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THE EFFECT OF TEMPERATURE ON THE COMPETITIVE INHIBITION OF SORBOSE TRANSFER IN HUMAN ERYTHROCYTES BY GLUCOSE

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SUMMARY

1. A simple method is described for the determination of the parameter K_i, the dissociation constant of a sugar by its inhibition of transfer of a second sugar across the human erythrocyte membrane.

2. Integrated rate equations are derived on the assumption that the transport is measured of a sugar with a very low affinity for the membrane in the presence of a high-affinity sugar, both sharing the same facilitated system.

3. The experimental data fit linear plots as predicted by the theory while the use of three low-affinity sugars, L-sorbose, D-fructose and D-arabinose indicate the generality of the method.

4. The temperature dependence of the inhibition constant K_i for glucose inhibition of sorbose uptake is studied and it is shown that the value of K_i passes through a minimum in the region of 25–30°. Some thermodynamic features of the carrier-inhibitor reaction are calculated from the temperature coefficient of K_i namely $\Delta H°$, $\Delta G°$ and $\Delta S°$.

5. It is postulated that hydration of glucose is the dominant factor at low temperatures, accounting for the negative values found for $\Delta H°$ and $\Delta S°$ for the dissociation of the glucose-carrier complex while at the higher temperatures hydration contributes less to the dissociation process, accounting for the more positive values found for $\Delta H°$ and $\Delta S°$.

6. The total energy involved, ΔH_T, in the temperature range 10–40° is found to be about 18 kcal·mole^{-1}.

INTRODUCTION

Studies of the kinetic parameters of the facilitated diffusion system in the human red cell membrane[1] have revealed the complexity of the mechanism by which monosaccharides are transported. The values obtained for the constants which characterize the system were shown to be dependent on the experimental technique used for their measurement. It has been customary, in previous studies, to define simply two parameters, v_{max_o}, the maximal transfer rate obtainable when one side of the membrane is

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saturated with the substrate while the other side is completely unsaturated and K_m, an apparent half-saturation constant, which is the sugar concentration resulting in an influx or efflux equal to half the maximal value v_{maxo}. LEVINE AND STEIN[1] have described various methods for measuring the above constants and also for determining the following additional parameters: K_s, the carrier-substrate dissociation constant: r, the ratio of the rates of transfer of loaded and unloaded carrier: v_{maxe}, the limiting transfer rate when both sides of the membrane are saturated with permeant and K_i, the dissociation constant of a sugar measured by its inhibition of transfer of a second sugar. Methods of obtaining the inhibition constant K_i that have been used previously, include the uptake of radioactively labelled sorbose in the presence of various concentrations of inhibitor at a fixed time[1] and the measurement of the rate of uptake of sorbose in the presence of inhibitor by means of a light-scattering method[2-4].

The finding that K_i and K_m differed significantly[1] was consistent with that noted previously by SEN AND WIDDAS[5]. More recently, in a detailed, analytical and experimental study, Miller established further evidence that the various affinity constants yielded values dependent on the type of experiment. For example, a 10-fold difference was found between K_m, the half-saturation constant of a sugar determined by the method of WIDDAS[5] and K_i, the value obtained by its inhibition of sorbose transport.

A preliminary investigation into the temperature dependence of the affinity constants suggested that the temperature dependences of K_m and K_s (or K_i) are opposite in sign[1]. Whereas K_m decreases with decrease in temperature, as originally reported by WIDDAS[5], the carriers-ubstrate dissociation constant K_s was found to increase as the temperature decreases in the range 25–5°. In addition, the inhibition constant, K_i, characterising the inhibition by glucose of sorbose entry, appeared to increase as the temperature dropped from 25 to 13°. However, a comprehensive set of data for the temperature dependence of K_i has hitherto not been obtained. Such an unusual finding warranted further investigation and the present work was therefore undertaken to provide more extensive data on the temperature dependence of the inhibition constant, K_i. This parameter is more readily interpreted than K_m since it is, in our experimental situation, a simple dissociation constant uncomplicated by the transfer process. Also the temperature dependence of K_i provides valuable information concerning the interactions involved between substrate, membrane and solvent.

In this paper we describe a simple method of finding the inhibition constant K_i and its temperature dependence in the range 10–40°. We make use of an integrated rate equation which gives a straight-line plot, permitting the evaluation of K_i from the examination of slopes in the presence and absence of one concentration of inhibitor. The theoretical justification for the method is described and the results are interpreted on a molecular basis.

THEORY OF THE METHOD

Suppose that the cells have been equilibrated with a sugar having a high affinity for the membrane carrier (*e.g.* glucose) and that in the presence of this sugar, the transport is measured of a second sugar (*e.g.* sorbose) with a very low affinity. The subscripts i and o indicate inside and outside the cells. In the case of the carriers,

these subscripts refer to the concentrations in the membrane facing the inside and outside of the cells, respectively. We introduce the following notation: t, time in min; I_i, I_0, concentrations of glucose (first sugar or inhibitor) in units of moles/l; S_i, concentration of sorbose (second sugar) at time t inside the cell, measured in units of counts/min of radioactively-labelled sorbose (see section on procedure); S_0, constant concentration of sorbose outside (5 mM); C_i, C_0, concentrations of free carrier; C_{si}, C_{s0}, concentrations of carrier-complex with sorbose; C_{Ii}, C_{I0}, concentrations of carrier-complex with glucose (the carriers, free or complexed, remaining in the membrane phase); k_2, k_4, rate constants for the translocation of complex and free carriers, each being the same for the two sugars; $r = k_2/k_4$, ratio of the two rate constants.

$$K_s = \frac{C_i \cdot S_i}{C_{si}} = \frac{C_0 \cdot S_0}{C_{s0}}, \qquad K_i = \frac{C_i \cdot I_i}{C_{Ii}} = \frac{C_0 \cdot I_0}{C_{I0}} \tag{1}$$

the two dissociation constants of the sugar complexes, where $K_s/K_i \gtrsim 100$.
The fixed total concentration of carriers is

$$\begin{aligned} \text{total } C &= C_i + C_0 + C_{si} + C_{s0} + C_{Ii} + C_{I0} \\ &= C_i \left(1 + \frac{S_i}{K_s} + \frac{I_i}{K_i}\right) + C_0 \left(1 + \frac{S_0}{K_s} + \frac{I_0}{K_i}\right) \end{aligned} \tag{2}$$

substituting Eqn. 1. The net inward fluxes of the two sugars read

$$\frac{dS_i}{dt} = k_2(C_{s0} - C_{si}) = \frac{k_2}{K_s}(C_0 \cdot S_0 - C_i \cdot S_i) \tag{3}$$

and

$$\frac{dI_i}{dt} = k_2(C_{I0} - C_{Ii}) = \frac{k_2}{K_i}(C_0 \cdot I_0 - C_i \cdot I_i). \tag{4}$$

Volume changes of the cell are neglected here because the 5 mM sorbose present in the medium is less than 2 % of an isotonic concentration. We assume the steady-state condition that the flows are equal in the two directions, *i.e.*

$$k_2 C_{si} + k_2 C_{Ii} + k_4 C_i = k_2 C_{s0} + k_2 C_{I0} + k_4 C_0 \tag{5}$$

which can be written as

$$C_i \left(1 + r\frac{S_i}{K_s} + r\frac{I_i}{K_i}\right) = C_0 \left(1 + r\frac{S_0}{K_s} + r\frac{I_0}{K_i}\right) \tag{6}$$

From Eqns. 2 and 6, we can solve for C_0 and C_i to obtain

$$C_0 = \text{tot } C \left(1 + r\frac{S_i}{K_s} + r\frac{I_i}{K_i}\right) \Big/ D \tag{7}$$

and

$$C_i = \text{tot } C \left(1 + r\frac{S_0}{K_s} + r\frac{I_0}{K_i}\right) \Big/ D \tag{8}$$

where

$$D = \left(1 + \frac{S_i}{K_s} + \frac{I_i}{K_i}\right)\left(1 + r\frac{S_o}{K_s} + r\frac{I_0}{K_i}\right) + \left(1 + \frac{S_0}{K_s} + \frac{I_0}{K_i}\right)\left(1 + r\frac{S_i}{K_s} + r\frac{I_i}{K_i}\right). \tag{9}$$

In our experimental situation $S_i/K_s \ll I_i/K_i$ and $S_0/K_s \ll I_0/K_i$, so that we can neglect the terms in Eqns. 7–9 proportional to $1/K_s$.

Eqn. 4 then becomes:

$$\frac{dI_i}{dt} = \frac{k_2 \text{ tot } C(I_0 - I_i)}{2K_i + (1 + r)I_0 + \left(1 + r + \frac{2rI_0}{K_i}\right)I_i} \tag{10}$$

This, in fact, describes the transport of one sugar (glucose) in the absence of a second sugar (sorbose) *i.e.* the transport of the glucose is practically unaffected by the presence of the sorbose (*cf.* ref. 1 where Eqn. 10 is given by Eqn. A.11 and refs. 4, 6 where this property of the glucose transport is confirmed). Eqn. 10 has the solution

$$I_i = I_0 + (I_i^0 - I_0) \exp(-\alpha t) \exp[-\beta(I_i - I_i^0)] \tag{11}$$

where I_i° is the initial value of I_i and

$$\alpha = \frac{k_2 \text{ tot } C}{2K_i\left(1 + \frac{I_0}{K_i}\right)\left(2 + r\frac{I_0}{K_i}\right)}, \qquad \beta = \frac{1 - r + \frac{2rI_0}{K_i}}{2K_i\left(1 + \frac{I_0}{K_i}\right)\left(1 + r\frac{I_0}{K_i}\right)}. \tag{12}$$

For the experimental conditions which lead to Eqn. 10, Eqn. 3 becomes

$$\frac{dS_i}{dt} = \frac{k_2}{K_s}\frac{\text{tot } C}{D}\left[(S_0 - S_i) + \frac{r}{K_i}(S_0 I_i - I_0 S_i)\right] \tag{13}$$

where in Eqn. 9 for D, we again neglect the terms in $1/K_s$. As explained below in the procedure, we shall be concerned with values of t significantly greater than $1/\alpha$ (say $\gtrsim 5/\alpha$) when $I_i = I_0$ very nearly. Then Eqn. 13 simplifies to

$$\frac{dS_i}{dt} = \frac{v_{\text{maxe}}(S_0 - S_i)}{K_s(1 + I_0/K_i)} \tag{14}$$

where $v_{\text{maxe}} = \frac{1}{2} k_2 \text{ tot } C$, is the maximum velocity that sorbose would attain at saturating concentrations if this were experimentally feasible.

The solution of Eqn. 14 can be written in the form

$$\ln\frac{S_0}{S_0 - S_i} = \frac{v_{\text{maxe}}t}{K_s\left(1 + \frac{I_0}{K_i}\right)} \tag{15}$$

where $S_i = 0$ at $t = 0$. In the absence of the inhibitor Eqn. 15 becomes

$$\ln\frac{S_0}{S_0 - S_i} = \frac{v_{\text{maxe}}t}{K_s} \tag{16}$$

Plots of $\ln[S_0/(S_0 - S_i)]$ against t of Eqns. 15 and 16 are linear in t and the ratio of the slopes obtained in the presence and absence of inhibitor equals $1/[1 + (I_0/K_i)]$ from which K_i can easily be calculated.

Some thermodynamic features of the carrier-inhibitor reaction can be calculated from the temperature coefficient of K_i in the following way. We assume the chemical potential of the inhibitor inside or outside the cell can be written as

$$\mu_I = \mu_I^0(P,T) + RT \ln I \tag{17}$$

where μ_I° is the standard chemical potential depending on pressure P and temperature T with corresponding expressions for the chemical potentials of the complex and

carrier. The condition of equilibrium with respect to the reaction carrier–inhibitor complex $\rightleftharpoons$ inhibitor + carrier reads

$$\mu_{CI} = \mu_I + \mu_C \tag{18}$$

which yields

$$RT \ln K_i = \mu_{CI}^0(P,T) - \mu_I^0(P,T) - \mu_C^0(P,T) = -\Delta G^0(P,T). \tag{19}$$

Thus ΔG° is the standard Gibbs' free energy of dissociation of the carrier-inhibitor complex. We now calculate the corresponding enthalpy change by

$$\Delta H^0(P,T) = \frac{\partial \left(\frac{\Delta G^0}{T}\right)}{\partial \left(\frac{1}{T}\right)} = -R \frac{\partial \ln K_i}{\partial \left(\frac{1}{T}\right)} \tag{20}$$

and the change in entropy by

$$\Delta S^0 = \frac{\Delta H^0 - \Delta G^0}{T} \tag{21}$$

Thus the Gibbs' free energy, enthalpy and entropy changes are obtained from K_i and its variation with T.

EXPERIMENTAL

Materials

The human erythrocytes were obtained from out-dated transfusion blood, preserved in acid–citrate–dextrose medium. Before use, the cells were washed at least three times in a 10-fold volume of "buffered saline" (1 % NaCl–0.025 M phosphate buffer, pH 7.4). The plasma and the top layer of cells were removed in the course of these washings. The radioactive sugars were universally labelled ^{14}C compounds obtained from the Radiochemical Centre, Amersham. Unlabelled sugars were either Analar or B.D.H. grade and were dissolved in the buffered saline. The composition of the scintillation counting mixture was 0.4 % 2,5-diphenyloxazol (PPO), 0.04 % dimethyl 1,4-bis-(5-phenyloxazolyl-2)benzene (POPOP) in a xylene base. Ethanol was added to the scintillation counting mixture in the proportion of 30 vol. ethanol to 68 vol. of the scintillation mixture.

Procedure

Aliquots of washed cell suspensions were equilibrated with glucose by the addition of 3 ml glucose solution to 2 ml cell suspension which had previously been brought to 25 % haematocrit. For the sorbose-uptake measurements in the absence of glucose, 3 ml buffered saline was added without glucose. At zero time, 1 ml of ^{14}C-labelled sorbose medium was added to the 5 ml cell suspension bringing the final concentration of sorbose to 5 mM. At a given time, t, the translocation was terminated by the abrupt addition of 35 ml ice-cold stopping medium (2 mM $HgCl_2$, 1.25 mM KI, 1 % NaCl), shown earlier to be an effective quencher of sugar movement[1]. The time intervals for the uptake were varied, depending on the temperature of the experiment. In general, the longest time interval was arranged so that approx. 25–35 % of the equilibrium value was attained during that time.

The amount of radioactive sorbose trapped in the extracellular space was found by mixing the labelled sorbose with the stopping solution and adding the mixture to the cell suspension, this amount being subtracted from the total counts. In addition, aliquots of cells were equilibrated with labelled sorbose medium until equilibrium was reached followed by the addition of the stopping medium. The time required to reach equilibrium with sorbose was found to be approx. 2 h at 37°. The concentration of sorbose inside the cells of the equilibrated sample could then be equated to S_0, the concentration of sorbose in the medium outside the cells. Thus the data, in the form of radioactive counts/min inside the cells at time t, could be used directly as concentrations without the need to calculate the intracellular volume.

Following the addition of the stopping medium, the cells were separated by centrifugation and the supernatant was discarded. The inside of the tube was carefully dried with filter paper without disturbing the pellet of cells. The radioactive sugar was extracted with 2 ml of 10% trichloroacetic acid and filtered. Aliquots (0.2 ml) of the filtrate were mixed with 10 ml of ethanolic scintillator and counted in a Packard TriCarb liquid scintillation spectrometer.

RESULTS

Figs. 1 and 2 are linear plots of sorbose uptake as a function of time at 20 and 25°, respectively. The figures show good linearity of the experimental data as predicted by the theory. For the data shown in Fig. 1 uptake was measured over a 10-min interval and the distribution ratio of sorbose (concentration inside/concentration on the outside) was approx. 0.40 at the end of 10 min. The slope_0 in the absence of inhibitor, that is $\log_{10}[S_0/(S_0-S_i)]/t = 0.0205$ while in the presence of inhibitor the $\text{slope}_i = 0.0132$. Since the concentration of the inhibitor was 3.75 mM, from the relation $\text{slope}_0/\text{slope}_i = 1 + (I/K_i)$, $K_i = 6.8$ mM. In Fig. 2, it can be seen that the slope in the absence of inhibitor at 25° has increased to 0.045 while the distribution ratio at 10 min is approx. 0.65. In general as already mentioned, time intervals were arranged so that distribution ratios reached at the longest time were usually in the range 0.25–0.35. The slopes are seen to decrease with increase in the concentration of glucose. The value of K_i, which should be constant when calculated from any of the slopes, appears to increase from 7.53 mM at a glucose concentration of 5.33 mM to

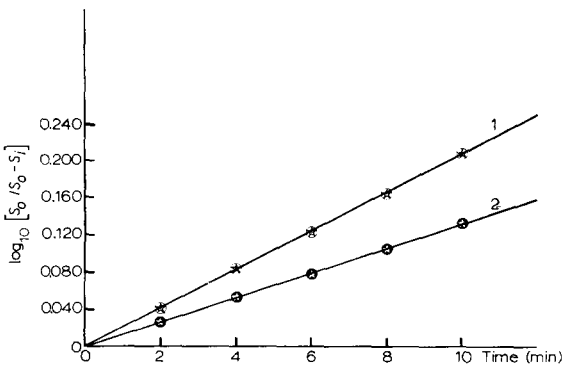

Fig. 1. The measurement of K_i of glucose at 20°. See text for definition of symbols. 1, sorbose uptake in the absence of inhibitor; 2, sorbose uptake in the presence of 3.75 mM glucose.

8.4 mM at 21.3 mM glucose. This effect was more marked at high glucose concentrations in the region of 60 mM suggesting a departure from ideality. Extrapolation to zero glucose concentration yields a value for K_i which is independent of concentration. Since this effect was found to be negligible at very low concentrations, all the work relating to the temperature dependence of K_i was carried out with 3.75 mM glucose.

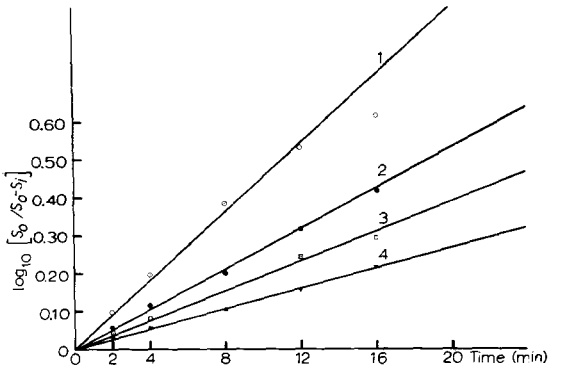

Fig. 2. Effect of varying glucose concentration on sorbose uptake. 1, sorbose uptake in the absence of inhibitor; 2, 3 and 4, sorbose uptake in the presence of 5.33 mM, 10.7 mM and 21.3 mM, glucose, respectively.

According to the theoretical model, other monosaccharides with low affinity for the red cell membrane should be equally suitable for the determination of the inhibition constant of a high-affinity sugar provided they share the same transport system. To test this assumption and to demonstrate that the value obtained for K_i by the inhibition of sorbose is not dependent on a property of sorbose itself, D-arabinose or D-fructose was substituted for the sorbose. The results obtained by the inhibition of D-arabinose gave values for the K_i of glucose close to those found by the sorbose method. D-Fructose also gave similar values indicating the generality of the method. The slopes obtained for the uptake of D-arabinose at 25° were very similar to those for D-fructose indicating that the affinities of both D-arabinose and D-fructose for the red cell membrane are much the same. Both sugars, however, show less affinity than sorbose.

It was of interest too, to find the contribution from the simple diffusion process to the uptake of the low-affinity sugars. Since sorbitol, a hexahydric alcohol, is not considered to belong to the group of sugars which shares the facilitated diffusion system, it was substituted for the sorbose and its entry measured in the same way. As expected, uptake of sorbitol was very slow amounting to about 3 or 4 % of sorbose entry under the same experimental conditions. It was not expected, however, that glucose would affect the simple diffusion of sorbitol across the membrane. Yet a small inhibitory effect in the presence of glucose was noted. The nature of this effect is uncertain since it is not altered appreciably by varying the glucose concentrations.

Fig. 3 records the mean values of K_i for glucose inhibition of sorbose at the temperatures indicated. The striking feature about the variation of the inhibitor constant K_i with temperature is the minimum through which it passes in the temperature range 25–30°. From a plot of $\log_{10} K_i$ *vs.* T^{-1} it is possible to calculate $\Delta H°$ using Eqn. 20. The standard Gibbs energy change and entropy change for the dissociation

of the carrier-inhibitor complex may be calculated using Eqns. 19 and 21, respectively. The calculation of ΔH° is subject to an uncertainty due to the difficulty of measuring the gradient of the curve of $\log_{10} K_i$ *vs.* T^{-1}. We estimate the uncertainty in ΔH° to be no more than $\pm$ 0.5 kcal/mol. Fig. 4 shows the values of ΔG°, ΔH° and $T\Delta S^\circ$ as a function of temperature. It can be seen that ΔG° is almost independent of temperature over the temperature range of the experiments while ΔH° and ΔS° depend very markedly on temperature, almost paralleling each other, the change of sign at 28° being a consequence of the minimum in the $\log_{10} K_i$ *vs.* T^{-1} plot.

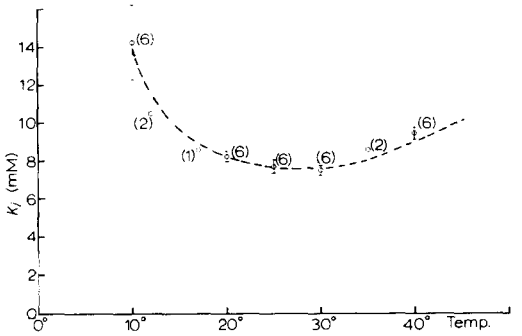

Fig. 3. The effect of temperature on K_i, the inhibition constant of glucose. The number of experiments is shown in parentheses.

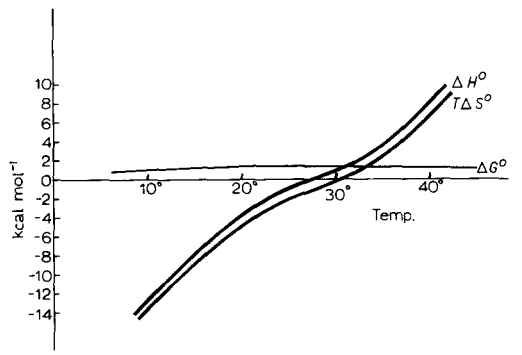

Fig. 4. Values of ΔG°, ΔH° and $T\Delta S^\circ$ as a function of temperature.

DISCUSSION

One possible interpretation of the data on the temperature dependence of K_i is to evoke the role that hydration of glucose plays in the dissociation of the carrier–glucose complex. In the translocation process the complex must pass through the hydrophobic region of the membrane and this process would be expected to occur more easily if the complex were not appreciably hydrated. Extensive hydration would not only raise the effective molecular weight of the complex but would impart to it a more hydrophilic character and both of these factors would reduce the ease of translocation[7]. If then we postulate that the glucose molecule is to some extent dehydrated in the complex then when the complex dissociates and the glucose molecule passes into the aqueous environment it will become hydrated. The extent of hydration will depend

on the temperature, being less at higher temperatures. In general an hydration process is exothermic and results in a reduction in entropy, whereas a dissociation process is usually endothermic and is accompanied by an increase in entropy. Considering the values of ΔH° and ΔS° in the light of the above comments the hydration effect would contribute an exothermic term to ΔH° and would result in a negative entropy change on dissociation due to the ordering of the water molecules which hydrate the glucose molecules. Since at a low temperature ΔH° and ΔS° for the dissociation of the glucose–carrier complex are negative it would appear that the hydration of the glucose molecule is the dominant factor under these conditions. As the temperature is raised the hydration would be expected to contribute less to the dissociation process and if this were so ΔH° and ΔS° would become more positive as is observed experimentally.

Perhaps the nearest analogy to this effect is the transfer of hydrocarbon moieties from a hydrophobic to a hydrophilic environment as occurs when proteins denature[8] or ionic micelles dissociate[9]. These processes involving 'hydrophobic bonding' occur with a decrease in entropy due to ordering of the water molecules around hydrocarbon chains. The enthalpy change on dissociation of ionic micelles[9], changes with temperature in a very similar manner as found in our measurements although the magnitudes here are larger, as would be expected, since ordering of water molecules around hydrocarbon chains will involve weaker intermolecular forces than the hydrogen bonding of water molecules to glucose.

The total amount of energy involved in the temperature range 10–40° is given by the equation

$$\Delta U_T \approx \Delta H_T = \int_{10^\circ}^{40^\circ} \left(\frac{\partial \Delta H^0}{\partial T}\right)_P \mathrm{d}T = \int_{10^\circ}^{40^\circ} \Delta C_p \mathrm{d}T$$

where the specific heat $\Delta C_p = C_p(\text{glucose}) + C_p(\text{carrier}) - C_p(\text{complex})$. Since the calculation of ΔH_T involves the second derivative of the $\log_{10} K_i$ *vs.* T^{-1} curve followed by graphical integration only a very rough estimate may be made. The value we obtain for ΔH_T is 18 kcal·mole^{-1}. If it is assumed that the dominant temperature-dependent contribution to the enthalpy is the hydration process then this corresponds to the formation of between 3 and 4 hydrogen bonds. This figure may be an underestimate since it only corresponds to a part of the temperature interval over which the process could occur. The magnitude, however, is not inconsistent with the proposed interpretation.

The variation of K_i with temperature reported here throws some doubt on the validity of comparing the affinities of sugars for the membrane carrier at a single temperature[10] since the profile of the K_i *vs.* temperature plot may well vary with the structure of the sugar.

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REFERENCES

1 M. Levine and W. D. Stein, *Biochim. Biophys. Acta*, 127 (1966) 179.
2 D. M. Miller, *Biophys. J.*, 8 (1968) 1329.
3 D. M. Miller, *Biophys. J.*, 8 (1968) 1339.

4 W. F. WIDDAS, *J. Physiol. London*, 125 (1954) 163.
5 A. K. SEN AND W. F. WIDDAS, *J. Physiol. London*, 160 (1962) 392.
6 M. LEVINE, D. L. OXENDER AND W. D. STEIN, *Biochim. Biophys. Acta*, 109 (1965) 151.
7 W. D. STEIN, *The Movement of Molecules across Cell Membranes*, Academic Press, New York, 1967, Chapter 3.
8 W. KAUZMAN, *Adv. Protein Chem.*, 4 (1959) 1.
9 M. N. JONES, G. PILCHER AND L. ESPADA, *Chem. Thermodyn.*, 2 (1970) 333.
10 V. P. CIRILLO, *J. Bacteriol.*, 95 (1968) 603.