THE DETECTION OF THE ENTHALPY OF BINDING OF D-GLUCOSE OF HUMAN RED BLOOD CELL MEMBRANES BY MICROCALORIMETRY

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1. Introduction

The transport of monosaccharides across the plasma membrane of the human erythrocyte occurs by facilitated diffusion for which several models have been proposed [1–4]. The monosaccharides are believed to combine with a component of the cell membrane sometimes referred to as the 'carrier' to form a complex which facilitates the movement of the sugar across the membrane. The proces has been extensively studied by kinetic methods and thermodynamic parameters for the formation of the sugar complex have sometimes been deduced from the temperature coefficient of K_m [5–10], assumed equal to K_S, the dissociation constant for the D-glucose–carrier complex, although in the absence of a complete knowledge of the mechanism involved in the process the precise relationship between K_S and K_m cannot be unambiguously stated. The values of K_m and their temperature dependence have been found to vary greatly with the method of measurement to the extent that there is conflicting data concerning the sign of the enthalpy changes derived from the temperature coefficients of K_m [7].

We report here a study of the application of the technique of microcalorimetry to the direct measurement of the enthalpy change associated with the specific binding of D-glucose to suspensions of human erythrocyte membranes (ghosts). Although the precision of the measurements was limited by the ghost concentration attainable and the thermal effects associated with mixing in the microcalorimeter, the data are consistent with an *endothermic* process having a half saturation constant in the region of 3–10 mmolal at 15°C.

2. Experimental

Whole blood was obtained from polycythaemic patients and collected in ACD mixture. Erythrocyte ghosts were prepared by the method of Dodge et al. [11]. During the course of the work 15 batches of ghosts were prepared with concentrations ranging from 1.08% to 3.53% (w/w) depending on the length of time of the final centrifugation. The average protein content of the ghosts was found to be 49.6% (w/w) which compares well with literature values [11,12] and the average haemoglobin content was 12.1% (w/w). The latter figure is rather higher than the values reported by Dodge et al. [11] but taking a value of 35 g of haemoglobin per gram of membrane in the intact cells, corresponds to removal of 99.7% of cellular haemoglobin. Protein content was determined by the Lowry method [13] and haemoglobin by the pyridine haemochromogen method [11]. In calculating the enthalpy changes per gram of non-haemoglobin protein the individual values for each batch of ghosts were used.

It was essential to demonstrate that the ghost suspensions did not contain any sealed vescicles impermeable to D-glucose so that apart from the volume excluded by the membranes themselves the entire volume of the ghost suspensions was non-specifically available to sugars. Two experiments were carried out to demonstrate the absence of a permeability barrier. In the first the rate of penetration into ghosts of [^{14}C] sorbitol, a non-transported sugar, relative to [^{14}C] sorbose, which is transported, was measured over a period of 150 min. In these experiments a solution of the labelled sugar was mixed with a ghost suspension and after known time intervals a stopper solution (2 mM $HgCl_2$ + 1.25 mM KI in isotonic saline)

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was added. The ghost suspensions were immediately centrifuged at 25 000 *g*, supernatant was removed and the activity in the ghost pellet determined. For both labelled sugars the amount of radioactivity trapped in the ghosts was found to be constant independent of time.

In the second experiment [^{14}C] glucose was incubated with a ghost suspension for various intervals of time up to 90 min. The overall D-glucose concentration in the suspension was 33 mM and the first measurement was made one min after mixing. The volume into which the D-glucose diffused was calculated from the measured change in activity and compared with that calculated assuming the entire volume of the suspension was available. From 11 experiments the percentage of the total volume of the suspension available to the glucose was found to be 104.9 ± 4.8% and independent of the time of incubation. The results of these experiments confirm that no sealed impermeable vesicles existed in the ghost preparations.

In the inhibition studies the ghosts were treated with 1-fluoro-2,4-dinitrobenzene (FDNB) at a concentration of 2×10^{-3} M for 2 hr under the conditions described by Krupka [14].

For all the enthalpy measurements the ghosts were dialyzed against unbuffered saline containing 1 mM Ca^{2+} ions, ionic strength 0.15 and pH 7.4. The sugar solutions were prepared with the saline dialysate. All the solutions contained 0.2% sodium azide to inhibit microbial growth. Enthalpy measurements were made with an LKB 10700 batch microcalorimeter which utilises the twin-vessel principle [15], each vessel being divided into two compartments.

3. Results and discussion

At least four processes could contribute to the enthalpy change observed when a monosaccharide solution is mixed with a suspension of ghosts; (i) the enthalpy of dilution of the ghosts, (ii) the enthalpy of dilution of the sugar, (iii) the enthalpy of interaction or non-specific binding of the sugar with the ghosts and (iv) the enthalpy of specific binding of the sugar to the carrier in the cell membrane.

It is possible to utilize the twin-vessel principle of the LKB microcalorimeter to obtain directly the difference in enthalpy on mixing D-glucose with a ghost suspension in the 'live' microcalorimeter vessel and L-glucose with a ghost suspension in the reference or 'dead' vessel. Because D-glucose has a high affinity for the 'carrier' while L-glucose has a very low affinity [16] such a differential experiment will largely eliminate the enthalpies of dilution of ghosts and sugars and the enthalpies of non-specific binding and give directly the enthalpy of specific binding of D-glucose to the ghosts. Before discussing the results of these differential experiments it is relevant to first consider the magnitudes of the enthalpies of dilution of ghosts and sugars.

The enthalpy of dilution of ghost suspensions was measured for the following process at 15°C, 4 g (x% w/w ghost suspension in saline) + 2 g (saline) → 6 g (2x/3% w/w ghost suspension) where x varied in the range 1–3% w/w.

It was found that this enthalpy change was small but varied from one batch of ghosts to another. The results for 8 batches of ghosts varied from +1.24 mJ to − 1.21 mJ with a mean value of +0.05 mJ. The reference cell in these experiments contained the premixed suspension.

The integral enthalpies of dilution at 15°C of D-glucose and L-glucose were measured for the process;

$$x \text{ g (y mmolal glucose–saline)} + z \text{ g (saline)} \rightarrow (x + z) \text{ g} \left(\frac{xy}{x + z\,(1 + y\, M_G\, 10^{-6})} \right) \text{mmolal glucose-saline}$$

where M_G is the molecular weight of glucose. A premixed glucose solution was used as blank in the reference vessel. For D-glucose was 89.7 mmolal and for L-glucose y was 100.3 mmolal. Both x and z were varied in the range 0.5 to 4.0 g to obtain a range of final glucose concentrations. The enthalpy changes measured were in the range 2 to 4 mJ. The integral enthalpies of dilution were exothermic and were linear when plotted against the final glucose concentration. Extrapolation of the least square lines to zero final glucose concentration gives the integral enthalpies of dilution at infinite dilution which were used to obtain the relative apparent molal enthalpies of dilution (ϕL_2) shown plotted in fig. 1 [17]. There is no significant difference between the results for D- and L-glucose and the equation;

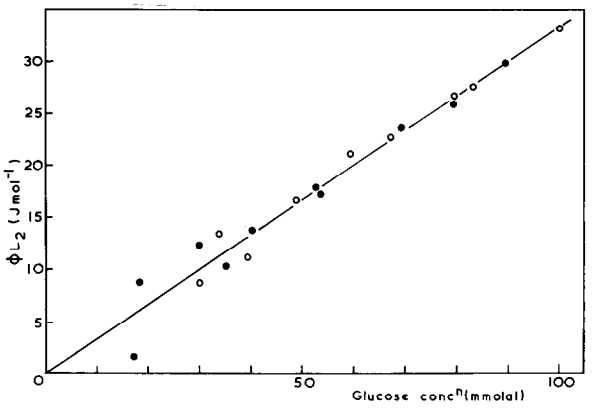

Fig. 1. The relative apparent molal enthalpy of dilution (ϕL_2) of glucose isomers as a function of glucose concentration at 15°C; (●—●—●) D-glucose; (○—○—○) L-glucose.

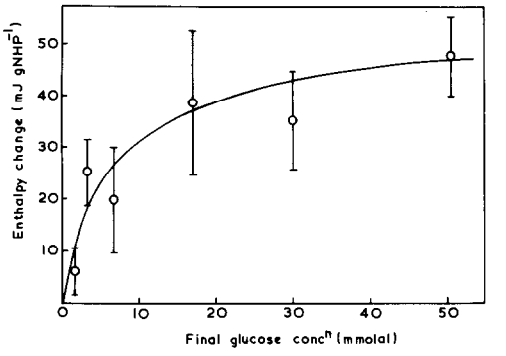

Fig. 2. The enthalpy of specific binding of D-glucose to human erythrocyte ghosts at 15°C (mJ per g of non-haemoglobin protein) as a function of the final glucose concentration. The error bars denote the standard deviations.

$\phi L_2 = 334.1\ (\pm\ 23.4\ m_G\ J\ mol^{-1}$, where m_G is the final glucose molality, represents the data.

The enthalpy of specific binding of D-glucose to ghosts at 15°C was measured in a series of experiments in which the process,

4 g (1–3% ghost suspension) + 2 g (x mmolal D-glucose–saline) → 6 g (0.66 to 2% ghosts, nominally x/3 mmolal D-glucose–saline)

occurred in the 'live' vessel and the analogous process with L-glucose occurred in the reference vessel. The value of x was varied over the range 5 to 150 mmolal so that the final glucose concentration was in the range 1.7 to 50 mmolal. The microcalorimeter vessels were charged with known weights of the solutions. A correction was made for any small differences in the enthalpies of dilution of glucose arising from slight inequalities in weights in the two vessels using the ϕL_2 expression discussed above.

The enthalpy changes measured were in the range 0.66 to 2.0 mJ. The microcalorimeter was operated at a very high sensitivity (3 μV range) and the frictional effects arising on mixing ghost suspension gave rise to 0.5 ± 0.13 mJ. Unfortunately these factors limited the reproducibility we were able to achieve. Fig. 2 shows the enthalpy of specific binding calculated per gram of non-haemoglobin protein at 15°C. The data are consistent with an *endothermic* binding process having a half-saturation constant in the region of 3–10 mmolal. This estimate of the half-saturation constant is in line with the values of K_m between 1 and 10 mM reported by various workers using several different techniques. The sign of the enthalpy change agrees with that predicted using the kinetic method of inhibition of sorbose entry by glucose [6] and the equilibrium exchange experiments of Levine and Stein [9] and Jung et al. [10] but not with the equilibrium exchange experiments of Lacko et al. [8]. The infinite cis procedure [5,7] also predicts an enthalpy change opposite in sign to that we observe.

The effect of inhibitors of the facillitated diffusion of sugars into human erythrocytes on the enthalpy of specific binding at 15°C is shown in table 1.

Table 1
Effect of inhibitors on the enthalpy of specific binding of D-glucose to human erythrocyte ghosts at 15°C (final D-glucose concentration 30 mmolal)

Treatment	ΔH_B/g NHP mJ g^{-1}	No. of expts.
Untreated	34.9 ± 9.7	6
FDNB treated	4.0 ± 13.2	12
in presence of 0.27 mM phloretin	40.8 ± 10.5	4

The enthalpy change is reduced almost to zero by the irreversible inhibitor FDNB as would be expected if the 2,4-dinitrophenyl derivative of the 'carrier' does not bind D-glucose. In contrast phloretin at a concentration of 180 times its K_1 of 1.5×10^{-6} M [18] has no effect within the experimental uncertainty. Phloretin has been described as a competitive inhibitor, although the measurements indicate that both V_{max} and K_m for glucose vary with phloretin concentration [19]. There is evidence that it must be present on the opposite side of the membrane from which transport is proceeding for inhibition to occur, i.e. it is a trans-inhibitor [18]. It is known to bind non-specifically to erythrocyte membranes [20] and to affect the permeability of a variety of non-electrolytes depending on their hydrophilicity [21]. The microcalorimetric data indicates that phloretin does not interact with the active binding site of the membrane component responsible for the transport of D-glucose.

Both the saturation behaviour illustrated in fig. 2 and the inhibition effects suggest that the enthalpy change we observe in the differential experiments arises from the association of D-glucose with a component of the monosaccharide transport system of the human erythrocyte membrane. Clearly the observed enthalpy change may include contributions from desolvation, binding and conformational changes. Estimates of the number of specific D-glucose binding sites per cell range from 2 to 5×10^5 [22–24]; if we take a figure of 6×10^{-13} g of non-haemoglobin protein per ghost [11] and our saturation enthalpy of about 50 mJ (g NHP)$^{-1}$ the enthalpy of binding lies in the range 91 to 36 kJ mol^{-1}. The lower figure is very close to that found from the kinetic method of inhibition of sorbose entry into erythrocytes by glucose [6] at 15°C. Unfortunately the level of precision of the data is not high and the possibility that several distinct binding sites exist cannot be ruled out.

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