The Interaction of 3-Deoxy-3-fluoro-D-glucose with the Hexose-Transport System of the Human Erythrocyte

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1. By using an optical method the kinetic parameters of hexose transport across the human erythrocyte membrane were determined for several sugars. The series of half-saturated constants is as follows: 3-deoxy-3-fluoro-D-glucose = 3-O-methyl-D-glucose < D-glucose < D-mannose < 3-deoxy-D-glucose < D-galactose < L-arabinose. 2. Estimations of the dissociation energy of the 3-deoxy-3-fluoro-D-glucose-carrier and D-glucose-carrier complexes suggest that the binding of the two sugars to the transport system is equivalent. 3. Incubation of the erythrocytes with 3-deoxy-3-fluoro-D-glucose results in a small but significant release of F⁻ anion. Cells treated in this way lose their ability to transport glucose.

A number of deoxyfluoro analogues of carbohydrates and related compounds have been used as pseudo-substrates to study enzyme-ligand interactions (Eisenthal *et al.*, 1972; Barnett, 1972), and the exploration of the potential of 3-deoxy-3-fluoro-D-glucose as a metabolic deceptor in a number of glucose-utilizing systems has been reviewed (Taylor, 1972). The active transport of 3-deoxy-3-fluoro-D-glucose across the hamster intestine (Barnett *et al.*, 1969) prompted us to investigate the interaction of this sugar with the carrier-mediated transport system of the human erythrocyte. In a comprehensive substrate-specificity study, it has been shown by Barnett *et al.* (1973) that 3-deoxy-3-fluoro-D-glucose can bind to the hexose carrier of the human erythrocyte, although its transport across the membrane was not demonstrated.

Although the transport parameters that have been obtained for glucose appear to vary with the method used (Miller, 1968*a,b*) the relative affinities of glucose analogues for the carrier system seem to be independent of this transport-parameter variation (Barnett *et al.*, 1973; Le Fevre, 1961; Kahlenberg & Dolansky, 1972). Consequently, the optical method of Sen & Widdas (1962) was chosen for our studies. This method has the advantage that it allows the use of non-radioactive sugar and it depends on the transfer of glucose and its analogues across the membrane. A disadvantage is that a number of kinetic approximations are made in the derivation of the rate equation.

The aim of this investigation was (a) to discover

whether 3-deoxy-3-fluoro-D-glucose was able to enter the erythrocyte by a mechanism similar to that by which glucose enters; (b) on the assumption that the half-saturated constant determined in these experiments approximates to a binding constant, to determine this parameter at several temperatures and estimate the dissociation energy of the sugar-carrier complex: any difference in binding of 3-deoxy-3-fluoro-D-glucose and glucose should be reflected in this estimate; (c) to compare our results with data already derived independently by different methods.

Materials and Methods

Materials

3-Deoxy-D-glucose, m.p. 105–107°C, was prepared by the method of Anet (1960). 3-Deoxy-3-fluoro-D-glucose, m.p. 112–113°C (Foster *et al.*, 1967), was separated from contaminating glucose by chromatography on a column (50 g; 33 cm × 4 cm) of silica gel (0.05–0.2 mesh) (Merck, Darmstadt, W. Germany) eluted with ethyl acetate-ethanol (3:1, v/v); 20 ml fractions were collected and portions monitored by t.l.c. on plates (20 cm × 20 cm) coated with a 0.25 mm layer of silica gel G according to Stahl (Shandon Scientific Co. Ltd., Camberley, Surrey, U.K.) and detected with H₂SO₄-ethanol (1:1, v/v) on heating for 10 min at 120°C. The purity of the 3-deoxy-3-fluoro-D-glucose was finally checked by paper chromatography on Whatman no. 1 paper by using butan-1-ol-ethanol-water (5:1:4, by vol., upper layer) and developed with aniline hydrogen phthalate (Partridge, 1948).

All other sugars and general reagents were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. or Koch-Light Ltd., Colnbrook, Bucks., U.K.

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Penicillin G and streptomycin sulphate were obtained from Sigma (London) Chemical Co., London S.W.6, U.K.

Methods

Human erythrocyte preparation. Samples (10ml) of blood were collected from volunteers by venepuncture and mixed with 2.5ml of acid-citrate-dextrose [100ml of 2% (w/v) disodium hydrogen citrate plus 20ml of 15% (w/v) glucose] (Wright, 1965). The blood could be stored in this condition for up to 3 weeks at 4°C. The cells were separated by centrifugation (3000 rev./min for 5 min at 20°C; MSE bench centrifuge), and the plasma and top layer of cells were removed. They were resuspended in saline [1% (w/v) NaCl-4mM-sodium phosphate, pH 7.4]. Washed packed erythrocytes (1 part) were suspended in a solution of the penetrating sugar in saline (7 parts) and incubated at 37°C for 30 min to give the following equilibrium concentrations: glucose, 3-*O*-methylglucose and 3-deoxy-3-fluoroglucose, 100mM; 3-deoxyglucose, mannose, galactose and L-arabinose, 200mM.

Sugar exit. A portion (15ml) of the test sugar (up to 50% of the pre-loaded concentration) was measured into a Perspex cuvette (6cm high × 4cm wide × 3cm deep) which was surrounded by a copper water jacket and mounted in the cell housing of a Pye Unicam SP.500 series 2 spectrophotometer. The solution was stirred by a paddle stirrer positioned so as not to obscure the light path. A portion (0.2ml) of the washed erythrocyte suspension was added and the slit-width control of the spectrophotometer rapidly adjusted to bring the E_{700} on to the scale 0–0.1. Amplification of the Pye Unicam SP.500 output by a Pye Unicam SP.45 concentration read-out unit gave full-scale deflexion on the Pye Unicam SP.22 chart recorder at 0.1 E_{700} unit. The absorbance scale was calibrated with the cellular concentration of sugar by equating the change in absorbance between zero time and at osmotic equilibrium with the known change in internal cell concentration.

Total F^- in the incubation media was measured by the method of Woodward *et al.*, (1970) by using 1M-sodium phosphate buffer at pH 5.5, instead of acetate-citrate. Free F^- ion was similarly determined but the hydrolysis step was omitted.

Glucose metabolism of human erythrocytes. The erythrocyte metabolism of glucose and 3-deoxy-3-fluoro-D-glucose was determined by their ability to reduce methaemoglobin (Jaffé, 1969). Blood was obtained by venepuncture and used on the same day, washed cells being prepared as described above with the exception that buffered saline (9 parts of 0.9% NaCl to 1 part of 0.11M-sodium phosphate buffer, pH 7.3) was used. The cells were equilibrated at 37°C for 20min with an equal volume of 1% (w/v)

NaNO₂ in buffered saline. The cells were washed free of NaNO₂ by five changes of 10 vol. of the saline and finally resuspended in the same buffer to a haematocrit of 20–30%. In those experiments where the pentose phosphate pathway was being investigated, 2ml of the cell suspension was mixed with 1ml of 72M-Methylene Blue in saline. When glycolysis was required to operate the dye was omitted and replaced with 1ml of buffered saline containing 5mg of penicillin G and 5mg of streptomycin sulphate.

Immediately after mixing, a 0.2ml sample was taken and the cells were disrupted with a mixture of 2ml of 0.1M-sodium phosphate buffer, pH 6.8, and 3ml of 1% (w/v) Triton X-100. The flasks containing the whole cells were stoppered with cottonwool plugs and shaken (100 oscillations/min) at 37°C. Further 0.2ml samples were taken at intervals and solubilized as described above.

The methaemoglobin in the cell-free sample was assayed by the method of Evelyn & Malloy (1938) as modified by Dacie & Lewis (1968). The methaemoglobin reduced by cells incubated without substrates in buffered saline was subtracted from the experimental values.

Theory

Sen & Widdas (1962) originally derived the mathematical expression for the exit of sugars from pre-loaded erythrocytes. This was later modified to give a simplified rate equation (Miller, 1969):

$$\frac{1}{v} = \frac{[X_0]}{V_{\max} \cdot K_x} + \frac{1}{V_{\max}}$$

where v is the rate of sugar exit, $V_{\max}$ is the maximum rate of transfer, $[X_0]$ is the external concentration of hexose and K_x is the half-saturation constant of the sugar-carrier complex. Thus a linear plot of $1/v$ against $[X_0]$ should give $1/V_{\max}$ as the intercept on the y axis and $-K_x$ as the intercept on the x axis.

The rate of exit can be followed spectrophotometrically (Sen & Widdas, 1962). On mixing pre-loaded cells into a dilute solution of the sugar, they immediately swell as water enters to maintain osmotic equilibrium. The exit of the internal sugar is accompanied by a loss of water and consequent shrinkage. This results in an increase in absorbance because of changes in the refractive index of the cells (Tedeschi & Harris, 1955).

ΔE was calculated from the slope of the plot of $\log_{10} K_x$ against $1/T$ and the dissociation energy ΔH^* from $\Delta E = -(\Delta H^* + RT)/2.303R$ (see Fig. 3).

Results and Discussion

Fig. 1 shows the straight-line plot of the reciprocal rate of sugar exit at 37°C against $[X_0]$ for glucose and its analogues modified at C-3. The lines were fitted to

Fig. 1. Exit of 3-deoxy-3-fluoro-D-glucose, 3-deoxy-D-glucose and D-glucose from pre-loaded erythrocytes into low concentrations of the appropriate sugar at 37°C

○, 3-Deoxy-3-fluoro-D-glucose; ●, D-glucose; ▲, 3-deoxy-D-glucose. For experimental details see under 'Sugar exit' in the Materials and Methods section. For K_x and V_{max} , see Table 1.

Fig. 2. Determination of transport parameters for 3-deoxy-3-fluoro-D-glucose at four temperatures

●, 23.0°C; K_x 1.05 mM, V_{max} 173 mmol·litre⁻¹·min⁻¹. ■, 28.0°C; K_x 1.56 mM, V_{max} 274 mmol·litre⁻¹·min⁻¹. □, 32°C; K_x 1.79 mM, V_{max} 450 mmol·litre⁻¹·min⁻¹. ○, 37°C; K_x 2.3 mM, V_{max} 600 mmol·litre⁻¹·min⁻¹. For experimental details see the Materials and Methods section.

the data and the transport parameters calculated by using the unweighted method of least squares.

Fig. 2 shows a similar plot of 3-deoxy-3-fluoro-D-glucose exit into dilute solutions of that sugar at four temperatures. Fig. 3 shows $\log K_x$ of 3-deoxy-3-fluoro-D-glucose at these temperatures plotted against $1/T$. This gives a slope of $-\Delta E$ from which the dissociation energy ΔH^* can be calculated (Dixon & Webb, 1964).

The present data indicate that 3-deoxy-3-fluoro-D-glucose is transported across the erythrocyte membrane by a mechanism similar to that of glucose. However, it was necessary to confirm that it was the glucose carrier that was responsible. In deriving the carrier equation it was assumed that the movement of the carrier between the membrane surface was independent of any ligand (Miller, 1969). Consequently, the rate of transfer of a sugar across the lipid phase of the membrane should be dependent only on the affinity of the sugar for the carrier. The rate of transport is proportional therefore to the number of occupied carrier sites. When the carrier is fully saturated with the sugar the maximum transport velocity is obtained. This should be independent of the sugar being transported. The similarity of the V_{max} for the transport of D-glucose, 3-deoxy-3-fluoro-D-glucose and 3-deoxy-D-glucose (Table 1) suggests that these sugars are all transported by the same carrier. However, 3-O-methyl-D-glucose, which has a K_x value in the region of that of 3-deoxy-3-fluoro-D-

glucose, achieves a maximum velocity only one-half that of comparable sugars. Similarly, L-arabinose, although having a much higher K_x value, also achieves a lesser maximum velocity. It is therefore apparent that certain structural features of the transported sugar can affect the rate of transfer of the sugar-carrier complex. Additional confirmation of the transport of the 3-deoxy-3-fluoro-D-glucose carrier was obtained by showing that the exit of 3-deoxy-3-fluoro-D-glucose from the cells could be inhibited by dilute solutions of glucose. Thus the apparent K_i for glucose inhibition of 3-deoxy-3-fluoro-D-glucose exit at 25°C was 2.7 mM.

The change in the apparent affinity of 3-deoxy-D-glucose with respect to D-glucose suggested that the 3-hydroxyl group was involved in the binding of glucose to the carrier. Since 3-deoxy-3-fluoro-D-glucose was not only bound to the carrier but transported, and with an apparent affinity marginally higher than glucose, the F atom is probably stereospecifically involved in binding. This is consistent with the models proposed by both Barnett *et al.* (1973) and Kahlenberg & Dolansky (1972).

To provide further evidence for the suggestion by Barnett *et al.* (1969) that F may be hydrogen bonding with the carrier the dissociation energies of the

Fig. 3. Estimations of the 3-deoxy-3-fluoro-D-glucose-carrier dissociation energy

$-\Delta E$ (kJ/mol): $\circ$, 9.1; $\bullet$, 12.5; $\blacktriangle$, 14.7. ΔH^* (kJ/mol) at 37°C: $\circ$, 39.1; $\bullet$, 54.6; $\blacktriangle$, 30.2. ΔE is obtained from the slope of the plot of $\log K_x$ versus $1/T$, and ΔH^* from the relationship $\Delta E = -\Delta H^* + (RT/2.303R)$, where R is the gas constant and T is the absolute temperature.

glucose-carrier and 3-deoxy-3-fluoro-D-glucose-carrier complexes were compared. Determination of the values of K_x for each of the sugars at four temperatures allowed such estimations (Fig. 3). The values for ΔH^* for D-glucose and 3-deoxy-3-fluoro-D-glucose were calculated as 47.8 ± 4.1 (4) and 41.1 ± 7.1 (3) kJ/mol ($\pm$ S.E.M.) respectively and thus did not differ significantly ($P > 0.05$, Student's t test). These results are similar to the value of 42 kJ/mol for D-glucose obtained by Sen & Widdas (1962). They argued that this value represented the sum of the hydrogen-bond forces between the sugar and the carrier. Thus the similarity of the D-glucose and 3-deoxy-3-fluoro-D-glucose dissociation energies lends support to the hypothesis (Barnett, 1972; Taylor, 1972) that the C-F bond can participate in hydrogen bonding between the ligand and certain biological receptors.

Although the transport of glucose has been reported to be up to 250 times faster than glucose metabolism (Widdas, 1954), it was necessary to confirm that the possible 3-deoxy-3-fluoro-D-glucose metabolism did not interfere with its own transport. Accordingly, the catabolism of 3-deoxy-3-fluoro-D-

Table 1. K_x and V_{max} values of monosaccharides at 37°C

Data were obtained as described in the Materials and Methods section (see Fig. 1).

Sugar	K_x (mM)	V_{max} (mmol·litre ⁻¹ ·min ⁻¹)
D-Glucose	3.9	640
3-Deoxy-3-fluoro-D-glucose	2.3	600
3-O-Methyl-D-glucose	2.1	340
3-Deoxy-D-glucose	15.3	795
D-Mannose	12.4	724
D-Galactose	36.8	620
L-Arabinose	45.0	425

Fig. 4. Regeneration of haemoglobin in erythrocytes incubated with 3-deoxy-3-fluoro-D-glucose and Methylene Blue

This gives a measure of the reduction of NADP⁺ by the pentose phosphate pathway (Jaffé, 1969). $\circ$, 0.5 mM 3-Deoxy-3-fluoro-D-glucose; $\bullet$, 5.0 mM 3-deoxy-3-fluoro-D-glucose; $\triangle$, 50.0 mM 3-deoxy-3-fluoro-D-glucose; $\blacktriangle$, 0.25 mM D-glucose. % haemoglobin = [haemoglobin] $\times$ 100/([methaemoglobin] + [haemoglobin]). For experimental details see the Materials and Methods section.

glucose by the pentose phosphate pathway and glycolysis was estimated. When erythrocytes are incubated with Methylene Blue hexose catabolism occurs largely via the pentose phosphate pathway (Huennekens *et al.*, 1958). Without Methylene Blue, however, glycolysis predominates (Jaffé, 1969).

Fig. 5. Regeneration of haemoglobin in erythrocytes by the glycolysis of glucose and 3-deoxy-3-fluoro-D-glucose

This gives a measure of the reduction of NAD^+ by the glycolytic pathway (Jaffé, 1969). ○, 1 mM-Glucose; ●, 100 mM-3-deoxy-3-fluoro-D-glucose. For experimental details see the Materials and Methods section.

Fig. 4 shows that there was little significant catabolism of 3-deoxy-3-fluoro-D-glucose by the pentose phosphate pathway, although it was at 100 times the concentration at which glucose gave a positive response. Similarly, there was little glycolysis of 3-deoxy-3-fluoro-D-glucose by cells in the absence of Methylene Blue (Fig. 5). However, after incubation for 24 h about 1% of the 50 mM-3-deoxy-3-fluoro-D-glucose solution had been degraded, as indicated by the presence of free F^- ion. Such cells when tested for their ability to transport the sugar were shown to have lost this function completely. However, erythrocytes incubated for this period with glucose or buffered saline both retained the transport faculty. Some preliminary studies indicate that this time-dependent inhibition requires at least 5 h at 37°C to have any significant effect on the transport characteristics and consequently would not affect the transport data above.

Our results therefore confirm some of the data derived independently by different methods. It has been shown that 3-deoxy-3-fluoro-D-glucose is transported by the glucose carrier of the erythrocyte,

and it appears that the F atom mimics the hydrogen-bonding role of the glucose hydroxyl group at C-3.

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