determined by the method of Krupka (1971).

Results

Inhibition of Glucose Exit

Exit of glucose from preloaded erythrocytes was progressively inhibited by increasing concentrations of the various sugar acetals in the media. The concentration of acetal necessary to double the exit time (K_i) was determined from plots of exit time *vs.* acetal concentration (Sen & Widdas, 1962), as illustrated in Fig. 1. To ascertain the mode of inhibition of the sugar acetals,

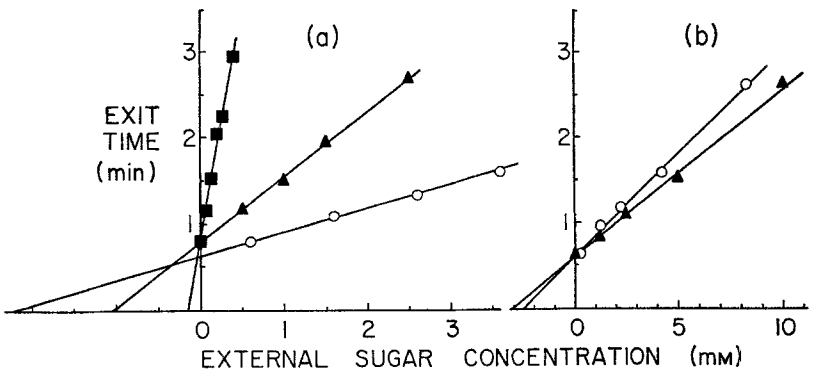


Fig. 1. Inhibition of D-glucose exit by external sugars. Initial intracellular glucose level = 100 mM. Ordinate is extrapolated "exit time" as used by Sen and Widdas (1962); abscissa indicates varying external concentration of glucose (○) or of: (Panel *a*) methyl-4,6-O-benzylidene-D-glucose, ▲; 4,6-O-benzylidene-D-glucose, ■; (Panel *b*) 1,2:5,6-di-O-isopropylidene-D-glucofuranose, ▲. Each panel covers concurrent experiments on a single suspension; each point is the mean of three determinations. Lines were fitted by least-squares regression. Similar determinations were carried out for all sugar acetals listed in Table 1

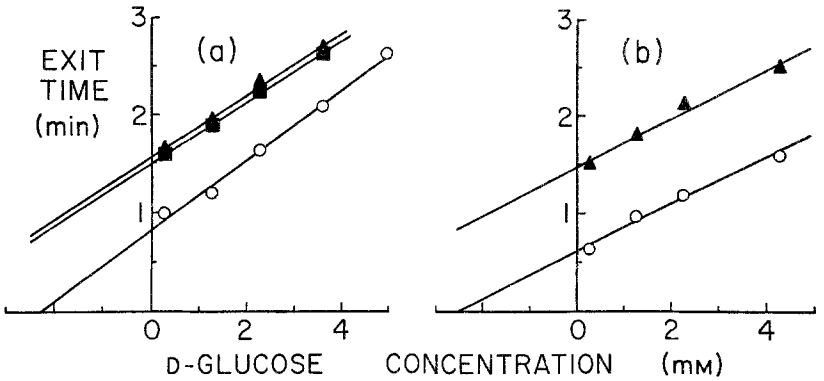


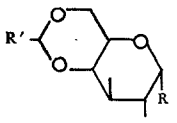
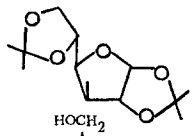
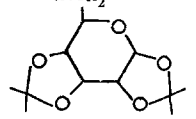
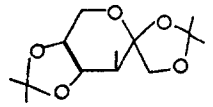
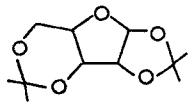
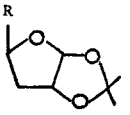
Fig. 2. Effects of fixed levels of sugar acetals on exit of glucose into varied low concentrations of glucose. Cells pre-equilibrated with D-glucose at 100 mM were transferred into the optical cuvette containing the low glucose concentrations indicated on the abscissa, with or without fixed concentrations of methyl-4,6-O-benzylidene-D-glucopyranose (1 mM), 4,6-O-benzylidene-D-glucopyranose (0.1 mM), or 1,2:5,6-di-O-isopropylidene-D-glucofuranose (5 mM). Arrangement of panels and symbols identical to that of Fig. 1. Similar determinations were carried out for all sugar acetals listed in Table 1

exit times were determined in the presence of constant acetal and varying D-glucose concentrations. When the results were plotted according to Sen and Widdas (1962), it was found that *all* of the acetals caused an increase in the apparent half-saturation concentration (K_m) for D-glucose, indicating that the mode of inhibition was competitive (Fig. 2). The derived data are summarized in Table 1, where the degree of interaction of the sugar acetals with the glucose transport system is expressed as R, the ratio of $K_{m\ \text{glucose}}/K_{i\ \text{acetal}}$.

Permeability of the Erythrocyte Membrane to Sugar Acetals

Photoelectric measurement of entry rates was restricted to the isopropylidene acetals (Ip-acetals) because of the low solubility of the benzylidene acetals in buffered saline. No evidence of saturation was observed for entry of the Ip-acetals up to a concentration of 120 mM, a concentration which exceeds the K_i by 2- to 40-fold (depending on the acetal). In addition, the entry rates were unaffected by (1) pre-equilibrating the cells with 30 mM D-glucose, (2) pre-equilibrating the cells with other Ip-acetals, or (3) 65 μM phloretin or 10 μM $HgCl_2$ (concentrations sufficient to block D-glucose entry). Thus, the Ip-acetals do not utilize the glucose transport system for entry, but appear to penetrate by a simple diffusion process. The entry data were analyzed by the method of Widdas (1954) and the resultant rate constant for diffusion, k, was converted to P, the permeability constant

Table 1. Cyclic sugar acetals tested for inhibition of sugar transport

Structure	Name	R (K_m/K_i)	P (cm/sec)
	R = OH, R′ = C_6H_5 4,6-O-benzylidene-D-glucopyranose	17.8	N.T.
	R = CH_3, R′ = C_6H_5 methyl-4,6-O-D-benzylidene-glucopyranoside	3.4	N.T.
	R = OH, R′ = CH_3 4,6-O-ethylidene-D-glucopyranose	0.62	3.4×10^{-7}
	1,2:5,6-di-O-isopropylidene-D-glucofuranose	0.91	5×10^{-4}
	1,2:3,4-di-O-isopropylidene-D-galactopyranose	0.59	5×10^{-4}
	1,2:4,5-di-O-isopropylidene-D-fructopyranose	0.55	5×10^{-4}
	1,2:3,5-di-O-isopropylidene-D-xylofuranose	0.32	5×10^{-4}
	R = $COOCH_3$ 5-O-carbomethoxy-1,2-O-isopropylidene-D-xylofuranose	0.30	N.T.
	R = $CHOHCH_2OOCCH_3$ 6-O-acetyl-1,2-O-isopropylidene-D-glucofuranose	0.12	6.8×10^{-6}
	R = CH_2OH 1,2-O-isopropylidene-D-xylofuranose	0.09	8.6×10^{-6}
	R = $CHOHCH_2OH$ 1,2-O-isopropylidene-D-glucofuranose	0.05	3.9×10^{-7}

R is the ratio of D-glucose K_m/acetal K_i (Sen-Widdas constants determined concurrently on the same cell suspension, the glucose K_m's all falling within the range 2.25 to 2.75 mM). P is the permeability constant measured according to Widdas (1954). All acetals listed behaved as competitive inhibitors of glucose exit. N.T. = Not Tested.

(Baker & Rogers, 1972). These data are included in Table 1. Only mono-Ip-acetals had diffusion rate constants which could be measured at 23 °C (10^{-7} to 10^{-6} cm sec^{-1}). The equilibration of the di-Ip-acetals across the cell membrane was too rapid to be resolved ($>10^{-5}$ cm sec^{-1}). An estimate of P for the di-Ip-acetals was made by the method of Stein (1967) utilizing the mono-Ip-acetal data and was found to be approximately 5×10^{-4} cm sec^{-1}.

FDNB Inhibition

The rate constant k for FDNB inactivation of the transport system was 2.3×10^{-2} min^{-1} (Table 2). 2-deoxy-glucose increased the rate of inactivation ($k = 7.2 \times 10^{-2}$ min^{-1}) while maltose and ethylidene-glucose had a slight protective effect ($k = 2.0 \times 10^{-2}$ min^{-1}). 4,6-O-benzylidene-D-glucopyranose strongly protected the transport system from FDNB attack ($k = 0.66 \times 10^{-2}$ min^{-1}).

Table 2. Effects of various sugars on the rate constant (k) for FDNB inactivation of the glucose transport system[a]

Sugar	k (min^{-1})
None	2.3×10^{-2}
Maltose (100 mM)	2.0×10^{-2}
Ethylidene-glucose (100 mM)	2.0×10^{-2}
4,6-O-benzylidene-glucose (10 mM)	6.6×10^{-3}
2-deoxy-glucose (50 mM)	7.2×10^{-2}

[a] Analysis was performed according to the procedure given by Krupka (1971).

Discussion

Cyclic sugar acetals contain two types of ring systems fused to the parent sugar molecule through ether linkages: a nonplanar, five-membered, 1,3-dioxolane ring and/or a six-membered *m*-dioxane ring with a chair conformation (Mills, 1955). In this study the isopropylidene (Ip) acetals contained one or more 1,3-dioxolane rings fused to a furanose or pyranose ring while the 4,6-O-acetals (benzylidene, ethylidene) had a *m*-dioxane ring fused to a pyranose ring.

The tricyclic di-Ip-acetals ($R > 0.3$) have a greater affinity for the glucose transport receptor than the bicyclic mono-Ip-acetals ($R < 0.3$). The K_i is not distinctly related to the conformation of the parent sugar (Table 1) but rather to both a reduction in hydrogen-bonding capability (increased hydrophobicity) and the stereochemistry of the cyclic acetal. This is clearly illustrated by the addition of a second ring system to both mono-Ip-glucose ($R = 0.05$) and mono-Ip-xylose ($R = 0.09$) to form the corresponding di-Ip-acetals ($R = 0.91$ and 0.32, respectively). The mono-Ip-acetals of glucose and xylose are analogous structures, differing only in the number of carbinol groups in the "R" group (*see* Table 1), while the di-Ip-acetals are not such comparably related structures. Thus, di-Ip-xylose contains three different ring systems locked into a rigid structure while the di-Ip-glucose has a "dangling" dioxolane ring (*see* Table 1) which could more easily participate in hydrophobic bonding.

The importance of hydrophobic interactions in the binding of di-Ip-acetals to the transport receptor is illustrated by di-Ip-fructose in which addition of two 1,3-dioxolane rings results in a more than 300-fold increase in affinity: di-Ip-fructose, $K_i = 0.0045$ M; D-fructose, $K_m = 1.5$ M (LeFevre & Marshall, 1958). Similarly, di-Ip-galactose shows a 10-fold increase in affinity over D-galactose. However, both of these acetals have pyranose rings with conformations generally presumed to have little or no affinity for the transport receptor (LeFevre, 1961): di-Ip-fructose, $1C$; di-Ip-galactose, *skew* (Durette & Horton, 1971).

All of the isopropylidene acetals (1,3-dioxolane ring) penetrate the cell membrane by an apparently simple diffusion. In addition, 4,6-O-ethylidene-D-glucose, which contains a *m*-dioxane ring, also has been shown to utilize a diffusion pathway for entry (Baker & Widdas, 1973). It appears that the sugar acetals are not translocated by the sugar transport system but do possess the ability to bind to the transport receptor. The diffusion properties of these derivatives could be the result of either the cross-linking bridge(s) or the reduction in hydrogen-bonding capability. Information on the permeation properties of di-O-alkyl-sugars could resolve this question.

The acetals with the greatest affinity were those containing a *m*-dioxane ring fused to a pyranose ring through the 4 and 6 hydroxyls, locking the sugar into the Cl conformation (Durette & Horton, 1971), the conformation with the greatest affinity (LeFevre, 1961). A *m*-dioxane ring system is found in di-Ip-xylose linking the 3 and 5 hydroxyls. The affinity of these acetals was found to be dependent upon the nature and configuration of the parent sugar (glucopyranose > methyl-glucopyranoside > xylofuranose) and on the nature of the substituent attached to the *m*-dioxane ring (phenyl > methyl). In fact, the phenyl residue can impart a high affinity ($R = 3.4$) to α-methyl-glucoside which as such does not measurably interact with the transport system (Chen & LeFevre, 1965). The K_i for benzyl alcohol inhibition of glucose exit was 7.5 mM ($R = 0.3$) indicating that the phenyl ring alone is not responsible for the high affinity of the benzylidene acetals. Likewise, the *m*-dioxane ring structure in itself does not impart a high affinity, as is evident from consideration of di-Ip-xylose ($R = 0.32$). The benzylidene acetals appear to combine with the receptor site on a 1:1 basis when the data are analyzed by the method of LeFevre (1961), emphasizing the simple competitive displacement action of these sugars. 4,6-O-benzylidene-glucose affords substantial protection against FDNB inactivation of the transport system, the inactivation being only 28% of that in the presence of FDNB alone (Table 2), and thus is the most potent protector found to date.* The

* With the exception of cytochalasin B (R. Bloch, 1973). *Biochemistry* **12**:4799.

protective effect can be attributed to the presence of the phenyl ring since 4,6-O-ethylidene-glucose which has a methyl group in the same position (Table 1) is a weak protector (Baker & Widdas, 1973). The phenyl group offers a unique starting point for the attachment of a potential protein-alkylating group and thus, 4,6-O-benzylidene-glucose appears to be a favorable glucose analogue for the synthesis of an active-site-directed reagent for the erythrocyte glucose transport system.

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