J. Membrane Biol. 3, 83–92 (1970)

The Temperature Dependence of the Facilitated Transport of D(+)-Glucose Across the Human Red Cell Membrane

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Received 31 March 1970

Summary. The rate of exit of D(+)-glucose from human red cells was measured as a function of the extracellular glucose concentration over the temperature range 12 to 47 °C. The results were analyzed at each temperature, according to the kinetic model of Widdas and of Rosenberg and Wilbrandt, in terms of the apparent maximum exit rate (V_{max}) and the apparent dissociation constant (K_m) of the carrier-glucose complex. When the values of V_{max} and K_m were obtained by the same graphical method as that used by Sen and Widdas, the results were very similar to theirs insofar as the effect of temperature is concerned. In particular, the apparent standard enthalpy of dissociation (ΔH_m) of the carrier-glucose complex does not vary with temperature, whereas the apparent activation energy (E_{max}) for the translocation of the carrier increases strongly with decreasing temperature. It is shown that the explanation of these findings given by Dawson and Widdas is internally inconsistent. Furthermore, the graphical method as used by these authors is unreliable at higher temperatures, where K_m is large and consequently underestimates K_m. An improved modification of the method, suggested by Bolis, Luly and Wilbrandt, overcomes this difficulty and leads to more reliable values for V_{max} and K_m. These new results show that E_{max} decreases, and ΔH_m increases, as the temperature is raised. This behavior is shown to be consistent with the modified kinetic model for sugar transport proposed by Wilbrandt, in which the translocation rate of the loaded carrier is assumed to be different from that of the empty carrier. The changes in E_{max} and ΔH_m with temperature are the result of the difference in true activation energies for the translocation of the loaded and empty carrier.

The temperature dependence of glucose transport across the erythrocyte membrane was first observed by Masing (1914), who concluded that the transport process involved a chemical reaction because of the high temperature coefficient. Later workers have confirmed that sugar penetration is highly activated. Recently, Faust (1960) and Sen and Widdas (1962) have examined more fully the effect of temperature on the facilitated transport of glucose across the human red cell membrane, and Dawson and Widdas (1964) have attempted an analysis of the temperature coefficients in terms of kinetic models for the transport process. This analysis would appear to

be incomplete in certain respects, and it seemed desirable both to obtain further experimental evidence and to look at other kinetic models in the light of the thermodynamic data.

Materials and Methods

Human blood was collected either into standard acid citrate dextrose (ACD; 1.47% glucose, 0.48% citric acid, 1.32% sodium citrate) or into heparin (5 IU/ml). The erythrocytes were washed three times with 20 volumes of isotonic saline with sodium phosphate buffer at pH 7.4.

The glucose permeability was determined by the indirect osmotic method of Wilbrandt (1955). The washed erythrocytes were equilibrated at 37 °C with isotonic glucose (300 mM), and the exit of glucose into solutions of isotonic saline containing low concentrations of glucose was followed at the desired temperature by suspending one volume of glucose-loaded cells in 20 volumes of the exit medium. Samples were drawn over short time intervals for determination of the osmotic fragility. To minimize osmotic loss during the determination, the fragility measurements were carried out at 5 °C. From the fragility curves, the exit of glucose was then calculated (Wilbrandt, 1955). The exit solutions contained 0.0125, 0.025, 0.0375, 0.05, 0.075 and 0.1 isotonic glucose. The exit rates for cells collected in citrate were followed at 5 °C temperature intervals from 12 to 47 °C. For cells collected in heparin, a less extensive series of experiments was carried out at 10 °C intervals. At the lower temperatures, the higher external glucose concentrations were not studied, since the exit times under those conditions are very long. The results are based on the mean of eight experiments at each external concentration at each temperature.

The results at each temperature were first analyzed by the method of Sen and Widdas (1962) to obtain estimates of the parameters V_{max} and K_m in the model of Wilbrandt and Rosenberg (1961). In view of the limitations of this graphical method, V_{max} and K_m were recalculated from the experimental data by a modified method, described by Bolis, Luly and Wilbrandt (*in preparation*), as briefly outlined below.

Results and Discussion

The model of Widdas (1954) and of Rosenberg and Wilbrandt (1955), which will be referred to as model I, treats the facilitated transport of sugar across the erythrocyte membrane as the net result of an exit flux and entry

flux mediated by an enzyme-like carrier characterized by a half-saturation constant K_m and by a maximum rate V_{max}. If the internal and external concentrations of sugar are S_1 and S_2, respectively, the net efflux rate (V) is given by

$$\frac{1}{V}=\frac{1}{V_{max}}\cdot\frac{(S_1+K_m)(S_2+K_m)}{(S_1-S_2)K_m}. \tag{1}$$

If S_1 is always much greater than both K_m and S_2, a plot of $1/V$ against S_2 will be linear and will show an intercept at $S_2=0$ corresponding to $1/V_{max}$, and an intercept at $(1/V)=0$ corresponding to $-K_m$. This method was used by Sen and Widdas (1962). It can be shown from their reported results that the Arrhenius plot of V_{max} as a function of temperature (T) is strongly curved, with the apparent activation energy for carrier transport (E_{max}) decreasing at higher temperatures. A plot of $\log K_m$ against $1/T$ from their results is linear, corresponding to a constant value of the apparent enthalpy of dissociation (ΔH_m) of the carrier-glucose complex over the range of temperatures studied.

When our experimental data were analyzed by the same graphical method, we found essentially the same results, namely an E_{max} diminishing with increase in temperature and a constant ΔH_m. As we shall show below, this result is inconsistent with any kinetic model yet proposed for facilitated transport, and the validity of the graphical method was accordingly re-examined. Firstly, at high T (47 °C), K_m becomes approximately 1/10 of S_1. For results using small values of S_2, the plot will then give an intercept at $S_2=0$ of $\frac{1}{V_{max}}(1+K_m/S_1)$. At high temperatures, therefore, the value of V_{max} is underestimated by up to 10%. However, even with this correction, the marked curvature of the plot of $\log V_{max}$ against $1/T$ remains. Rather more serious is the error in calculating K_m, arising from the fact that S_2 is not everywhere negligible with respect to S_1. It is readily shown from Eq. (1) that, for constant S_1,

$$\frac{d\frac{1}{V}}{dS_2}=\frac{1}{K_m V_{max}}\frac{(S_1+K_m)^2}{(S_1-S_2)^2}. \tag{2}$$

The plot of $1/V$ against S_2 will therefore be increasingly nonlinear as S_2 becomes large, as pointed out by LeFevre (1962). A good linear plot of experimental results can be obtained by working with very small S_2 values, but the extrapolation of the line to $1/V=0$ constitutes an extrapolation to values of $-S_2$ of the magnitude of K_m, and hence across a region where the

function cannot be linear. It follows that a linear extrapolation is unreliable, particularly at higher temperatures, and K_m is underestimated.

The defects of the graphical method described above are avoided in a modified method described by Bolis, Luly and Wilbrandt (*in preparation*). Rearranging Eq. (1), we have

$$F \equiv \frac{S_1 - S_2}{V} = \frac{1}{V_{max}} \frac{S_1 + K_m}{K_m} (S_2 + K_m). \tag{3}$$

A plot of F against S_2 should be strictly linear with an intercept at $S_2 = 0$ given by $(S_1 + K_m)/V_{max}$, and an intercept at $F = 0$ given by $-S_2 = K_m$. This graphical method was therefore used to obtain V_{max} and K_m. To avoid arbitrariness, however, the intercepts were calculated directly from the experimental data, using the method of least squares (regression line). The results so obtained are shown in Figs. 1 and 2 for all experimental temperatures. These results differ in value from those of Sen and Widdas. However, as pointed out above, the new results have the same form of dependence on temperature as those of Sen and Widdas. Since the following

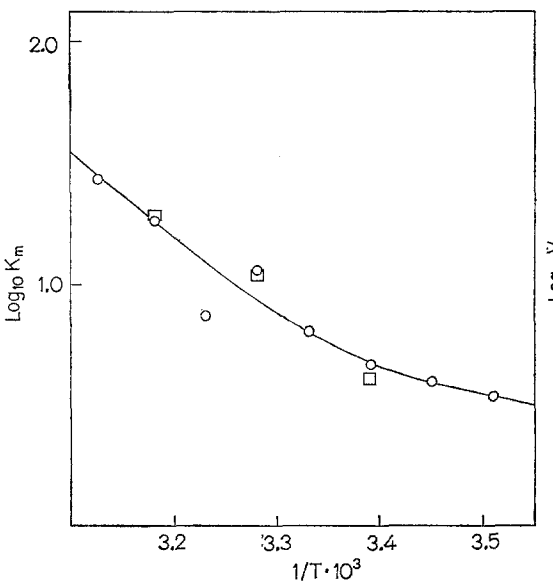

Fig. 1

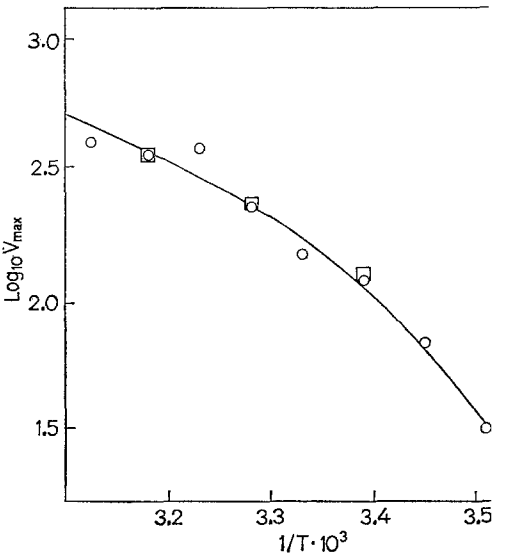

Fig. 2

Fig. 1. $\text{Log}_{10} K_m$ as a function of $1/T$. The experimental values are for cells collected into ACD (○) and into heparin (□). K_m has the units of mM. Each point represents the average from eight experiments

Fig. 2. $\text{Log}_{10} V_{max}$ as a function of $1/T$. The experimental values are for cells collected into ACD (○) and into heparin (□). V_{max} has the units of mM · min^{-1}. Each point represents the average from eight experiments

analysis of kinetic models is based on a consideration of the shapes of the graphs of the logarithms of V_{max} and K_m as a function of reciprocal temperature, both sets of data lead to the same conclusions, despite the differences in the actual values.

It will be observed from Fig. 1 that the strong trend of the results is that the $\log V_{max} - \frac{1}{T}$ plot is convex upwards, whereas the $\log K_m - \frac{1}{T}$ plot is concave upwards. With increasing temperature, therefore, E_{max} decreases, whereas ΔH_m increases. It will be seen that the results for cells collected in heparin are not significantly different from those for cells collected in ACD at the pH of these experiments (pH 7.4), and we accordingly treat all the results together from the point of view of obtaining the temperature coefficients.

The kinetic model I, on which the calculations of V_{max} and K_m (and hence of E_{max} and ΔH_m) were based, supposes four processes – the association and dissociation of glucose with the carrier on either side of the membrane, and the rate-limiting transport of loaded and empty carrier. The association-dissociation processes are taken as identical on both sides of the membrane, and the carrier translocation rate constant is assumed to be the same for the loaded and empty carrier. It is of particular interest to decide whether the variation of E_{max} with T corresponds to a genuine heat capacity of activation, or whether the variation arises from some feature of model I modified in later analyses of the kinetics of the facilitated transport of glucose. If it could be shown that modified kinetic treatments will explain the variation of E_{max} with T in terms of one or more activation energies which do not vary with temperature, then conclusions drawn from calculations of the apparent heat capacity of activation would be rendered suspect. Similarly, the variation of ΔH_m with T should be examined in the light of the modified kinetic models.

Dawson and Widdas (1964) have, in effect, made one such analysis of model I, using the results of Sen and Widdas (1962). In reexamining the arguments of Dawson and Widdas, and in analyzing E_{max} and ΔH_m in terms of the modified model of Wilbrandt (1967), it is assumed at the outset that the activation energies of the individual rate processes in the later models are constant with temperature. If this assumption does not give an account of E_{max} and ΔH_m varying with T, we may conclude that one or more of the individual steps does in fact have a significant heat capacity of activation, and that the measurements of the variation of K_m and V_{max} with temperature do not, at this stage, enable a choice between these models to be made.

Dawson and Widdas (1964) discuss the temperature coefficients of V_{max} and K_m in terms of a relaxation of one of the assumptions of model I. This model requires that dissociation and association of glucose with the carrier be fast processes, and that the translocation be rate-limiting. Under these conditions, K_m is the true Michaelis constant for the glucose-carrier complex, provided that the rate constants of the empty and loaded carrier transport processes are identical. Dawson and Widdas suggest that the assumption of rapid association and dissociation be relaxed, and that consequently the calculated K_m is no longer the true Michaelis constant. Furthermore, V_{max} will then depend not only on the rate constant for translocation (assumed to be the same for empty and loaded carrier), but also on the rate constants for association and dissociation of the complex. Their analysis therefore contains three rate constants corresponding to carrier translocation (a), dissociation (d) and association (b), respectively, using their notations. V_{max} and K_m are then expressed as various combinations of these rates, as required by the different assumptions made about the relative magnitudes of the rate processes. Making the assumption that the activation energies of individual steps are constant, we find that it is not possible to cover the kinetic and temperature results as given by Sen and Widdas (1962) on the arguments of Dawson and Widdas (1964). To take a concrete case, if d is not too different from a, then K_m becomes $(a+d)/b$ and V_{max} becomes $K^1 ad/2(a+d)$, where K^1 is a constant. It is then readily shown that

$$\frac{d \ln K_m}{d \frac{1}{T}} = \frac{d \ln (a+d)}{d \frac{1}{T}} - \frac{d \ln b}{d \frac{1}{T}} \tag{4}$$

and

$$\frac{d \ln V_{max}}{d \frac{1}{T}} = \frac{d \ln a}{d \frac{1}{T}} + \frac{d \ln d}{d \frac{1}{T}} - \frac{d \ln (a+d)}{d \frac{1}{T}}. \tag{5}$$

Since the Sen and Widdas (1962) calculations from experiment for K_m show the left-hand side of Eq. (4) constant, and the third term of the equation is constant by definition, the second term is therefore constant. Thus, in Eq. (5) the left-hand side is also constant, which is contrary to the conclusions of Sen and Widdas (1962). Likewise, the other redefinitions of K_m and V_{max} given by Dawson and Widdas (1964) will not allow E_{max} to vary with T whilst ΔH_m remains constant, unless it is supposed that one or more of the terms like $d \ln a / d \frac{1}{T}$ can vary with T.

Thus, on the basis of the K_m and V_{max} values as given by Sen and Widdas (1962), the Dawson and Widdas (1964) modifications to model I are ruled out. However, if the left-hand side of Eq. (4) is no longer constant (i.e., if ΔH_m varies with temperature), then it is readily seen that the Dawson and Widdas (1964) modifications will predict the form of the results shown in Figs. 1 and 2. Put in another way, the kinetic proposals of Dawson and Widdas are at variance with the values of Sen and Widdas for K_m as a function of T.

We turn next to another refinement of the model I such that the translocation step remains rate-limiting, but the translocation rates of the empty and loaded carrier are assumed to be different. Various forms of this refined model have been analyzed, notably by Wilbrandt (1967). We shall follow the detailed account given by Stein (1967) in which (using his notation) the carrier translocation rate constants are given by k_2 for the loaded carrier, and by k_3 for the empty carrier. For the procedure employed in this paper and in that of Sen and Widdas (1962) for calculating K_m and V_{max} from the experimental data, it follows from the model that

$$K_m = \frac{1+r}{2r} K_s \tag{6}$$

and

$$V_{max} = \frac{2}{1+r} k_2 \,\mathrm{tot}\, E \tag{7}$$

where $r = k_2/k_3$, K_s represents the true Michaelis constant for the complex and tot E represents the total amount of carrier per unit area of membrane. From Eqs. (6) and (7) we obtain

$$\frac{d \ln K_m}{d \frac{1}{T}} = \frac{d \ln K_s}{d \frac{1}{T}} - \frac{1}{1+r} \cdot \frac{d \ln r}{d \frac{1}{T}} \tag{8}$$

and

$$\frac{d \ln V_{max}}{d \frac{1}{T}} = \frac{d \ln k_2}{d \frac{1}{T}} - \frac{r}{1+r} \cdot \frac{d \ln r}{d \frac{1}{T}}. \tag{9}$$

Eqs. (8) and (9) are readily rewritten, on multiplying throughout by R, the gas constant, to give

$$\Delta H_m = \Delta H_s + \frac{1}{1+r}(E_3 - E_2) \tag{10}$$

and

$$E_{max} = E_2 + \frac{r}{1+r}(E_3 - E_2) \tag{11}$$

where ΔH_s is the enthalpy of dissociation of the glucose-carrier complex, and E_2 and E_3 are the activation energies for the translocation of the empty and loaded carriers, respectively.

At physiological temperatures, there is evidence that r has a value of three (Stein, 1967). It follows that, according to the model, the difference between ΔH_m and ΔH_s is smaller at physiological temperatures than the difference between $E_{\max}$ and E_2. Furthermore, if r increases with decreasing temperature, as seems likely from the small amount of evidence available (Stein, 1967), then $(E_3 - E_2)$ is positive. Therefore, $E_{\max}$ should increase in magnitude as the temperature is lowered, in agreement with experiment. At the same time, assuming ΔH_s is constant, the magnitude of ΔH_m will decrease as the temperature is lowered, to an extent equal to the increase in $E_{\max}$. This change of ΔH_m with temperature is found experimentally (*see* Fig. 1). Eqs. (8)–(11) require that the plot of $\log K_m$ against $1/T$ should be as concave upwards as the $\log V_{\max}$ against $1/T$ plot is concave downwards. The results of Figs. 1 and 2 are in qualitative agreement with this requirement. It is interesting to note that in the case of glucose transfer across the membrane of the fetal guinea pig, the plot of $\log K_m$ against $1/T$ is concave upwards whilst the plot of $\log V_{\max}$ against $1/T$ is concave downwards (Dawson & Widdas, 1964). In this case, the concave shapes are not symmetrical with each other, as would be required by Eqs. (8)–(11) above, although it should be noted that the calculation of K_m and $V_{\max}$ in the paper of Dawson and Widdas may be somewhat in error, as pointed out above.

We therefore conclude that the experimental results presented here can be explained simply as arising either from relaxing the condition of fast association and dissociation of the complex, as suggested by Dawson and Widdas (1964), or from postulating that the carrier transfer rate depends on whether it is carrying glucose or not. Correspondingly, it is not necessary to propose that the translocation process is accompanied by a large heat capacity of activation, or that the dissociation process of glucose with the carrier is characterized by a large heat capacity change.

The form of the collected K_m and $V_{\max}$ results as a function of temperature does not, of itself, permit a choice between the modifications proposed by Dawson and Widdas (1964) and those put forward by Wilbrandt (1967). The later kinetic scheme is, however, in accordance with conclusions of Levine, Oxender and Stein (1965), and, despite several difficulties discussed by Miller (1968) and by Levine and Stein (1966), it is regarded as generally acceptable. We may accordingly treat the current experimental results in terms of the later theory, as covered by Eqs. (6)–(11).

The values of ΔH_m and E_{max} which can be derived from the data of Figs. 1 and 2 are not accurate enough to permit a full analysis of the application of Eqs. (10) and (11). We may, however, assume a reasonable value of r, based on the data given by Stein (1967), and make a consistent analysis of the results. When r is chosen as unity at 45 °C, the data lead to the following values of the significant constants in Eqs. (10) and (11):

$$\Delta H_s = 6 \text{ Kcal/mole}; \quad E_3 = 22 \text{ Kcal/mole}; \quad E_2 = 4 \text{ Kcal/mole}.$$

The analysis also gives the reasonable value of 2.5 for r at 37 °C, and a value of 20 at 15 °C.

The thermodynamic quantities quoted above are subject to considerable error but are consistent with the data. The striking result is the very small value of E_2, the activation energy for the translocation of the empty carrier. The value of E_2 is, in fact, comparable with that for ordinary diffusion in water. Since the rates k_2 and k_3 are comparable at physiological temperatures, it appears that the activation of translocation for the loaded carrier is accompanied by a large positive entropy of activation, this being almost 60 cal/deg · mole greater than that for the activation of the translocation of the empty carrier.

For the future, certain testable predictions are readily made from the conclusions given above. Firstly, further estimates of r will confirm or reject the substantial increase in r proposed for low T. Secondly, isotopic exchange experiments between glucose solutions of identical concentration on either side of the membrane should show a large temperature dependence at high sugar concentrations (carrier saturated in both directions), corresponding to an activation energy of about 22 Kcal/mole, invariant with temperature. For low glucose concentrations, on the other hand, the apparent activation energy will be lower, and variable with temperature. Other features of the proposals will also be illuminated by more accurate and reproducible experiments on the transfer rates as a function of temperature in the future. It is possible that direct calorimetric experiments could resolve the most important outstanding issue – the sign of the heat of formation of the glucose-carrier complex. Levine and Stein (1966) confirm that K_m decreases with temperature, but conclude that the formation of the complex is endothermic, whereas our analysis (and that of Sen & Widdas, 1964) requires the formation to be exothermic. This discrepancy poses a serious difficulty for currently accepted kinetic models.

This work was supported by N.A.T.O. Research Grant SA 5-2-O5B (370) 1049 and by Schweiz. Nationalfonds (Project Nr. 4048).

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