Evidence for Two Asymmetric Conformational States in the Human Erythrocyte Sugar-Transport System

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6-*O*-Methyl-, 6-*O*-propyl-, 6-*O*-pentyl- and 6-*O*-benzyl- β -D-galactose, and 6-*O*-methyl-, 6-*O*-propyl- and 6-*O*-pentyl- β -D-glucose inhibit the glucose-transport system of the human erythrocyte when added to the external medium. Penetration of 6-*O*-methyl- β -D-galactose is inhibited by β -D-glucose, suggesting that it is transported by the glucose-transport system, but the longer-chain 6-*O*-alkyl- β -D-galactoses penetrate by a slower β -D-glucose-insensitive route at rates proportional to their olive oil/water partition coefficients. 6-*O*-*n*-Propyl- β -D-glucose and 6-*O*-*n*-propyl- β -D-galactose do not significantly inhibit L-sorbose entry or β -D-glucose exit when present only on the inside of the cells whereas propyl- β -D-glucopyranoside, which also penetrates the membrane slowly by a glucose-insensitive route, only inhibits L-sorbose entry or β -D-glucose exit when present inside the cells, and not when on the outside. The 6-*O*-alkyl- β -D-galactoses, like the other non-transported C-4 and C-6 derivatives, maltose and 4,6-*O*-ethylidene- β -D-glucose, protect against fluorodinitrobenzene inactivation, whereas propyl β -D-glucopyranoside stimulates the inactivation. Of the transported sugars tested, those modified at C-1, C-2 and C-3 enhance fluorodinitrobenzene inactivation, where those modified at C-4 and C-6 do not, but are inert or protect against inactivation. An asymmetric mechanism is proposed with two conformational states in which the sugar binds to the transport system so that C-4 and C-6 are in contact with the solvent on the outside and C-1 is in contact with the solvent on the inside of the cell. It is suggested that fluorodinitrobenzene reacts with the form of the transport system that binds sugars at the inner side of the membrane. An Appendix describes the theoretical basis of the experimental methods used for the determination of kinetic constants for non-permeating inhibitors.

The transport of hexoses across the human erythrocyte membrane takes place by 'facilitated diffusion', a saturable process which does not consume energy but which involves combination of the sugar with a specific site or sites on the membrane. Studies over many years have established the specificity requirements of the process, and similar results have been obtained whether the measurement was of transport (LeFevre, 1961; Sen & Widdas, 1962), or of the ability to inhibit transport of another sugar, such as L-sorbose (Barnett *et al.*, 1973*a*) or the ability selectively to displace β -D-glucose from membrane fragments (Kahlenberg & Dolansky, 1972). In the more recent studies, which have implicated hydrogen bonds in sugar-transport-site binding, the inhibitory sugar was present on both sides of the membrane.

Baker & Widdas (1973*a*) have shown that 4,6-*O*-ethylidene- β -D-glucose inhibits sugar transport asymmetrically. When added outside the cells it does not penetrate the membrane by the glucose-transport system, but inhibits the system competitively. However, the sugar will slowly penetrate the cells by another route, and does not inhibit the transport

process when present only on the inner surface of the cells. We now report a group of similar non-transported hexoses with lipophilic groups either at C-1 or C-6. Their behaviour suggests that the specificity for binding is different on the inner and outer surfaces of the membrane and that the transport system is asymmetric. We also report some observations on the fluorodinitrobenzene inactivation of the glucose-transport system, which extend the observations of Bowyer & Widdas (1958), Krupka (1971, 1972), Shimmin & Stein (1970) and Edwards (1973), and which appear to confirm that the transport system can exist in different conformational states. Preliminary reports of some of this work have been published (Barnett *et al.*, 1973*b,c*).

Materials and Methods

O-Alkyl derivatives of sugars

6-*O*-Propyl- β -D-glucose. 3,5-*O*-Benzylidene-1,2-*O*-isopropylidene- α -D-glucopyranose (2.5 g) was dissolved in dry dioxan (15 ml) containing freshly powdered

NaOH (3 g). *n*-Propyl bromide (15 ml) was added and the mixture stirred at 70°C overnight. After cooling, the mixture was poured into ice-water and diethyl ether mixture. The ether layer was washed with water and dried over Na₂SO₄. Removal of the solvent gave 3,5-*O*-benzylidene-1,2-*O*-isopropylidene-6-*O*-propyl- α -D-glucopyranose, which was recrystallized from ethanol, m.p. 72°C, $[\alpha]_D^{25}$ 8.2° (c 0.56 in ethanol). A portion (1.8 g) was dissolved in ethanol (10 ml) and water (10 ml) and Amberlite IR 120 (H⁺ form; 5 g) added. The mixture was stirred at 70°C for 5 h and filtered to remove the resin, which was washed with water. Filtrate and washings were combined and washed with diethyl ether and the aqueous solution was evaporated to dryness to give 6-*O*-propyl-D-glucose, recrystallized from ethanol, m.p. 122–123°C.

6-*O*-Pentyl-D-glucose, m.p. 72–75°C, was made by a similar procedure. By using 1,2:3,4-di-*O*-isopropylidene- α -D-galactopyranose or 1,2:5,6-di-*O*-isopropylidene- α -D-glucopyranose (Koch–Light Ltd., Colnbrook, Bucks., U.K.), syrupy 6-*O*-methyl-D-galactose, 6-*O*-propyl-D-galactose, m.p. 58–60°C, 6-*O*-pentyl-D-galactose, m.p. 110–112°C, syrupy 6-*O*-benzyl-D-galactose, 3-*O*-propyl-D-glucose, m.p. 138–140°C, 3-*O*-pentyl-D-glucose, m.p. 133–135°C, and syrupy 3-*O*-benzyl-D-glucose were obtained by the same procedure.

Sugars

Phenyl β -D-glucopyranoside, 6-deoxy-D-glucose, 6-deoxy-D-galactose, 4,6-*O*-ethylidene-D-glucose, 1,2:5,6-di-*O*-isopropylidene- α -glucopyranose, 1,2:3,4-di-*O*-isopropylidene- α -D-galactopyranose and phenyl α -D-glucuronide were obtained from Koch–Light Ltd. Other sugars were obtained from British Drug Houses Ltd., Poole, Dorset, U.K., or were made by the methods used by Barnett *et al.* (1973a).

L-[U-¹⁴C]Sorbitose, D-[6-³H]glucose, 6-deoxy-D-[1-³H]galactose, [1-³H]galactose and NaB³H₄ were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Phenyl β -D-[6-³H]glucopyranoside. Phenyl β -D-glucuronide (100 mg) was dissolved in methanol (0.3 ml) and a solution of diazomethane in ether added dropwise until a precipitate formed. The solvents were removed and the process repeated until the supernatant was a permanent yellow colour. Removal of the solvent gave phenyl β -D-glucopyranoside methyl ester, m.p. 131–132°C. The ester (50 mg) was dissolved in water (0.5 ml) and NaB³H₄ (2 mg, 4 mCi) added in water (0.5 ml). The solution was left at room temperature for 1 h, deionized with Amberlite IR 120 (H⁺ form; 1 g) and evaporated to dryness. The residue was purified by paper chromatography to give the product (yield 22 mg; m.p. 167°C; 4 mCi/mmol).

***n*-Propyl β -D-[1-³H]glucopyranoside.** D-[1-³H]Glu-

cose (2 g, 500 μ Ci) was converted into the penta-acetate by the action of HClO₄ and acetic anhydride (Krüger & Roman, 1936). Treatment of the penta-acetate with 5 ml of 45% (w/v) HBr in acetic acid gave 2,3,4,6-tetra-*O*-acetyl- α -D-glucopyranosyl bromide, which was dissolved in propan-1-ol (50 ml) and stirred with Ag₂O (6 g) overnight in the dark. The silver salts were filtered off and the propan-1-ol was removed. The residue was dissolved in chloroform and washed with 5% (w/v) Na₂S₂O₃ and water and dried over CaCl₂. Removal of the solvent gave *n*-propyl 2,3,4,6-tetra-*O*-acetyl- β -D-[1-³H]glucopyranoside, recrystallized from ethanol, m.p. 84°C [Timmell (1964) gives 96°C for the unlabelled compound]. Catalytic deacetylation by 0.01 M-sodium methoxide gave the product, recrystallized from ethanol–diethyl ether, m.p. 96–97°C [Timmell (1964) gives 101–103°C].

Unlabelled *n*-propyl β -D-glucopyranoside and *n*-propyl β -D-galactopyranoside, m.p. 97–99°C, $[\alpha]_D^{25}$ –9° (c 1.2 in water), were made in the same way.

1,2:3,4-Di-*O*-isopropylidene- α -D-[6-³H]galactopyranose. 1,2:3,4-Di-*O*-isopropylidene- α -D-galactodialdose (1 g) (Godman *et al.*, 1968) was dissolved in ethanol (3 ml). NaB³H₄ (12 mg, 12 mCi/mmol) was added in water (0.5 ml) at 0°C with magnetic stirring. The mixture was left at room temperature for 1 h, and unlabelled NaBH₄ (200 mg) added. After 30 min the product was poured into water and extracted into chloroform. The aqueous layer was extracted with a further 50 ml of chloroform containing unlabelled product (5 g). The chloroform layers were combined, washed with water and dried over Na₂SO₄. Removal of the solvent gave the product (5 g; 6 μ Ci/mmol), which was stored in dry dioxan at –15°C until required.

6-Deoxy-6-iodo-D-galactose (Raymond & Schroeder, 1948) and 6-*O*-methyl-, 6-*O*-propyl-, 6-*O*-pentyl- and 6-*O*-benzyl-D-[6-³H]galactose were made from 1,2:3,4-di-*O*-isopropylidene- α -D-[6-³H]galactopyranose by the established methods (see Corbett & McKay, 1961).

Stopping solutions

Two stopping solutions were used: mercury stopper, 1% NaCl, 2 mM-HgCl₂, 1.25 mM-KI; phloretin stopper; 1% NaCl, 10 μ M-HgCl₂, 1.25 mM-KI, to which was added a solution of phloretin, to give a final concentration of 0.1 mM-phloretin and 1% (v/v) ethanol.

Phosphate-saline buffer

This was 25 mM-sodium phosphate buffer, pH 7.4, in 1% (w/v) NaCl.

Chromatography

All sugars were tested for purity by chromatography as described by Barnett *et al.* (1973a). Most of the sugars were finally purified by preparative chromatography on Whatman no. 3 paper in butan-1-ol-ethanol-water (49:11:19, by vol.) and all those used were chromatographically pure.

Human erythrocyte suspensions

These were prepared as described (Barnett *et al.*, 1973a).

Penetration rates of D-glucose and D-galactose derivatives

The radioactively labelled sugar (25 or 50mM, $1\mu\text{Ci}$ in 1ml) in NaCl-sodium phosphate buffer, pH 7.4 (25mM-phosphate and 1% NaCl), was added to flasks containing washed erythrocyte suspensions (4ml, 25% packed-cell volume) in the same buffer at 25°C. The final sugar concentration was 5 or 10mM. Penetration was stopped at various time-intervals by the addition of 30ml of ice-cold mercury stopping medium. Samples were centrifuged rapidly and the pellet was rapidly washed with a further 30ml of ice-cold stopping solution. Tubes were dried with tissue and the pellets extracted with 10% (w/v) trichloroacetic acid (4ml). At zero time stopping medium was added before sugar, and one flask was incubated for an 'infinite' time (3–4h) so that the system attained equilibrium and the radioactivity of the cells corresponding to 5 or 10mM final concentration could be measured. Penetration could then be expressed as mmol/min per cell unit where 1 cell unit is that number of cells whose cell water volume is 1 litre at equilibrium. This represents effectively the intracellular concentration in mM.

Penetration rates were also measured in the presence of D-glucose (50mM). D-Glucose was preincubated with the cell suspension for 10min before addition of the labelled sugar, which was also 50mM in D-glucose.

Inhibition constants

Inhibition constants were measured by three methods.

(a) Inhibition of L-sorbose penetration at 25°C. The method of Levine *et al.* (1971) was adapted as previously described (Barnett *et al.*, 1973a). The method was also adapted to measure the effect of maintaining the inhibitory sugar predominantly on the inside or on the outside of the cells. For experiments in which the inhibitor was predominantly outside the cell, a zero preincubation time was used and 2.5ml of the erythrocyte suspension in the

phosphate-saline buffer was added to a solution of the inhibitor and [^{14}C]sorbose in 2.5ml of the phosphate-saline buffer. For experiments in which the inhibitor was predominantly inside the cells, these were preincubated at 25°C for 2h with the inhibitor and then rapidly washed with $2 \times 30\text{ml}$ of the phosphate-saline buffer. The pellets were rapidly resuspended in 4ml of the buffer and L- ^{14}C]sorbose was added to start the penetration. Both of these adaptations are only suitable for slowly penetrating inhibitors. A kinetic treatment of the inhibition for non-penetrating inhibitors is given in Appendix (A).

(b) Inhibition of the net influx of D-glucose at 15°C. Sugars were tested as inhibitors of net D- ^3H]glucose entry into erythrocytes at 15°C under conditions in which the initial rate of entry could be measured. Fig. 1 shows the rate of entry of both 2 and 20mM-D-glucose measured as the percentage filling after various times. The rate of entry was linear for at least 15s and the initial rate was taken as the concentration of sugar accumulating in this time. The inhibition was then analysed by a conventional reciprocal plot.

Fig. 1. Rate of entry of D-glucose into human erythrocytes at 15°C

Results are expressed as % filling of the cells by sugar. ●, 2mM-D-Glucose; ▲, 20mM-D-glucose. Entry was followed by using [^3H]glucose, and equilibrium was obtained in 5 min at each concentration.

A kinetic treatment adapted from that of Stein (1967) is shown in Appendix (B). Constant concentrations of inhibitor and [^3H]glucose (2–20 mM, 0.4 $\mu\text{Ci}/\text{flask}$) were dissolved in one phosphate–saline buffer (4 ml) at 15°C. Similar flasks contained no inhibitor. Washed erythrocyte suspensions in the same buffer (1 ml, 50% packed volume) were added and the flasks shaken turbulently for 15 s. Ice-cold phloretin stopping medium was added and the cells were washed and extracted with trichloroacetic acid as described above for the penetration of D-glucose and D-galactose derivatives. A sample at zero time was obtained by adding stopper solution before erythrocytes to 2 and 20 mM-glucose, and an equilibrated sample was obtained by leaving an incubation with 20 mM-D-glucose for 30 min at 25°C. It was found that more radioactivity appeared to equilibrate into the cells at 2 mM-glucose, and this was ascribed to a small amount of metabolism of the glucose.

After subtraction of the zero-time blank, which accounted for sugar trapped in the extracellular space, the apparent molarity in the intracellular space could be determined from the comparison of the radioactivity after 15 s with that of the fully equilibrated 20 mM-D-glucose sample. Initial rates were expressed as mm/15 s.

(c) Inhibition of the 'zero-trans' exit of D-glucose. The method of Karlsh *et al.* (1972) was modified to measure the inhibition of glucose exit by inhibitors inside the cell. The kinetic treatment is given in Appendix (C). The exit of D-glucose is rapid and is most conveniently measured under conditions which lead to the use of an integrated rate equation. Washed cells (50% packed-cell volume) were pre-loaded with 40 mM-inhibitor or -malonamide and 80 mM-D-glucose in phosphate–saline buffer at 25°C for 2 h. Solutions were then centrifuged and resuspended in iso-osmotic phosphate–saline buffer (24 mM-sodium phosphate buffer, pH 7.4, in 147 mM-NaCl) containing the same solutions at 25°C together with [^3H]glucose (50 μl , 5 μCi) for 10 min. The solution was then re-equilibrated to 18°C. Efflux of glucose was measured by pipetting 0.2 ml of the pre-loaded cells with vigorous magnetic stirring into 100 ml of exit medium (40 mM-inositol, 190.5 mM-NaCl, 20 mM- Na_2HPO_4 adjusted to pH 7.4 with HCl) at 18°C. Then 10 ml of the mixture was expelled into 30 ml of ice-cold phloretin stopper at 20, 30, 40, 50, 60 and 70 s and at 5 min by using an automatic syringe. The amount of radioactivity at zero time was found by adding 20 μl of the erythrocyte suspension to 30 ml of phloretin stopper with 10 ml of exit medium. The 5 min value gave an estimate of extracellular space. The apparatus used in this part of the experiment was maintained at 18°C by working in a temperature-controlled room.

The cell suspensions in phloretin stopper were centrifuged and the pellets lysed by addition of water

(1 ml) with vigorous mixing. A sample (0.5 ml) was added to 20% (w/v) trichloroacetic acid (0.5 ml) and the mixture centrifuged and 0.5 ml of the supernatant was counted for radioactivity in 10 ml of NE 250 scintillation fluid (Nuclear Enterprises, Edinburgh, U.K.). Each exit experiment was repeated at least three times and the means of the points at each time were used to calculate K_m and V values.

The apparent K_m for D-glucose was identical in the absence of malonamide (inositol is omitted from the exit medium), and agreed with the literature value (Karlsh *et al.*, 1972).

Fluorodinitrobenzene inactivation of L-sorbose transport in the presence of sugars

The sugar (10–100 mM) was preincubated in phosphate–saline buffer with the erythrocytes (25% packed-cell volume) at 25°C for 10 min (longer for sugars known to penetrate slowly). Fluorodinitrobenzene in ethanol was then added to give a final concentration of 2 mM and 4% (w/v) ethanol. After 1 h cells were washed twice with the phosphate–saline buffer (30 ml) and then resuspended in phosphate–saline buffer (4 ml). L- [^{14}C] Sorbose (25 μmol , 1 ml) was added and the cells were shaken at 25°C for 10 min. Ice-cold mercury stopper solution (30 ml) was added and the cells were centrifuged, extracted with trichloroacetic acid and the supernatants after centrifugation were counted for radioactivity. One flask was treated with stopping solution at zero time to give a measure of extracellular space. The control rate of inactivation in the absence of sugar was measured in each experiment. This gave the base from which stimulation or protection against inactivation could be measured. To ensure that washing after fluorodinitrobenzene inactivation was complete, sorbose transport was measured in cells that had been preincubated with the sugar but not with fluorodinitrobenzene. In all cases except that of propyl β -D-glucopyranoside, for which correction was made, washing was sufficient.

Olive oil/water partition coefficients

^3H -labelled sugars (about 20 mg) were added to centrifuge tubes containing 1 ml of water and 1 ml of olive oil. The tubes were vigorously shaken for 2 min to form a suspension, and the suspension was separated by centrifugation at 1000g, for 5 min. A portion (0.1 ml) of the oil layer was carefully removed and counted for radioactivity. The oil layer was removed and 0.1 ml of the water layer counted for radioactivity. Counts were corrected for the different quenching characteristics of the two media.

Results

Determination of the rate and mode of penetration of substituted hexoses into erythrocytes

Sugars penetrated the membrane by two distinct routes, which were respectively inhibited or not inhibited by 50mM-D-glucose. The time-course of penetration by some of the sugars tested in the presence and absence of glucose is shown in Figs. 2(a) and 2(b), and the initial rates for all the sugars tested are shown in Table 1. Penetration of 6-deoxy-D-galactose, 6-deoxy-6-iodo-D-galactose and 6-O-methyl-D-galactose was inhibited by D-glucose, indicating that they penetrate the membrane by the glucose-transport system. The rates of penetration of these sugars decrease with size of the substituent at C-6 and it seems probable that the decrease in rate is due to steric hindrance. 6-O-n-Propyl-D-galactose penetrates even more slowly and the rate is indepen-

dent of the presence of glucose. This sugar and the sugars with larger C-6 substituent groups do not penetrate on the glucose carrier, but instead their penetration rates relate more to their lipophilicity as measured by their oil/water partition coefficients. Both n-propyl β -D-glucopyranoside and phenyl β -D-glucopyranoside also penetrate the membrane by this alternative route.

Inhibition of L-sorbose or D-glucose entry into, and D-glucose exit from, human erythrocytes by sugars which do not penetrate the membrane on the glucose-transport system

Sugars were first tested for inhibition of L-sorbose transport, in many cases under conditions in which the inhibitor was predominantly on either the inside or outside of the erythrocyte. The results are shown in Table 2. This method is incapable of distinguishing

Fig. 2. Penetration of [6-³H]galactose derivatives (a) and [³H]galactose derivatives (b) into human erythrocytes at 25°C in the presence and absence of 50 mM-D-glucose

For details see the text (a) 5 mM-D-fucose (6-deoxy-D-galactose) alone (○) and in the presence of D-glucose (●); 5 mM-6-deoxy-6-iodo-D-galactose alone (△) and in the presence of D-glucose (▲). (b) 10 mM-6-O-methyl-D-galactose alone (○) and in the presence of D-glucose (●); 10 mM-6-O-propyl-D-galactose in the presence or absence of D-glucose (△); 10 mM-6-O-pentyl-D-galactose in the presence or absence of D-glucose (□).

Table 1. Olive oil/water partition coefficients and penetration rates of several sugars into human erythrocytes at 25°C

For details see the text.

Sugar	10 ³ × Partition coefficient	Concn. (mM)	Rate (mM/min)	Inhibition by 50 mM-glucose (%)
6-Deoxy-D-galactose	—	5	8	64
6-Deoxy-6-iodo-D-galactose	—	5	3	59
6-O-Methyl-D-galactose	—	10	0.5	78
6-O-n-Propyl-D-galactose	1.6	10	0.1	0
6-O-n-Pentyl-D-galactose	5.4	10	0.6	0
6-O-Benzyl-D-galactose	2.9	10	0.4	0
n-Propyl β -D-glucopyranoside	0.7	10	0.1	0
Phenyl β -D-glucopyranoside	5.5	10	0.35	0

Table 2. Inhibition of penetration by D-glucose or L-sorbose of the human erythrocyte membrane by hexoses substituted at C-1 or C-6 which are predominantly either inside or outside the cells

For details see the text. The sugars used in these experiments penetrated the membrane relatively slowly and were predominantly on one side of the membrane. The modified kinetic derivation described in the Appendix has therefore been used for calculation where K_m (inhibited) = K_m (uninhibited) $(1 + 1/2K_i)$. N.D., No detectable inhibition.

Sugar	Apparent inhibition constants (K_i) (mM)				
	L-Sorbose entry		D-Glucose entry		D-Glucose exit
	Outside cell	Inside cell	Outside cell	Inside cell	Inside cell
6-O-Propyl-D-glucose	17	90	—	—	N.D.
6-O-Pentyl-D-glucose*	1.1	—	—	—	—
6-O-Propyl-D-galactose	17	N.D.	24	—	N.D.
6-O-Pentyl-D-galactose*	1.5	—	5	—	—
6-O-Benzyl-D-galactose*	1.2	6	3	—	—
Propyl β -D-glucopyranoside	N.D.	9	N.D.	Inhibited	20
Phenyl β -D-glucopyranoside*	6	0.5	N.D.	—	—
Propyl β -D-galactopyranoside	N.D.	90	—	—	—
Propan-1-ol	∞	—	∞	—	—
Pentan-1-ol	30	—	∞	—	—
Benzyl alcohol	11	—	Non-competitive		—

* These sugars penetrate the membrane significantly during the time-course of the L-sorbose entry measurement.

Fig. 3. Double-reciprocal plots for the net entry of 2–20 mM D-glucose into human erythrocytes at 15°C in the presence and absence of 6-O-alkyl-D-galactoses

Cells were incubated for 15 s with D-glucose with or without the inhibitor, and the entry was stopped by addition of 'stopper'. D-Glucose alone, ●; with 10 mM 6-O-benzyl-D-galactose, ○; 10 mM 6-O-pentyl-D-galactose, △; and 40 mM 6-O-propyl-D-galactose, □.

between competitive and non-competitive inhibition and also uses a long preincubation time during which some of the more lipophilic sugars seemed to cause non-specific inhibition. Table 2 shows that both pentan-1-ol and benzyl alcohol, although not propan-1-ol, also inhibit this system. Therefore the sugars were also tested for inhibition of entry by a rapid net D-glucose entry method in which the nature of the inhibition could be determined from double-

reciprocal plots. In this system pentan-1-ol no longer inhibited, although benzyl alcohol was still a non-competitive inhibitor. All the 6-O-alkylgalactoses were shown to be competitive inhibitors (Fig. 3) when present on the outside of the cell, and their effectiveness as inhibitors increased with increasing chain length in the alkyl substituent, so that 6-O-benzyl- and 6-O-propyl-D-galactose were very good inhibitors (Table 2).

Because the rate of penetration of the 6-O-alkyl-D-galactoses is slow, particularly for 6-O-propyl-D-galactose, it was possible to investigate the inhibition of transport when the inhibitor was predominantly on either the inside or the outside of the cell. It was found that 6-O-benzyl- and 6-O-propyl-D-galactose and 6-O-propyl-D-glucose were very poor inhibitors when predominantly on the inside of the cell (Fig. 4, Table 2) when transport was measured by the L-sorbose entry method. In contrast *n*-propyl and phenyl β -D-glucopyranoside inhibited L-sorbose entry when present on the inside of the cells but only very poorly when present on the outside of the cells. It should be noted that whereas the propyl derivatives will remain on one side of the cell membrane during the course of these experiments (15 min), the penetration rate of the aromatic derivatives is comparable with the incubation time. By using 6-O-propyl-D-galactose and *n*-propyl β -D-glucopyranoside these results were confirmed with the D-glucose net entry method.

To confirm and quantify the inhibition at the inner face, the D-glucose-exit method of Karlisch *et al.* (1972) was used. Fig. 5 shows the rates of exit of D-glucose

Fig. 4. Inhibition of L-sorbose entry into human erythrocytes at 25°C in the presence and absence of D-glucose derivatives predominantly inside or outside the cells

For details see the text. Penetration of L-sorbose in the absence of added sugar, □; in the presence of 10 mM-propyl β-D-glucopyranoside outside the cells, ●; or on both sides (equilibrated), ○; in the presence of 15 mM-6-O-propyl-D-glucose outside the cells, ▲; or only inside the cells, Δ. S_0 , Sorbose concentration outside the cell at time zero or inside the cell at equilibrium; S_t , sorbose concentration inside the cell at time t .

in the presence of propyl β-D-glucopyranoside, 6-O-propyl-D-glucose, or malonamide. Propyl β-D-glucopyranoside was an effective inhibitor of glucose exit, whereas 6-O-propyl-D-glucose was not. In our hands, the derived K_m value for D-glucose in the presence and absence of inhibitor was subject to a large standard error primarily owing to the obligatory use of an integrated rate equation for the determination of kinetic constants, but the inhibition appeared to be competitive in that K_m rather than V was altered, and the inhibition constant, K_i , was about 20 mM, which correlated well with that found by the L-sorbose method.

Inactivation of the transport system by fluorodinitrobenzene in the presence of substituted hexoses

A preliminary experiment was carried out by using different concentrations of fluorodinitrobenzene and different times of incubation in the presence and absence of 20 mM-D-glucose in order to find conditions giving a good stimulation of inactivation. The increment in inactivation in the presence of sugar appeared to be fairly constant with increasing percentage inhibition with concentrations between 1 and 4 mM-fluorodinitrobenzene and times of 15 min to 1 h. The conditions chosen, 2 mM-fluorodinitro-

Fig. 5. Exit of D-[3H]glucose from human erythrocytes pre-loaded with 80 mM-D-glucose into an 'infinite' volume of iso-osmotic saline in the presence of substituted glucoses or malonamide

For details see the text. ○, 40 mM-Malonamide; ▲, 40 mM-propyl β-D-glucopyranoside; ■, 40 mM-6-O-propyl-D-glucose.

benzene for 1 h at 25°C, gave an inactivation of 23 % in the absence of glucose and 36 % in its presence. The stimulation phenomenon is saturable, so that by plotting the reciprocal of the increment in inactivation against the reciprocal of the sugar concentration a straight line is obtained and an apparent K_i value can be calculated. This was done only for D-glucose, when a value of 5 mM was obtained. In all other cases a high (40 mM) concentration of the sugar was used and the percentage increase in the fluorodinitrobenzene-inactivation measured. Many of the sugars protected against inactivation, as shown by the negative sign. The results are shown in Table 3.

Discussion

The structural requirements of sugars for binding to the human erythrocyte sugar-transport system have been given recently by two groups of workers. Kahlenberg & Dolansky (1972) measured the relative inhibition of the binding of D-glucose compared with inhibition of the binding of L-glucose to isolated erythrocyte membrane fragments, whereas Barnett *et al.* (1973a) measured the inhibition of L-sorbose entry into intact cells. Both methods measure the sum of the binding at the two sides of the membrane and the models produced are in good agreement except for the suggestion of a lipophilic binding region close to C-6 in the intact membrane not present in the membrane fragments. The increase in affinity of the 6-O-alkyl-D-galactose derivatives with the lipophilicity of the substituent group appears to confirm the presence of such an area on the outside surface of the cell (Table 2).

Table 3. *Effect of the presence of substituted hexoses on the extent of fluorodinitrobenzene inactivation of the sugar-transport system in the human erythrocyte*

Cells were preincubated with 2 mM-fluorodinitrobenzene in the presence and absence of 40 mM-sugar for 1 h at 25°C and the percentage inactivation was measured by the L-sorbose entry method.

Sugar	% inactivation in the presence of sugar minus % inactivation in its absence	Transported	Binds to transport system inside or outside cell
D-Glucose	14.8 ± 0.3	Yes	Both
1-Deoxy-D-glucose	12.4 ± 0.2	Yes	Both
3-Deoxy-D-glucose	7.2 ± 1.1	Yes	Both
D-Galactose	-0.9 ± 0.4	Yes	Both
6-Deoxy-D-galactose	-5.6 ± 0.1	Yes	Both
6-Deoxy-D-glucose	-2.3 ± 0.4	Yes	Both
6-Deoxy-6-fluoro-D-galactose	-8.5 ± 0.2	—	—
4,6-O-Ethylidene-D-glucose	-10.6 ± 0.4	No	Outside
6-O-Propyl-D-galactose	-12.6 ± 0.4	No	Outside
6-O-Propyl-D-galactose (80 mM)	-23.0 ± 0.6	No	Outside
Propyl β-D-glucopyranoside (20 mM)	12.5 ± 1.4	No	Inside
Phloretin*	Protects	—	Both
Phlorrhizin*	Activates	No	Inside
Methyl α-D-glucopyranoside*	Activates	No	—
Maltose	Protects	No	Outside
Cellobiose*	Protects	No	Outside
2-Deoxy-D-glucose*	Activates	Yes	Both

* Data from Krupka (1972), concentration variable.

Further investigation of the *O*-alkylgalactoses showed that only 6-*O*-methyl-D-galactose penetrates the membrane by the glucose-transport system (Table 1). The rates of transport of the higher homologues were roughly proportional to their oil/water partition coefficients and were uninhibited by D-glucose, although the sugars were competitive inhibitors of D-glucose entry (Fig. 3). These sugars are therefore comparable with 4,6-*O*-ethylidene-D-glucose (Baker & Widdas, 1973a), which also penetrates the membrane by an alternative route, and maltose, which also inhibits the glucose-transport system, but does not penetrate the cell membrane (Chen & LeFevre, 1965; Lacko & Burger, 1962).

Baker & Widdas (1973b) have used the relatively slow penetration of 4,6-*O*-ethylidene-D-glucose to measure the apparent affinity of the sugar for the transport system on the two sides of the cell. They found that although this sugar was a good inhibitor of D-glucose exit when present on the outside of the cell, it was not an effective inhibitor when present on the inside of the cell. The same behaviour is apparent with the non-penetrating 6-*O*-alkyl-D-galactoses and 6-*O*-propyl-D-glucose. The 6-*O*-propylhexoses inhibit only when present on the outside of the cell. These sugars do not appear to interfere with the transport system non-specifically and penetrate the membrane slowly. The higher alkyl derivatives, which may exhibit some non-specific inhibition of the transport system, show

some inhibition when present primarily on the inside of the cell (Table 2), but this is far less than that found when the sugar is present on the outside. The binding of inhibitors to the transport system is therefore asymmetric, as indicated by Baker & Widdas (1973b).

Sugars substituted at C-1, the propyl and phenyl β-D-glucopyranosides, also penetrate the membrane by some route other than the sugar-transport system because their penetration is not inhibited by D-glucose (Table 1). These sugars were also tested for inhibition on the two sides of the cell membrane. In contrast with the 6-*O*-alkyl sugars, it was found that they inhibit only when present at the inner face of the cell. By using *n*-propyl β-D-glucopyranoside it was shown that the inhibition of D-glucose exit (Fig. 5) was probably competitive.

It therefore appears that the asymmetry of the sugar-transport system is such that hexose derivatives with large substituent groups at C-4 or C-6 can bind to the system on the outside of the cell, but not on the inside, whereas sugars with large substituents at C-1 can bind only on the inside and not at the outer face. Large substituents at either end of the sugar molecule will prevent transport. A model consistent with these observations is shown in Fig. 6.

When entering the cell the sugar first binds to a site on the outside of the membrane by using binding groups in the C-1 region of the molecule. Then part

Fig. 6. Possible model for sugar transport in the human erythrocyte

6-*O*-Propyl-D-glucose ($R = C_3H_7$; $R' = H$) can bind to the transport system in conformation A, but cannot be transported for steric reasons. Similarly, propyl β -D-glucopyranoside ($R = H$; $R' = C_3H_7$) can bind to conformation B but cannot be transported. D-Glucose can bind to both conformations, and if the transport site changes conformation from form A to form B, is effectively transported from outside to inside. There is some evidence that a hydrogen bond is formed between the C-4 hydroxyl group of D-glucose and the transport protein only in conformation B. Only some of the probable hydrogen bonds are shown.

of the membrane protein rearranges to give a second stable conformation around the binding site thus exposing the sugar to the inner solution. In this conformation it is the C-4 and C-6 regions of the sugar that are involved in binding. Rearrangement is prevented by a bulky group. This model would explain the observed apparent asymmetry of the transport system (cf. Baker & Widdas, 1973*b*) because the hydrogen bonds between sugar and membrane protein will probably be different in the two conformations, as will other non-bonding interactions. It is in effect a case of the theoretical allosteric model of Vidaver (1966), who showed that a conformational alteration in the transport barrier could give the same kinetics as a mobile carrier. A similar conformational 'flip' between two hydrogen-bonded conformations occurs between the two stable forms of haemoglobin.

The structural requirements for binding to the sugar-transport system given by both Kahlenberg & Dolansky (1972) and Barnett *et al.* (1973*a*) must be reinterpreted if an asymmetric model similar to that described here is correct. Both groups of workers used methods that could not distinguish between binding on the inner or outer surface of the cell. The methyl α - and β -D-glucopyranosides were inhibitors of D-glucose binding to membrane fragments (Kahlenberg & Dolansky, 1972), although neither sugar is transported or is an inhibitor on the outside of the erythrocyte. Therefore the significant amount of inhibition observed could be attributed to inhibition of glucose binding at the inner surface of the fragments.

We sought further confirmation of the proposed model by studying the fluorodinitrobenzene inactivation of the transport system in the presence of several substituted hexoses. Since the original observation that the presence of D-glucose stimulated rather than protected against inactivation (Bowyer & Widdas, 1958), the inactivation has been extensively studied (Shimmin & Stein, 1970; Krupka, 1971, 1972; Edwards, 1973) and several authors have suggested that the behaviour is caused by the ability of the membrane to exist in more than one conformational state.

The most extensive studies of the phenomenon have been by Krupka (1971, 1972). He showed that most sugars stimulated the inactivation, although by different amounts. Maltose, cellobiose and phloretin, all competitive inhibitors of the glucose-transport system, protected against inactivation by fluorodinitrobenzene. The glucoside of phloretin, phlorrhizin, which has been shown (Lepke & Passow, 1973) to inhibit D-xylose transport in erythrocyte 'ghosts' more strongly when on the inside of the membrane, was a stimulator. By studying the combined effects of maltose, a protector, and 2-deoxy-D-glucose, an activator, Krupka (1972) showed that both actions were by formation of a 1:1 complex between the sugar and the sugar-transport system. Baker & Widdas (1973*a*) have shown that 4,6-*O*-ethylidene-D-glucose also protects the sugar-transport system against fluorodinitrobenzene inactivation.

Table 3 shows that the ability to potentiate or protect against fluorodinitrobenzene inactivation does not correlate with the ability to transport the sugar. Instead, if non-transported sugars alone are considered there is a direct correlation of protection with binding on the outside of the cell and activation with binding on the inside. Under the conditions used 80 mM-6-*O*-propyl-D-galactose completely protected against inactivation. These observations suggest that the fluorodinitrobenzene, which rapidly penetrates the membrane and is therefore present on both sides, may act only on the 'inner facing' form of the transport system (conformation B, Fig. 6) but not necessarily close to the sugar-binding site. Non-transported compounds which stabilize this form, such as propyl β -D-glucopyranoside, phlorrhizin and possibly methyl α -D-glucopyranoside, cause the reactive residue to be exposed and therefore potentiate inactivation by fluorodinitrobenzene. In contrast, non-transported sugars which bind to the form of the membrane that binds sugars at the outer surface will stabilize this form of the transport system and decrease the rate of inactivation. It is difficult to predict which form of the carrier a transported sugar will expose, because this will depend on the relative dissociation constants on the two faces of the membrane, the ratios of the rate

for 'translocation' of the 'loaded carrier' to that of the 'unloaded carrier' (Levine & Stein, 1966), the ratio of the rate of association of the sugar with the transport system to both of these rates, and the type of experiment used. However, in the experimental situation used here, in which the sugar is allowed to equilibrate across the membrane, and extending the principle that the conformation binding sugars at the inner face is reactive towards fluorodinitrobenzene, those sugars which potentiate the fluorodinitrobenzene inactivation should have a relatively high affinity for the inner face of the membrane, whereas those which protect should have a relatively high affinity for the outer surface. Inspection of Table 3 shows that this correlates very well with the model in Fig. 6. Sugars with alterations from the D-glucose structure near to C-4 and C-6 which project out into the solution on the outside of the cell would be expected to have little effect on binding to the outer membrane, but a significant effect on binding to the inner membrane, where they would be in contact with the transport-system protein. They should therefore stabilize the form of the system that binds sugars at the outer face (conformation A, Fig. 6) and protect. The converse is true for sugars modified near C-1, which would stabilize the form binding sugars on the inner surface (conformation B, Fig. 6) and therefore activate.

The apparently anomalous observation by Edwards (1973), that 120 mM-glucose on the inside of the cells protects the system against fluorodinitrobenzene inactivation whereas the same concentration outside the cell potentiates inactivation, can be reconciled with this model if one considers the different experimental conditions used. The inactivation proceeded during transport of the glucose under conditions which approximated to a 'zero-trans' exit procedure and with a very rapid fluorodinitrobenzene inactivation over a short time-course. Because the rate of 'translocation' of the 'loaded carrier' is greater than that of the 'unloaded carrier', glucose on the inside of the cell may actually lead to an increase in the concentration of the form of the transport system (conformation A, Fig. 6) that binds sugars at the outer surface, that is, should lead to protection of the transport system against fluorodinitrobenzene inactivation. This explanation of this result was also suggested by Edwards (1973).

Bloch (1974) has shown that sodium tetrathionate inactivates sugar transport in the erythrocyte in a rather similar way to fluorodinitrobenzene. However, D-glucose protects against the inactivation, whereas maltose potentiates the inactivation. It seems possible, therefore, that sodium tetrathionate reacts preferen-

tially with the 'outer facing' form of the transport system.

Together with the inhibition data described above the experiments with alkylating agents appear to confirm the asymmetry of the human erythrocyte sugar-transport system.

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APPENDIX

Derivation of the Kinetic Parameters for an Asymmetric Carrier with Non-penetrating Inhibitors

This Appendix describes the theoretical basis of the methods used in the preceding paper. The existing theoretical treatments of the experimental methods required adapting to take account of the asymmetry of the transport system and the non-penetration of the inhibitors used. To relate to the theoretical treatments from which they have been adapted, the proofs assume a 'carrier' model for transport. The model used in the paper to explain the results, in which transport is effected by a conformational change in the membrane protein, leads to identical kinetics (see Vidaver, 1966) and each concept has a direct analogy. For instance the ratio of the rates of translocation of loaded/unloaded carrier (r) becomes the ratio of the rate of conformational change of the transport protein in the loaded and unloaded states.

(A) Measurement of inhibition constants by the penetration of L-sorbose

This treatment is closely adapted from the kinetic treatment of Levine *et al.* (1971), who assumed complete equilibration of the inhibitory sugar and that the affinity of the inhibitor for the transport site was much greater than that of L-sorbose. They also assumed a symmetrical carrier. The present kinetics will assume that the inhibitor is present only on the outside of the membrane, that the transport system is asymmetric, and that the affinity of the inhibitor for the transport system is much greater than that of L-sorbose. This is summarized symbolically in Scheme 1. The rate of entry of L- $[^{14}\text{C}]$ sorbose is determined in the presence and absence of inhibitor.

The subscripts o and i represent the outside and inside of the cells, t is the time in min, I_o is the concentration of the strongly binding non-penetrating

inhibitor in mol/litre, S_o and S_i are the respective concentrations of L-sorbose outside and inside the cell at time t . It is assumed that the concentration of L-sorbose outside the cells does not alter with time so that S_o is constant. C_o , C_i represent the concentrations of free carrier and CS_o , CS_i , the concentrations of carrier loaded with L-sorbose. CI_o is the concentration of carrier loaded with inhibitor. The rate constants are as shown in Scheme 1, and the ratio of the rate of translocation of loaded to unloaded carrier is r , k_{+2}/k_{+4} , in the forward direction and r' , k_{-2}/k_{-4} , in the reverse direction. We therefore have the following dissociation constants if it is assumed that k_{+1} , k_{-1} are much greater than k_{+2} , k_{+4} etc:

$$K_s = \frac{C_i \cdot S_i}{CS_i}; \quad K_s^* = \frac{C_o \cdot S_o}{CS_o}; \quad K_I = \frac{C_o \cdot I_o}{CI_o} \quad (1)$$

The total concentration of carrier is always:

$$\text{Total } C = C_i + C_o + CS_i + CS_o + CI_o \quad (2)$$

Substituting:

$$\text{Total } C = C_i \left[1 + \frac{S_i}{K_s} \right] + C_o \left[1 + \frac{S_o}{K_s^*} + \frac{I_o}{K_I} \right] \quad (3)$$

The rate of entry of L-sorbose is:

$$\frac{dS_i}{dt} = k_{+2}CS_o - k_{-2}CS_i$$

or substituting from eqn. (1):

$$= \frac{k_{+2}C_o \cdot S_o}{K_s^*} - \frac{k_{-2}C_i \cdot S_i}{K_s} \quad (4)$$

Scheme 1. Rate of entry of L-sorbose in the presence and absence of inhibitors

For details of subscripts and terminology see the text.

At any steady state, the total carrier moving in each direction must be equal, therefore:

$$k_{-2}CS_1 + k_{-4}C_1 = k_{+2}CS_0 + k_{+4}C_0$$

or substituting from eqn. (1):

$$k_{-2} \frac{C_1 \cdot S_1}{K_s} + k_{-4}C_1 = k_{+2} \frac{C_0 \cdot S_0}{K_s^*} + k_{+4}C_0 \quad (5)$$

Dividing out:

$$C_1 \left[1 + \frac{k_{-2}}{k_{-4}} \cdot \frac{S_1}{K_s} \right] = C_0 \left[1 + \frac{k_{+2}}{k_{+4}} \cdot \frac{S_0}{K_s^*} \right] \quad (6)$$

or

$$C_1 \left[1 + r' \frac{S_1}{K_s} \right] = C_0 \left[1 + r \frac{S_0}{K_s^*} \right] \quad (7)$$

Solving eqns. (3) and (7) for C_0 or C_1 :

$$C_0 = \text{Total } C \left[1 + r' \frac{K_s}{S_1} \right] / D \quad (8)$$

$$C_1 = \text{Total } C \left[1 + r \frac{S_0}{K_s^*} \right] / D \quad (9)$$

where

$$D = \left[1 + \frac{S_1}{K_s} \right] \left[1 + r \frac{S_0}{K_s^*} \right] + \left[1 + \frac{S_0}{K_s^*} \pm \frac{I_0}{K_i} \right] \left[1 + r' \frac{S_1}{K_s} \right]$$

Substituting for C_0 and C_1 in eqn. (4):

$$\frac{dS_1}{dt} = \frac{\text{Total } C}{D} \left\{ \left[\frac{k_{+2}}{K_s^*} \cdot S_0 \left(1 + r' \frac{S_1}{K_s} \right) \right] - \left[\frac{k_{-2}}{K_s} \cdot S_1 \left(1 + r \frac{S_0}{K_s^*} \right) \right] \right\} \quad (10)$$

This is a general equation for the entry of sugar into cells in the presence of an external, non-penetrating, competitive inhibitor.

But under the experimental conditions used $K_s^* \gg K_i$ and S_1/K_0 and $S_0/K_s^* \ll I_0/K_i$ or 1.

Therefore D simplifies to $[2 + I_0/K_i]$ neglecting all terms in S_1/K_s or S_0/K_s^* .

Again neglecting terms in S_0/K_s^* and S_1/K_s :

$$\frac{dS_1}{dt} = \frac{\text{Total } C}{D} \left[\frac{k_{+2}}{K_s^*} \cdot S_0 - \frac{k_{-2}}{K_s} \cdot S_1 \right] \quad (11)$$

But at equilibrium $dS_1/dt = 0$ and $S_0 = S_1$; therefore substituting in eqn. (11):

$$\frac{k_{+2}}{K_s^*} = \frac{k_{-2}}{K_s}$$

and so eqn. (11) simplifies to:

$$\frac{dS_1}{dt} = \frac{\text{Total } C}{D} \cdot \frac{k_{+2}}{K_s^*} (S_0 - S_1) \quad (12)$$

and substituting for D :

$$\frac{dS_1}{dt} = \frac{\text{Total } C}{2K_s^*} \cdot k_{+2} \left[\frac{S_0 - S_1}{1 + \frac{I_0}{2K_i}} \right] \quad (13)$$

When $I_0 = 0$:

$$\frac{dS_1}{dt} = \frac{\text{Total } C}{2K_s^*} \cdot k_{+2} (S_0 - S_1) \quad (14)$$

Integrating and substituting V for $\text{Total } C \cdot k_{+2}/2$:

$$\ln \frac{S_0}{S_0 - S_1} = \frac{V \cdot t}{K_s^* (1 + I_0/2K_i)} \quad (15)$$

An identical solution is obtained if the non-penetrating inhibitor is on the inside of the cell, or if the system is symmetrical.

(B) *Measurement of inhibition constants by D-glucose entry under conditions in which the internal concentration of glucose is effectively zero*

The theoretical treatment is based on that of Stein (1967). The symbols are identical with those used above and the model used is shown in Scheme 1. The initial rates of entry of D-glucose into cells were measured under conditions in which the internal concentration of glucose could be neglected. It is assumed that the transport system is asymmetric and that the inhibitor is present only on the outside of the cells. The general equation for entry of a sugar in the presence of a non-penetrating external inhibitor is given by eqn. (10) above.

$$\frac{dS_1}{dt} = \frac{\text{Total } C}{D} \left[\frac{k_{+2}}{K_s^*} \cdot S_0 \left(1 + r' \frac{S_1}{K_s} \right) - \frac{k_{-2}}{K_s} \cdot S_1 \left(1 + r \frac{S_0}{K_s^*} \right) \right] \quad (10)$$

where D has the same value as above:

$$D = \left(1 + \frac{S_1}{K_s} \right) \left(1 + r \frac{S_0}{K_s^*} \right) + \left(1 + \frac{S_0}{K_s^*} + \frac{I_0}{K_i} \right) \left(1 + r' \frac{S_1}{K_s} \right)$$

When the internal concentration of substrate is zero, $S_1 = 0$, this simplifies to:

$$\frac{dS_1}{dt} = \text{Total } C \frac{k_{+2}}{K_s^*} \cdot \frac{S_0}{1 + r \frac{S_0}{K_s^*} + 1 + \frac{S_0}{K_s^*} + \frac{I_0}{K_i}} \quad (16)$$

$$= \frac{\text{Total } C \cdot k_{+2} S_0}{2 K_s^* \left(1 + \frac{(1+r)S_0}{2K_s^*} + \frac{I_0}{2K_i} \right)} \quad (17)$$

$$\text{Let } \frac{dS_1}{dt} = v \text{ and } \frac{\text{Total } C \cdot k_{+2}}{2} = V$$

$$\frac{1}{v} = \frac{1}{V S_0} \left[1 + \frac{I_0}{2K_i} + \frac{(1+r) \cdot S_0}{2 K_s} \right] \quad (18)$$

$$= \frac{K_s^*}{V} \left(1 + \frac{I_0}{2K_i} \right) \frac{1}{S_0} + \frac{(1+r)1}{2V} \quad (19)$$

The solution is identical in a symmetrical system, and in both cases when $I_0 = 0$.

$$\frac{1}{V} = \frac{K_s^*}{V \cdot S_0} + \frac{(1+r)1}{2V}$$

Plotting $1/v$ against $1/s$ the x intercepts for the inhibited and uninhibited systems are respectively:

$$-\frac{1+r}{2K_s^*} \cdot \frac{1}{\left(1 + \frac{I_0}{2K_i}\right)} \quad \text{and} \quad -\frac{1+r}{2K_s}$$

(C) *Measurement of inhibition constants by D-glucose exit under conditions in which the external concentration of glucose is effectively zero*

In this method the non-penetrating inhibitor is on the inside of the cell, as is the substrate, and so the assumptions and equations are identical with those in

(B) and eqn. (19) holds in which K_s^* is replaced by K_s and r by r' and v by $-dS_i/dt$. However, the integrated form of the equation must be used experimentally. This is:

$$-V \cdot t = K_s \left(1 + \frac{I_0}{2K_i} \right) \ln \frac{S'_0}{S'_t} + \frac{(1+r')}{2} (S'_0 - S'_t) \quad (20)$$

where S'_0 is the internal concentration at time 0 and S'_t the internal concentration at time t . Corrections have to be made for volume changes in the cell and these were identical with those of Karlsh *et al.* (1972). In the absence of inhibitor, $I_0 = 0$ and eqn. (20) simplifies to the equation used by these authors.

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