Structural Requirements for Binding to the Sugar-Transport System of the Human Erythrocyte

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The structural requirements for binding to the glucose/sorbose-transport system in the human erythrocyte were explored by measuring the inhibition constants, K_i , for specifically substituted analogues of D-glucose when L-sorbose was the penetrating sugar. Derivatives in which a hydroxyl group in the D-glucose configuration was inverted, or replaced by a hydrogen atom, at C-1, C-2, C-3, C-4 or C-6 of the D-glucose molecule, all bound to the carrier, confirming that no single hydroxyl group is essential for binding to the carrier. The binding and transport of 1-deoxy-D-glucose confirmed that the sugars bind in the pyranose form. The relative inhibition constants of D-glucose and its deoxy, epimeric and fluorinated analogues are consistent with the combination of β -D-glucopyranose with the carrier by hydrogen bonds at C-1, C-3, probably C-4, and possibly C-6 of the sugar. Both polar and non-polar substituents at C-6 enhance the affinity of D-glucose derivatives relative to D-xylose, and D-galactose derivatives relative to L-arabinose, and it is suggested that the carrier region around C-6 of the sugar may contain both hydrophobic and polar binding groups. The spatial requirements at C-1, C-2, C-3, C-4 and C-6 were explored by comparing the relative binding of D-glucose and its halogeno and O-alkyl substituents. The carrier protein closely approaches the sugar except at C-3 in the D-glucose configuration, C-4 and C-6. D-Glucal was a good inhibitor, showing that a strict chair form is not essential for binding. 3-O-(2',3'-Epoxypropyl)-D-glucose, a potential substrate-directed alkylating agent, bound to the carrier, but did not inactivate it.

The presence or absence of a carrier system in the transport of sugars across the human erythrocyte membrane is still a subject for debate (Lieb & Stein, 1972), but all models of transport currently considered useful contain a protein with one or more sugar-binding sites. This binding of sugar to protein is required to explain the phenomena of saturation, specificity, competition between sugars and counterflow. In the present paper, this protein is termed a carrier.

A method of establishing the presence of such a carrier, which would allow subsequent investigation of its properties, would be discovery of a substrate-directed alkylating agent that would react specifically with the sugar-binding site of the carrier protein. Before one can design such a reagent, it is necessary to know the extent and type of binding between sugar and carrier, and to explore the spatial limits for substituents on the sugar molecule.

In a valuable review, LeFevre (1961) has collated much of the information on specificity known before 1961, and some additional information has been gathered subsequently (Sen & Widdas, 1962; Lacko & Burger, 1962; Baker & Widdas, 1971). The parameter of binding measured in the early studies was

effectively the apparent K_m for entry of sugars into the erythrocyte, which may contain a kinetic component of both binding and transport across the membrane. When the rate of translocation, k_{+2} in Scheme 1, is comparable with the rate of formation of the sugar-carrier complex, k_{+1} , the apparent K_m is no longer a good measure of the affinity of the carrier for the sugar. Further, methods based on overall transport do not give an indication of binding for sugars which bind but cannot translocate. We have therefore measured the apparent inhibition constants of the sugars, which may more accurately reflect the binding constant k_{-1}/k_{+1} .

It is generally accepted that L-sorbose enters the erythrocyte by the system that transports D-glucose, although it has a very low affinity for the carrier (LeFevre & Davies, 1951; Sen & Widdas, 1962). It has been shown by Levine *et al.* (1971) that if the entry of L-sorbose into the erythrocyte is measured in the presence and absence of a second sugar that binds strongly to the carrier, an inhibition constant K_i can be measured which represents, on the symmetrical carrier model where $k_{+1}/k_{-1} = k_{-3}/k_{+3}$, the true dissociation constant of the carrier-sugar complex. In the asymmetric carrier model, where this is not true,

Scheme 1. Kinetic constants for a simple carrier system

s_0 and c_0 represent the concentrations of the unbound sugar and carrier on the outer surface of the membrane, and $s_0 c_0$ the concentration of the sugar-carrier complex. The corresponding concentrations on the inside of the membrane have the subscript 1.

K_i approximates to the dissociation constant on the outside of the membrane if measurements are taken in the early part of the entry of L-sorbose.

In the present paper, the variations of the apparent K_i for several modified D-glucose derivatives, measured under identical conditions, have been related to modifications in the structure, in order to probe the nature and extent of the binding of sugar to carrier. Although the numerical values obtained may not correspond exactly to the true dissociation constants for the sugars, by using the same transported sugar and the same conditions, the K_i values obtained must reflect the relative binding of the inhibitory sugars. A similar investigation of the binding requirements for the transport of sugars in hamster intestine has established the usefulness of the comparison of the inhibition constants of carefully selected sugars for exploring the interaction of sugar and carrier (Barnett *et al.*, 1970). In the present paper, as in the previous study, use is made of the replacement of the hydroxyl group by hydrogen and fluorine atoms to investigate the presence and direction of hydrogen bonds between sugar and carrier.

Materials and Methods

Materials

Sugars. The following sugars were prepared by the methods of the authors given in parentheses: α -D-glucopyranosyl fluoride, decomp. 120–130°C (Helferich *et al.*, 1926); β -D-glucopyranosyl fluoride, m.p. 91–92°C (Micheel & Klemer, 1952); 1-deoxy-D-glucose, m.p. 138–140°C (Ness *et al.*, 1950); 2-chloro-2-deoxy-D-glucose, m.p. 149°C (Adamson & Foster, 1969); 2-O-methyl-D-glucose, m.p. 152–155°C (Oldham & Rutherford, 1932); 1,2-dideoxy-D-arabinohexose, chromatographically pure syrup (Gray & Barker, 1967); D-glucal, m.p. 48–49°C (Shafizadeh & Stacey, 1952); 3-deoxy-D-glucose, m.p. 110–112°C (Anet, 1960); 3-deoxy-3-fluoro-D-glucose, m.p. 112–113°C (Foster *et al.*, 1967); 3-bromo-3-deoxy-D-

glucose, m.p. 148°C, and 3-chloro-3-deoxy-D-glucose, m.p. 150–152°C (Barnett *et al.*, 1969); 3-O-propyl-D-glucose, m.p. 138–140°C; 3-O-allyl-D-glucose, m.p. 130–133°C (Gigg & Gigg, 1966); 6-deoxy-D-glucose, chromatographically pure syrup (Karrer & Boettcher, 1953); 6-deoxy-6-fluoro-D-glucose, m.p. 153°C (Blakley, 1960); 6-deoxy-6-fluoro-D-galactose, m.p. 156°C (Taylor & Kent, 1958); 6-deoxy-6-iodo-D-galactose, m.p. 108–111°C (Raymond & Schroeder, 1948); D-allose, m.p. 141–142°C (Sowa & Thomas, 1966). 3-O-(2',3'-Epoxypropyl)-D-glucose (Bessell & Westwood, 1972) was made from 3-O-allyl-D-glucose by methods similar to those described for the preparation of 2',3'-epoxypropyl β -D-glucoside (Barnett & Ralph, 1971). We thank Dr. J. H. Westwood for a kind gift of tetra-acetyl-3-O-(2',3'-epoxypropyl)- β -D-glucose, and also for indicating their synthetic route before publication.

6-O-Propyl-D-galactose. The 1,2:3,4-di-O-isopropylidene-D-galactopyranose (10g) was converted into the 6-O-propyl ether by propyl iodide and NaOH in dioxan by the method described for preparing 3-O-allyl-1,2:5,6-di-O-isopropylidene-D-glucofuranose (Corbett & McKay, 1961). The propyl ether was purified by chromatography on a column of Merck (Darmstadt, W. Germany) silica gel (0.05–0.2 mesh; 100g, 50 cm $\times$ 2.5 cm), eluted with ethyl acetate–light petroleum (b.p. 40–60°C) (1:1, v/v), yield 8.5g. The propyl ether (3.5g) was dissolved in ethanol (10ml) and stirred at 70°C for 5h with Amberlite IR-120 (H^+ form) resin (10g) and water (25ml). The resin was filtered off, the aqueous solution washed with diethyl ether, and the solvent evaporated under reduced pressure. The product contained a trace of galactose and was purified by preparative paper chromatography on Whatman 3MM paper by using butan-1-ol–ethanol–water (4:1:5, by vol., upper layer) as solvent.

4-O-Propyl-D-glucose. Methyl 2,3,6-tri-O-benzoyl- α -D-glucopyranoside was synthesized by the method of Bell (1934) and crystallized from a small amount of ethanol, m.p. 128°C, $[\alpha]_D^{20} +136^\circ$ (c 0.27 in $CHCl_3$).

After recrystallization from methanol containing a trace of water, a sample had m.p. 129°C, $[\alpha]_D^{25} +139^\circ$ (*c* 0.33 in CHCl_3). A portion (2g) was dissolved in propyl iodide (25ml) and stirred with silver oxide (3g) overnight at 70–75°C. Diethyl ether was added and the solution filtered. After washing with saturated NaHCO_3 and drying, the ether was removed. Methyl 2,3,6-tri-*O*-benzoyl-4-*O*-propyl- α -D-glucopyranoside, m.p. 146–150°C, $[\alpha]_D^{25} +142^\circ$ (*c* 0.09 in CHCl_3) was recrystallized from ethanol. It had no i.r. absorption corresponding to the hydroxyl group. The *O*-propyl ether (1g) was suspended in dry methanol (10ml) and dry chloroform (1ml) and 1M-sodium methoxide (0.2ml) were added. After being stirred for 6h at room temperature, the methanol was removed and the resulting syrup dissolved in diethyl ether and water. The aqueous layer was evaporated to dryness to give methyl 4-*O*-propyl- α -D-glucopyranoside, which was hydrolysed by 2M-HCl in a sealed tube at 105°C for 18h. The acidic solution was deionized with Amberlite MB-1 resin and evaporated to dryness. The resulting syrup contained traces of glucose as shown by paper chromatography, and was purified on Whatman 3MM paper to give 4-*O*-propyl-D-glucose, $R_{\text{glucose}} 3.6$, $M_{\text{glucose}} 0.2$. 3-*O*-Propyl-D-glucose had $R_{\text{glucose}} 3.8$, $M_{\text{glucose}} 0.8$. The low relative mobility of 4-*O*-propyl-D-glucose on electrophoresis in sodium borate is characteristic of 4-*O*-alkyl and 2-*O*-alkyl ethers of glucose (Foster, 1953).

2,2'-Dichloro-2-deoxy-D-*arabino*-hexose (2,2'-dichloro-D-glucose) and some 3-deoxy-3-fluoro-D-glucose were respectively the generous gifts from Dr. J. Adamson and Professor A. B. Foster, Chester Beatty Institute, and Dr. N. F. Taylor, University of Bath.

1,5-Anhydro-D-[6- ^3H]glucitol(1-deoxy-D-glucose). This (m.p. 139–140°C; 700 $\mu\text{Ci}/\text{mmol}$; lit. m.p. 142–143°C) was prepared from D-[6- ^3H]glucose by the method of Ness *et al.* (1950) for the unlabelled anhydro sugar. Although paper chromatography showed only one radioactive spot, the material was further purified by preparative chromatography before use.

Other sugars and general chemicals. These were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., or Koch-Light Ltd., Colnbrook, Bucks., U.K., and 6-amino-6-deoxy-D-glucose was obtained from Cambrian Chemicals Ltd., Croydon, Surrey, U.K.

Methods

Chromatography. All sugars were tested for purity by descending paper chromatography on Whatman no. 1 paper with butan-1-ol-ethanol-water (49:11:19, by vol.) or butan-1-ol-pyridine-water (9:2:1, by vol.) (for allose) as solvent. Non-reducing sugars were located by immersion in 0.5% HIO_4 in

acetone, followed by staining of the background with benzidine. Reducing sugars were located by alkaline AgNO_3 treatment. Sugars containing any impurity were purified on Whatman 3MM paper with the above solvents. T.l.c. of sugar analogues modified at C-1 was on Kieselgel G (Merck) (previously run in 0.2M-sodium acetate, and dried) with ethyl acetate-propan-2-ol-water (26:14:7, by vol.) as solvent. Sugars were located by treatment with anisaldehyde- H_2SO_4 -ethanol (1:1:18, by vol.) and heating for 15 min. All sugars used in transport experiments were chromatographically pure. T.l.c. of protected sugars was on Kieselgel G (Merck) in ethyl acetate-light petroleum (b.p. 40–60°C) (3:2, v/v). Spots were usually located in u.v. light.

Electrophoresis. The mobilities of 3-*O*-propyl- and 4-*O*-propyl-D-glucose relative to D-glucose were determined by the method of Foster (1953) on a Locarte high voltage electrophoresis apparatus, by using Whatman no. 1 paper and 0.2M-sodium borate buffer, pH 10, at 1800 V. Under these conditions glucose travelled 6cm in 2h. The origin moved towards the cathode and was marked with Blue Dextran.

Human erythrocyte suspension. This was prepared by a method similar to that of Levine *et al.* (1971). Out-dated transfusion blood, which had been stored at 4°C, was washed with 3 $\times$ 6 vol. of iso-osmotic NaCl-sodium phosphate buffer, pH 7.4, 25mm in phosphate and 1% in NaCl, at 37°C. The cells were centrifuged after each washing and the supernatant containing plasma protein and leucocytes was discarded. After final suspension at 50% packed cell volume in the buffer, the cells were stored overnight at 4°C. The cells were finally washed once more in the buffer at 37°C and resuspended at 50% packed cell volume in buffer at 25°C.

Inhibition constants. Inhibition constants were measured by the method of Levine *et al.* (1971) at 25°C. The sugar to be tested (1.5ml, usually 33.3mm) in 25mm-sodium phosphate buffer, pH 7.4, containing 1% NaCl, was added to 2.5ml of cell suspension previously adjusted to 50% packed cell volume. For measurement of sorbose uptake in the absence of inhibitor, the cells were pre-equilibrated with buffer containing 1% NaCl. The suspensions were pre-equilibrated for at least 10min, and at zero time 25mm-[^{14}C]sorbose (1ml, 0.4 μCi) was added to each. After 2, 4, 6, 8 or 10min 30ml of ice-cold stopping medium (2mm- HgCl_2 , 1.25mm-KI in 1% NaCl) was added. The cells were centrifuged, and the supernatants discarded. The pellets were carefully washed with stopping solution (30ml) without disturbing the pellet, and the inside of the tube was carefully dried. Then 10% (w/v) trichloroacetic acid (4ml) was added, and after centrifugation the supernatant (0.5ml) was added to 10ml of NE 250 dioxan-based scintillation fluid [Nuclear Enterprises (G.B.) Ltd., Edinburgh, U.K.], and counted for radioactivity in a Beckman

liquid-scintillation counter. A blank (zero time) was made by adding stopping fluid before the sorbose. This measured extracellular space and was subtracted from the counts for subsequent times. A measurement of the total intracellular space was made by equilibration of the sorbose at 37°C for 2 h.

In the absence of inhibitor, sorbose enters the cell, and as the concentration inside tends to equilibrium, the net entry of [^{14}C]sorbose decreases. If the concentration of added sorbose is s_o and the concentration inside after time t is s_i , then Levine *et al.* (1971) showed that for the symmetrical carrier model:

$$\ln \left(\frac{s_o}{s_o - s_i} \right) = \frac{V_{\max} \cdot t}{K_s}$$

where $V_{\max}$ is the maximum initial rate of entry at infinite substrate concentration and K_s is the dissociation constant of the sugar-carrier complex.

At equilibrium, the concentration outside the cell is equal to that inside, s_o is proportional to the counts obtained after equilibration ($c_{\text{equil.}}$) and s_i is proportional to the counts at time t (c_t); the expression therefore becomes:

$$\ln \left(\frac{c_{\text{equil.}}}{c_{\text{equil.}} - c_t} \right) = \frac{V_{\max} \cdot t}{K_s}$$

In the presence of an inhibitor that equilibrates across the membrane, Levine *et al.* (1971) showed that if the concentrations of substrate and inhibitor are comparable, and if $K_i \gg K_s$:

$$\ln \left(\frac{s_o}{s_o - s_i^*} \right) = \frac{V_{\max} \cdot t}{K_s \left(1 + \frac{I}{K_i} \right)}$$

that is:

$$\ln \left(\frac{c_{\text{equil.}}}{c_{\text{equil.}} - c_t^*} \right) = \frac{V_{\max} \cdot t}{K_s \left(1 + \frac{I}{K_i} \right)}$$

where K_i is the dissociation constant of the inhibitor-carrier complex, I is the concentration of inhibitor, s_i^* is the internal sorbose concentration and c_t^* the counts at time t in the presence of inhibitor.

D-Glucose mutarotated to equilibrium within 10 min in the buffer used and at 25°C. Other sugars used in this work were assumed to mutarotate to an equilibrium value within 10 min at 25°C. K_i values given are therefore for the anomeric equilibrium mixture of the sugar.

It was assumed, because 1-deoxy-D-glucose (K_i 80 mM) and D-galactose (K_i 85 mM) equilibrated across the erythrocyte membrane within 30 s, that all sugars tested as inhibitors equilibrate across the erythrocyte membrane within 10 min, except where stated in the tables. This assumption may be incorrect, particularly for sugars with substituents at C-4 and C-6 or those that showed no inhibition, but sugars

known to bind to the carrier, but not to penetrate the membrane on the glucose/sorbose carrier, such as 4,6-O-ethylidene-D-glucose, gave a K_i similar to that expected from their structure and previous work.

Results

Inhibition constants

Fig. 1 shows the straight-line plot of $\ln[s_o/(s_o - s_i)]$ {equivalent to $\ln[c_{\text{equil.}}/(c_{\text{equil.}} - c_t)]$ } against time for L-sorbose alone and in the presence of the D-glucose analogues modified at C-3. The rate of entry of sorbose depended slightly on the preparation, and in every case for the calculation of the inhibition constants, K_i (Tables 1–4), the rate of entry of sorbose in the presence of inhibitor was compared directly with the rate of sorbose entry measured at the same time in its absence. However, for the graphical presentation in Figs. 1 and 2, the average slope for the rate of sorbose entry from all experiments has been plotted,

Fig. 1. Penetration of L-sorbose into human erythrocytes in the presence of D-glucose and its analogues modified at C-3

5 mM-L-[^{14}C]Sorbose was incubated with human erythrocytes at 25°C in the presence and absence of: D-glucose, Δ ; 3-deoxy-3-fluoro-D-glucose, $\blacksquare$; 3-O-methyl-D-glucose, $\bullet$; 3-chloro-3-deoxy-D-glucose, $\square$; 3-deoxy-D-glucose, $\blacktriangle$; D-allose, $\circ$; all 10 mM. A function of the extent of filling was plotted against time. Uninhibited L-sorbose entry is represented by a bar, which shows the limits of the standard error, and the points are corrected as described in the text.

Fig. 2. Penetration of L-sorbose into human erythrocytes in the presence of D-glucose and its analogues modified at C-6

5mM-[¹⁴C]Sorbose was incubated with human erythrocytes at 25°C in the presence and absence of: 10mM-D-glucose, Δ ; 10mM-6-deoxy-D-glucose, $\bullet$; 10mM-6-deoxy-6-fluoro-D-glucose, $\square$; 20mM-D-xylose, $\circ$. A function of the extent of filling was plotted against time. Uninhibited L-sorbose entry is represented by a bar, which shows the limits of the standard error, and the points are corrected as described in the text.

together with the s.e.m. The small error in the points taken at the longest times reflects variations in slope rather than the error in determining the points, which usually lay precisely on a straight line. The points obtained in the presence of various inhibitors were then multiplied by the average slope for uninhibited sorbose entry, and divided by the actual slope for sorbose entry determined at the same time as the inhibited entry. In this way relative inhibition by the various D-glucose analogues can be directly compared in graphical form.

Usually inhibition constants were obtained in duplicate, and both values are shown in the tables. The reproducibility was excellent. Determinations were made initially with 10mM inhibitor, but in those cases in which the inhibition was small ($K_i > 50$ mM) a more accurate value was obtained by repeating the determination with 20mM inhibitor. This change did not markedly affect the value of the inhibition constant obtained for D-glucose.

Penetration experiments

The sugar was incubated with a suspension of erythrocytes and the penetration measured in the same way as the penetration of L-sorbose was measured during the inhibition experiments. Both D-[1-³H]galactose and 1,5-anhydro-D-[6-³H]glucitol (1-deoxy-D-glucose) penetrated completely within 30s at 25°C.

Discussion

Interaction of D-glucose with the carrier binding site must involve an equilibrium between the binding of the sugar to the solution and its binding to the carrier protein. The carrier may also have water molecules associated with the pocket that will receive the sugar.

The association of water with both sugar and carrier, and between sugar and carrier, will probably be by hydrogen bonds, but may also include covalent bonds (see Barnett *et al.*, 1970). The equilibrium lies towards carrier-sugar complex-formation, because if the hydroxyl groups are in the correct configuration on the sugar the hydroxyl groups and the complementary groups on the carrier will be in an exact stereochemical relationship. If such a binding hydroxyl group is replaced by a hydrogen atom or inverted, the affinity of the sugar for the carrier will decrease. Assuming that the stereochemistry is still retained, and that the substituent is not too large, replacement of the hydroxyl group by another electronegative atom or group, such as fluorine, may restore the affinity for the carrier if it is the protein that donates the hydrogen atom, but not if it was the hydroxyl group that originally donated the hydrogen atom. The electronegative atoms that can take part in a hydrogen bond have an electronegativity greater than about 2.5 (Pauling, 1940, p. 64), i.e. fluorine, oxygen (as hydroxyl or alkoxy group), chlorine, nitrogen and bromine, in decreasing order of effectiveness. Other atoms (e.g. hydrogen, iodine) do not usually take part in hydrogen bonding (see Pauling, 1940, pp. 284-334).

The affinity for the carrier is measured by K_i , which represents the dissociation constant of the carrier-sugar complex. When K_i is high, the affinity is low. At positions where hydrogen bonds appear to be formed, the change in K_i , when a hydroxyl group is replaced by a hydrogen atom, is about tenfold. Complete loss of affinity when the hydroxyl group has been replaced by the smaller hydrogen atom has been taken as an indication of the formation of a covalent bond with the hydroxyl group (Barnett *et al.*, 1970).

Study of Tables 1, 2, 3 and 4 shows that no single hydroxyl group is essential for binding to the carrier. Although neither of the methyl glucopyranosides was an inhibitor, 1-deoxy-D-glucose was both a good

inhibitor and was transported, showing that the hydroxyl group at C-1 is not essential. Transport of 1-deoxy-D-glucose has been reported (LeFevre, 1967), and it has been shown that the transport is inhibited by D-glucose (Evans *et al.*, 1969), but hitherto the possibility has remained that 1-deoxy-D-glucose might be transported by a separate glucose-sensitive system. The present results confirm that 1-deoxy-D-glucose binds to the D-glucose/L-sorbose-carrier system. Its high affinity for the system shows that the pyranose forms of the sugars bind to the carrier.

Similar analogues of D-glucose lacking a hydroxyl group in the D-*gluco* configuration at each of the other carbon atoms also bind to the carrier. Binding of the sugar to the carrier cannot therefore involve covalent bonds with any of the hydroxyl groups, but appears to involve hydrogen bonds to the hydroxyl group in the β -configuration at C-1 and in the D-*gluco* configuration at C-3, probably C-4 and possibly C-6, as shown by the following evidence.

Binding at C-1

Inspection of Table 1 shows that the replacement of the hydroxyl group at C-1 of D-glucose by a hydrogen atom in 1-deoxy-D-glucose gives a marked decrease

in affinity for the carrier, suggesting the presence of a hydrogen bond. Binding is considerably restored if a fluorine atom is introduced into the β -configuration, but not if it is introduced into the α -configuration, indicating that the hydrogen bond is to the hydroxyl group of β -D-glucopyranose. More rapid penetration of the β rather than the α form of D-glucose has been reported (Reinwein *et al.*, 1957; LeFevre & Marshall, 1958; Faust, 1960) but different interpretations have been placed on the meaning of this observation. LeFevre & Marshall (1958) maintain that at the concentrations used in the earlier experiments, this might mean that the α -anomer binds more effectively. The present results, obtained with fluorinated analogues that cannot mutarotate, suggest that the β -anomer of D-glucose will bind more effectively to the carrier.

T.l.c. of the solutions immediately before the addition of the L-sorbose showed that the extent of hydrolysis of the fluorides during the experiment was negligible. However, some of the erythrocytes used appeared to contain an α -glucosidase, which catalysed the hydrolysis of α -D-glucopyranosyl fluoride and methyl α -D-glucopyranoside. The results given are for erythrocytes that did not cause this hydrolysis.

Because the enhanced binding of the β form of the

Table 1. Inhibition constants (K_i) of D-glucose analogues modified at C-1 and C-2 and tested as inhibitors of L-sorbose entry into human erythrocytes

The K_i values were determined as described in the Materials and Methods section. N.D., No detectable inhibition.

Sugar	Concn. tested (mM)	Amount of β -pyranose form at equilibrium (%)	K_i (mM) (25°C)
D-Glucose	20	64	7.6
	10		6.3
			7.5
1-Deoxy-D-glucose	20	—	76
			81
α -D-Glucopyranosyl fluoride	10	0	77
			80
β -D-Glucopyranosyl fluoride	10	100	15.4
			15.5
Methyl α -D-glucopyranoside	10	0	N.D.
Methyl β -D-glucopyranoside	10	100	N.D.
2-Deoxy-D-glucose	10	52.5	3.2
2-Chloro-2-deoxy-D-glucose	10	—	8.3
			9.0
2,2'-Dichloro-2-deoxy-D-glucose	10	—	5.8
2-O-Methyl-D-glucose	20	—	119
D-Mannose	10	36	19
			20
1,2-Dideoxy-D-glucose	10	—	37.8
D-Glucal	10	—	26.3
			27.0

glucopyranosyl fluorides is presumably reflected in the relative binding of the free sugars, the percentage of the β -pyranose form of the sugar at equilibrium, wherever it is known (Angyal, 1968; Foster *et al.*, 1970), has been included in the tables. Fortunately the ratio of α to β forms in the majority of these sugars does not differ markedly from the ratio found for D-glucose.

Binding at C-2

Replacement of the hydroxyl group at C-2 by a hydrogen atom in 2-deoxy-D-glucose (Table 1) results in a small enhancement of affinity for the carrier, showing that there is no sugar-carrier bond at C-2. The small enhancement of binding can be rationalized if it is assumed that the hydroxyl group at C-2 of D-glucose is solvated in solution, but not on the carrier. The effect of this will be to confer a greater stability on the glucose in solution compared with 2-deoxy-D-glucose, which cannot be solvated at C-2. The energy of the carrier-bound forms of both sugars should, however, be similar. The equilibrium might therefore be expected to favour the sugar-carrier complex of 2-deoxy-D-glucose slightly more than that of D-glucose. A similar enhancement of affinity is found in the comparison of 1,2-dideoxy-D-glucose and 1-deoxy-D-glucose.

D-Glucal (1,2-dideoxy-D-arabino-hex-1-enopyranose) (Fig. 3) bound more strongly than 1,2-dideoxy-D-glucose, showing that the half-chair conformation, planar about C-1 and C-2, does not diminish binding to the carrier, but marginally increases it.

There appears to be some steric hindrance in the binding of both D-mannose and 2-O-methyl-D-glucose, suggesting that the protein of the carrier approaches fairly closely at both D-glucose and D-manno configurations, although in neither case was this tested with larger substituents that did not bind at all.

Binding at C-3

Table 2 shows that the replacement of the hydroxyl group at C-3 of D-glucose by a hydrogen atom causes a marked decrease in affinity for the carrier, and that the affinity is restored by substitution of a fluorine atom. These observations suggest a hydrogen bond from the carrier to the hydroxyl group at this position, a hydrogen atom of the protein being used as the bridge hydrogen. 3-O-Methyl-D-glucose, which can form a similar, though probably weaker, hydrogen bond, has an affinity intermediate between that of 3-deoxy-D-glucose and D-glucose, but closer to that of D-glucose.

D-Allose (the 3-epimer) had no measurable affinity for the carrier, and it must be presumed that this is

due to steric hindrance. This suggests a close approach of the protein residues of the carrier to the D-allo configuration at C-3 of the sugar, contrasting with the space available for substituents in the D-glucose configuration at C-3. Although 3-O-ethyl- and 3-O-propyl-D-glucose had a lower affinity than 3-O-methyl-D-glucose, indicating some steric hindrance, the affinity of 3-O-allyl-D-glucose was greater than that of 3-O-propyl- or 3-O-ethyl-D-glucose, showing that in this more planar molecule the steric hindrance was decreased.

Binding at C-4

4-Deoxy-D-glucose was not available, but D-galactose, the 4-epimer of D-glucose, had a considerably lower affinity for the carrier than D-glucose. Although the decrease in affinity could be due to steric constraints, as in D-allose, it seems more probable that it is due to the absence of a hydrogen bond between the sugar hydroxyl group at C-4 in the D-glucose configuration and the carrier, because there appears to be a large 'space' on the carrier near C-4 on the sugar.

Indeed, there is much evidence that the area around C-4 of the sugar is directed into solution. We have confirmed (Table 3) that the non-transported sugars, 4,6-O-ethylidene-D-glucose (Baker & Widdas, 1971), maltose and cellobiose (Chen & LeFevre, 1965; Lacko & Burger, 1962), which inhibit glucose exit, bind to the glucose/sorbose carrier. Lactose has an extremely low affinity for the carrier, confirming the results of Chen & LeFevre (1965). The affinity was much lower than that of cellobiose, which, as noted by Widdas (1972), differs only in the configuration of the C-4 hydroxyl group in the second sugar residue.

Fig. 3. 1,2-Dideoxy-D-arabino-hex-1-enopyranose (D-glucal)

The half-chair conformation shown is planar about the ring oxygen, C-1 and C-2. The disposition of the remaining groups resembles that of D-glucose in the C1 conformation (see Fig. 4).

Table 2. *Inhibition constants (K_i) of D-glucose analogues modified at C-3, tested as inhibitors of L-sorbose entry into human erythrocytes*

The K_i values were determined as described in the Materials and Methods section. N.D., No detectable inhibition.

Sugar	Concn. tested (mM)	Amount of β -pyranose form at equilibrium (%)	K_i (mM)
D-Glucose	10	64	6.3
			7.5
	20		7.6
3-Deoxy-D-glucose	10	56	71.5
			69.0
3-Deoxy-3-fluoro-D-glucose	10	55	6.9
			7.1
3-Chloro-3-deoxy-D-glucose	10	—	33
			32
3-Bromo-3-deoxy-D-glucose	10	—	30
			33
3-O-Methyl-D-glucose	10	—	12
			14
3-O-Ethyl-D-glucose	14.65	—	48
3-O-Propyl-D-glucose	10	—	59
3-O-Allyl-D-glucose	10	—	25
3-O-(2',3'-Epoxypropyl)-D-glucose	10	—	25
			27
D-Allose	10, 20, 40	65	N.D.

Table 3. *Inhibition constants (K_i) of D-glucose analogues modified at C-4, tested as inhibitors of L-sorbose entry into human erythrocytes*

The K_i values were determined as described in the Materials and Methods section.

Sugar	Concn. tested (mM)	Amount of β -pyranose form at equilibrium (%)	K_i (mM)
D-Galactose	20	65	85
			90
Maltose*	20	—	19
Cellobiose*	20	—	51
Lactose*	20	—	>250
4,6-O-Ethylidene-D-glucose*	10	—	7.0
4-O-Propyl-D-glucose	5	—	10.2

* These sugars do not penetrate the erythrocyte membrane by the D-glucose/L-sorbose carrier.

4-O-Propyl-D-glucose had an affinity for the carrier comparable with that of D-glucose, and maltotriose inhibits glucose exit (Lacko & Burger, 1962). All of these sugars can be regarded as C-4 substituents of glucose, and there must therefore be a large space around C-4 in the D-glucose configuration.

Binding at C-6

The sugar-carrier interaction at C-6 is not simple. Unlike all the other positions tested, where the sub-

stitution of an O-alkyl group for the hydroxyl group leads to decreased affinity for the carrier, 6-O-alkyl ethers appear to bind more effectively than the corresponding sugar (Table 4). Further, both polar and non-polar substituents at C-5 of D-xylose (glucose series) (Fig. 2) or of L-arabinose (galactose series) increase the affinity of the sugar, and the enhancement cannot be interpreted either in terms of hydrophobic binding or hydrogen bonding alone. Similar substituents in both series have the same pattern of relative affinities.

Table 4. Inhibition constants (K_i) of D-glucose analogues modified at C-6, tested as inhibitors of L-sorbose entry into human erythrocytes

The K_i values were determined as described in the Materials and Methods section. N.D., No detectable inhibition.

Sugar	Concentration (mM)	Amount of β -pyranose form at equilibrium (%)	K_i (mM)
D-Glucose	10	64	6.3
	20	64	7.6
6-Deoxy-D-glucose	10	—	6.7
	10	—	6.7
6-Deoxy-6-fluoro-D-glucose	8.7	—	1.2
	8.7	—	1.3
Glucose 6-phosphate	10	—	N.D.
6-Amino-6-deoxy-D-glucose	10	—	N.D.
D-Xylose	20	77	69
	20	77	70
D-Galactose	20	65	85
6-Deoxy-D-galactose	20	—	48
	20	—	51
6-Deoxy-6-fluoro-D-galactose	10	—	6.2
	10	—	7.7
6-Deoxy-6-iodo-D-galactose	10	—	8
6-O-Methyl-D-galactose	20	—	45
	20	—	47
6-O-Propyl-D-galactose	20	—	33.4
L-Arabinose	20	63*	N.D.

* Percentage of the α -L form, which corresponds to the stereochemistry of β -D-galactose.

The high affinities of both 6-deoxy-6-fluoro- and 6-deoxy-6-iodo-D-galactose, the similarity of the binding of glucose and galactose to their respective deoxy analogues, and the increase in affinity in the series D-galactose, 6-O-methyl-D-galactose and 6-O-propyl-D-galactose, suggest that both hydrogen bonding and hydrophobic binding may contribute. The unexpectedly high affinity of the iodo compound may suggest an interaction between the iodine atom and an aromatic ring, and the high affinities of the fluorinated compounds may suggest the possibility of hydrogen bonding. Models indicate that either a tryptophan or a tyrosine residue suitably placed with respect to C-6 of the sugar could fulfil both these functions, but other lipophilic materials cannot be excluded. It is possible that the fluorine atom behaves in a non-polar way, acting as an analogue of hydrogen rather than a hydroxyl group, but this would not explain why the affinity is greater than that of the corresponding deoxy sugar.

Neither D-glucose 6-phosphate nor 6-amino-6-deoxy-D-glucose, both charged at pH 7.4, had any affinity for the carrier. The region around C-6 will not, therefore, accept either a positively or negatively charged group.

General model for sugar binding

A general model for the binding of sugars to the human erythrocyte glucose/sorbose carrier is shown in Fig. 4. The model is based on the considerations discussed above and explains the relative affinities, not only of the sugars tested in the present paper, but also of those investigated elsewhere. Thus L-sorbose may bind as shown in Fig. 5, with the ring oxygen in the position normally occupied by the C-1 of D-glucose, and the methylene group at the normal ring oxygen position. The hydroxymethyl group is then at C-2 and normal binding can be expected at the other positions.

The correlation previously noted between the stability of the D-C1 (Reeves, 1951) conformation of the sugar and the affinity for the carrier (LeFevre, 1961) can be rationalized because it is clear that all of the hydrogen bonds between the sugar and the carrier are to hydroxyl groups in the β -D-glucopyranose configuration, which is also the most stable D-C1 form. Alteration, by epimerization, of one of these hydroxyl groups will destroy the hydrogen bond, and at the same time, but coincidentally, introduce a Reeves instability factor. The inability to form hydrogen bonds is thus paralleled by the number

Fig. 4. Model for the glucose-carrier complex in the human erythrocyte

For explanation of the model see the Discussion section.

Fig. 5. β -L-Sorbofuranose shown in the conformation in which it may bind to the glucose carrier

The hydroxymethyl group at C-1 of sorbose may fit in the position normally occupied by the hydroxyl group at C-2 of a hexose (see Fig. 3). In β -L-sorbofuranose (shown) this is in the *D-manno* configuration; in α -L-sorbofuranose it would take up the *D-gluco* configuration. The C-5 hydroxyl group of α -L-sorbofuranose fits in the space for the hydroxymethyl group at C-6 of *D*-glucopyranose, and the C-3 and C-4 hydroxyl groups can make hydrogen bonds.

of instability factors in the *D*-C1 form. It is probable, however, that glucose does bind in the C1 form, because this is its most favourable configuration, and the hydrogen bonds are probably oriented to combine with this form of the sugar.

To design a successful substrate-directed alkylating agent, it is important that the bulk of the alkylating group can be accommodated on the carrier binding site without markedly decreasing the effectiveness of the binding. The results in the present paper show that there is space in the *D-gluco* configuration at C-3, C-4 and C-6. For the preliminary tests C-3 was chosen because the relative binding of 3-*O*-allyl- and 3-*O*-

propyl-*D*-glucose suggested that some steric hindrance occurred in this position, and that the protein might form a pocket, whereas the evidence suggests that C-4 and C-6 point out into solution. 3-*O*-(2,3-Epoxypropyl)-*D*-glucose was synthesized and tested as an alkylating substrate, but although it bound well, it had the same K_i , 25 mM, whether it was pre-incubated with the erythrocytes for 10 min or 5 h. More reactive substituents and derivatives of C-4 and C-6 might be more successful alkylating substrates.

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