THE KINETICS OF SELECTIVE BIOLOGICAL TRANSPORT

I. Determination of Transport Constants for Sugar Movements in Human Erythrocytes

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ABSTRACT A method for the determination of the affinity constants and transfer rate constants of an equalizing selective transport system is derived from the simple transport mechanism, and applied to monosaccharide movements in human red cells. It is similar to the method of Widdas but does not require his approximations. Results are compared with those of the other workers.

A number of monosaccharides are known to penetrate human erythrocyte membranes. The movement of these sugars through the membrane has three important characteristics. Firstly, it usually occurs down a concentration gradient tending to *equalize* the internal and external concentrations. Secondly, the rate of movement is highly dependent on the chemical conformation of the sugar showing that it is a structurally *selective* mechanism. Finally, extensive studies of the kinetics of sugar movement has revealed examples of saturation and competition between sugars suggesting that such movement is not by diffusion of the sugar alone but rather is a result of the presence of a limited number of sites or agents within the membrane whose function is to *transport* the sugars across the membrane. Thus the erythrocyte-sugar system is a typical example of *equalizing selective transport.*

Widdas (1954) has derived an equation providing a very good quantitative description of the kinetics of transport in this system. This expression contains two characterizing constants, one giving the maximum rate of transport, and another the affinity of a given sugar for the transport sites. Two methods have recently been described whereby these constants may be determined. Using an optical method Sen and Widdas (1962*a*) measured the rate of loss of sugar from cells previously loaded with a relatively high concentration of sugar into a solution containing a lower concentration of the same sugar. Their method, however, contains approximations which result in low values, especially for sugars having a low affinity for the

transport system. LeFevre (1962) employing a curve-fitting method has determined the constants for six different sugars and has shown that although the affinity constants vary by as much as 400-fold the maximum rate constant is approximately the same for all sugars as predicted by Widdas' equation. This method is too complex for routine use, however.

The method to be presented here is similar to that of Widdas but does not require his simplifiying approximations.

THEORY

To ensure that all terms are rigorously defined and all assumptions explicitly stated, expressions will be derived from first principles.

Notation Erythrocytes do not possess a restricting cell wall and thus change volume with changes in internal or external osmotic pressure. For the present purposes however, all quantities will be expressed in terms of a "cell unit," defined as the number of cells whose isotonic solvent water volume is one litre (Miller, 1964).

The following notation will be employed:

x the amount of substrate or permeating species (number of osmols)
C internal substrate concentration (osmols/litre)
e the amount of non-penetrating species within a cell unit (number of osmols)
V the solvent water volume per cell unit (litres)
$\bar{C}$ external substrate concentration (osmols/litre)
E external osmolarity of non-penetrating species (osmols/litre)
K affinity (half saturation) constant (osmols/litre)
t time (minutes)

Assumptions The following assumptions are employed in subsequent derivations:

(*a*) Penetration of the cell membrane by the substrate, x, is achieved through the mediation of an agent or carrier, P, present only in the membrane phase, with which the substrate reversibly forms a complex, P_x. Only P and P_x move through the membrane so that the mechanism of permeation may be represented by the following schematic model:

Outer solution	—	x
		I ⇵ II
Outer surface	—	$x + P \rightleftharpoons P_x$
Membrane phase	—	⇵ III ⇵
Inner surface	—	$x + P \rightleftharpoons P_x$
		I ⇵ II
Inner solution	—	x

(*b*) The rate limiting process is considered to be III, the movement of P_x through the membrane, since it is much slower than either the diffusion of x to and from the membrane surface, I, or the formation and dissociation of the complex, II. This

assumption allows us to equate the concentration (or more strictly the activity) of x at each membrane surface to that in the bulk of the corresponding solution. Furthermore, we may define the concentrations of carrier and complex (denoted by $[\bar{P}]$ and $[\bar{P}_x]$ at the outer surface and, $[P]$ and $[P_x]$ at the inner surface) by means of the mass law expressions.

$$K = \frac{\bar{C}[\bar{P}]}{[\bar{P}_x]} = \frac{C[P]}{[P_x]} \tag{1}$$

(*c*) As noted in expression (1), K is the same on both sides of the membrane.

(*d*) The rates of movement of the free carrier inward, the free carrier outward, the complex inward, and the complex outward are all equal so that the total concentration, T, of carrier both free and complexed is constant throughout the mebrane or

$$T = [\bar{P}] + [\bar{P}_x] = [P] + [P_x] \tag{2}$$

Derivations Wilbrandt and Rosenberg (1961) have pointed out that the fundamental equation of transport is:

$$F_x = D([\bar{P}_x] - [P_x]) \tag{3}$$

where F_x is the flux of permeant into the cells in moles/cell unit and D is a constant for a given carrier system independent of the substrate. (Note that F_x is positive for inward flux and negative for outward flux). Furthermore, by definition of terms

$$dx/dt = F_x \tag{4}$$

so that by combining expressions (1) to (4) and letting $DT = k$ we obtain

$$\frac{dx}{dt} = k\left(\frac{\bar{C}}{K + \bar{C}} - \frac{C}{K + C}\right) = \frac{k\,K(\bar{C} - C)}{(K + \bar{C})(K + C)} \tag{5}$$

To integrate expression (5) we must make the substitution $C = x/V$. This cannot be done immediately, however, since V is dependent on C, due to the osmotic behaviour of the cell, and we must first express V in terms of C. Water is known to penetrate erythrocytes much more rapidly than sugars so that osmotic equilibrium may be assumed to obtain at all times. This means water will move in such a direction as to balance the osmotic pressure on both sides of the membrane by altering the concentration of osmotically active species within the cell. Thus

$$E + \bar{C} = \frac{e + x}{V} = \frac{e}{V} + C$$

and

$$V = \frac{e + x}{E + \bar{C}} = \frac{e}{E + \bar{C} - C} \tag{6}$$

With this expression, expression (5) may be integrated within the limits $x = x_o$

when $t = 0$, to give an equation similar to that of Sen and Widdas (1962*a*) and Harris (1964), but applicable to any tonicity:

$$\frac{E^2 k K t}{K + \bar{C}} = e(K + \bar{C})(E + \bar{C}) \ln \frac{(e\bar{C} - Ex_0)}{(e\bar{C} - Ex)} + E(E + K + \bar{C})(x_0 - x) \quad (7)$$

Since V is a litre when $C = \bar{C} = 0$ and $E = 310$ mOsM (*i.e.*, isotonic with serum), then e must be 310 mOsM. If we define this quantity as an isotone and express all other concentrations in the same units (*i.e.*, milliosmolarity/310 mOsM) then $e = 1$.

Widdas' method consists in measuring the change in x when x_0 is large and $\bar{C}$ is small. Under these circumstances x is found to drop linearly with the time during the first stages of the efflux as shown by the plot of equation (7) in Fig. 1*A*. Further-

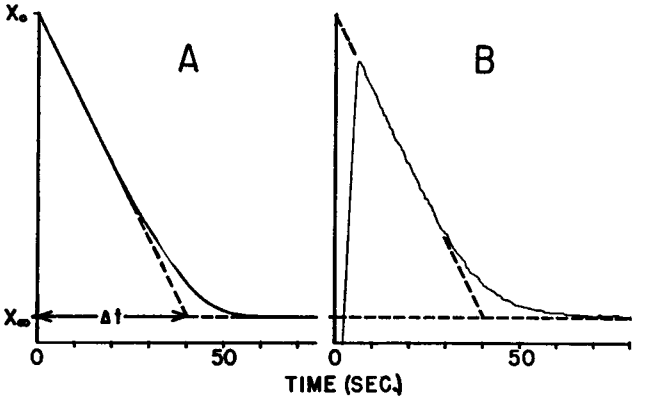

FIGURE 1 *A. Solid line* is the plot of x against t according to equation (7) with $E = 1$ isotone/litre, $k = 2.0$ isotone/minute, $K = 0.018$ isotone/litre, $x_0 = 0.410$ isotone/litre, and $\bar{C} = 0.0323$ isotone/litre. *B. Solid line* is the actual recording of light transmission through a suspension of cells loaded with glucose at a concentration of 0.410 isotone/litre (127.6 mM) and suspended in an isotonic medium containing added glucose to a concentration of 0.0323 isotone/litre (10 mM) at 37°C.

more, the slope of this portion should be equal to the initial rate of loss of sugar (*i.e.*, when $C = C_0$) or according to expression (5)

$$\left(\frac{dx}{dt}\right)_{t=0} = \frac{k K(\bar{C} - C_0)}{(K + C_0)(K + \bar{C})} \quad (8)$$

In order to raise the internal sugar concentration to C_0, the cells are first suspended in a sugar solution of concentration $\bar{C}_s$ and time allowed for the internal concentration to reach $\bar{C}_s$ giving an internal sugar content $x_0 = \bar{C}_s V = \bar{C}_s/E$ [see equation (6)]. At the start of the run the external sugar concentration is reduced to the required value $\bar{C}$. The cell immediately swells to give the initial internal concentration

$$C_0 = \left(\frac{E + \bar{C}}{E + \bar{C}_s}\right)\bar{C}_s \quad (9)$$

The sugar content then proceeds to drop as in Fig. 1 until the concentration reaches the external value $\bar{C}$ (at $t = \infty$) at which time the sugar content is $x_\infty = \bar{C}/E$. If the straight portion of the curve is extrapolated to the x_∞ line as in Fig. 1, then the time coordinate at the point of intersection will be the time required for the exit of $(\bar{C}_\bullet - \bar{C})/E$ isotones of sugar at the initial rate. Therefore,

$$-\left(\frac{dx}{dt}\right)_{t_0} = \frac{\bar{C}_\bullet - \bar{C}}{E\Delta t} = \frac{k\,K(C_0 - \bar{C})}{(K + C_0)(K + \bar{C})} \tag{10}$$

This equation only applies if $\bar{C}$ is a constant, which is virtually the case if the cell volume is small compared to the volume of suspending medium. For the case where $\bar{C} = 0$, the time interval is Δt_0 and equation (10) reduces to

$$\frac{\bar{C}_\bullet^{\,0}}{E\Delta t_0} = \frac{kC_0}{K + C_0} \tag{11}$$

These latter two expressions may be combined, provided C_0 is constant for all values of $\bar{C}$. To achieve this condition, the cells are equilibrated with the concentration as determined by expression (12) [derived from equation (9)].

$$\bar{C}_\bullet = \frac{EC_0}{E + \bar{C} - C_0} \tag{12}$$

Under these conditions we may divide equation (11) by equation (10) to give

$$A\Delta t = \frac{\Delta t_0}{K}\bar{C} + \Delta t_0 \tag{13}$$

where

$$A = \frac{C_0 - \bar{C}}{C_0}\cdot\frac{\bar{C}_\bullet^{\,0}}{C_\bullet - \bar{C}} = \frac{E(E + \bar{C} - \bar{C}_0)}{(E - C_0)(E + \bar{C})}$$

Thus by plotting $A\Delta t$ against $\bar{C}$ a straight line is obtained (Fig. 2) whose slope is $\Delta t_0/K$ and whose intercept is Δt_0. Furthermore, by rearranging equation (11) with $\bar{C}_\bullet^{\,0} = EC_0/(E - C_0)$ we obtain

$$k = \frac{1}{\Delta t_0}\left(\frac{K + C_0}{E - C_0}\right) \tag{14}$$

so that both k and K may be determined from such a plot.

The amount of sugar lost is less for greater values of $\bar{C}$, which means that Δt actually measured refers to the loss of a different quantity of sugar in each case. Multiplying by the factor A, however, increases each Δt to that value which it would have had, were the amount of sugar lost the same as during a measurement with $\bar{C} = 0$. Thus all $A\Delta t$ values refer to the same total amount of sugar transported.

EXPERIMENTAL

Blood was obtained fresh from the Canadian Red Cross Blood Bank and stored at

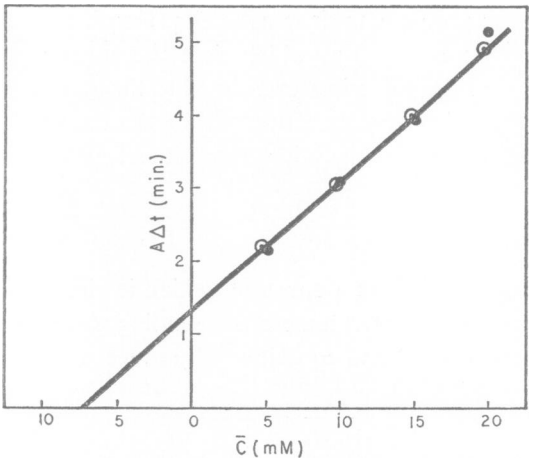

FIGURE 2 Plot of $A\Delta t$ *vs.* $\bar{C}$ for mannose at 20°C: ⊙ by the isotope method; ● by the light scattering method. Line drawn in accordance with a least-squares analysis of results from the isotope method.

2-4°C when not used the same day. The cells were removed by centrifugation and washed with the tris buffer solution described by LeFevre and McGinniss (1960). This solution, which is virtually isotonic, was used throughout all the experiments described here.

In determining the constants for a given sugar 4 to 5 different values for $\bar{C}$ were decided on, ranging around the expected value of K. This should give maximum accuracy to the determination according to LeFevre (1962). A value for C_0 well in excess of these concentrations was chosen and $\bar{C}_s$ determined in each case. Cells were then soaked in each of the appropriate loading solutions containing sugar at the predetermined value of $\bar{C}_s$ and transferred to the experimental solution containing sugar at concentration $\bar{C}$ in which the rate of exit was measured by one of the two methods to be described below. The values of Δt multiplied by their corresponding A values were subjected to a least-squares analysis yielding the values of intercept and slope required for the k and K determinations.

The two methods used to measure rates of exit were:

(*a*) *The light scattering method* This method is essentially the same as that used by Sen and Widdas (1962). The intensity of light shining through a thermostated, stirred suspension of cells was measured photometrically and the change with time recorded. As the amount of light scattered by the cells is proportional to their volume, which in turn is proportional to their sugar content, x may be taken as a direct function of light intensity. The procedure for a single Δt determination was as follows: Cells were soaked in a relatively large volume of loading solution at 37°C until equilibrium was reached, centrifuged, made up to about 30 per cent by volume

in fresh loading solution, and brought to the temperature at which the measurement was to be made. About 0.5 ml of this solution was added rapidly to about 70 ml of the experimental solution, giving an approximately 0.2 per cent cell suspension, and changes in light transmittance measured with time. The straight portion at the beginning of the curve was extended to the x_∞ line as described above, and Δt measured.

(*b*) *The isotope method* Cells (0.2 ml) equilibrated in the loading solution were centrifuged and made up to a predetermined volume with a solution at the same sugar concentration but containing a small amount (about 0.5 to 3 μc) of radioactive sugar and again allowed to come to equilibrium. At zero time an amount of buffer (approximately 10 ml) was added so as to dilute the sugar outside the cell to the appropriate concentration, $\bar{C}$. (Since isotopic equilibrium was attained before dilution, no exchange diffusion should occur.) Samples (1 ml) were taken at various time intervals following dilution, added to 10 ml of cold $HgCl_2$-NaCl solution (which quenches sugar movements) described by LeFevre (1962), and centrifuged at 0°C in a Hopkins vaccine tube. After removal of the supernatant, the tube was rinsed with water above the cells without disturbing the packed cells themselves. NH_4OH(3M) was added to dissolve the cell membrane and to dilute the solution to about 0.1 ml. This solution was transferred by means of a Hamilton syringe to a 1-inch filter paper placed on a planchette where it spread out evenly and was dried at room temperature in air. The radioacitivity of these samples were determined by means of an end window counter.

About six samples were taken during the first half of the sugar loss and two more at a time sufficient to give the final equilibrium value. These last two values were averaged and subtracted from the others which were then plotted against time. This process served to correct for background and any radioactive supernatant carried over with the cells. Those points forming a straight line were subjected to a least squares analysis giving the intercept on the time axis, t.

RESULTS

Determination of constants for glucose, mannose, and galactose were made at 37°C by the light scattering method and for mannose and galactose at 20°C by both methods. A typical plot of $A\Delta t$ against $\bar{C}$ is given in Fig. 2 for data derived from mannose experiments by the isotope method, with results from the light scattering method included for comparison. Each point on the graph is an average of at least four determinations. Table I summarizes results obtained by both methods.

DISCUSSION

The method of Sen and Widdas (1952*a*) requires that $\bar{C}$ and K be small relative to unity. The present method does not require these conditions and is therefore applicable to a wider range of sugars. According to theory, the approximate method should give lower values of K than the present method so that taking this into account, the

TABLE I

CONSTANTS (WITH STANDARD DEVIATIONS) DETERMINED FOR SUGAR TRANSPORT IN HUMAN ERYTHROCYTES BY TWO METHODS

Temperature	Sugar	K Isotones/litre		k Isotones/minute		Method*
°C						
37	Glucose	0.018 ± 0.003		1.9 ± 0.3		L
	Mannose	0.040 ± 0.003		2.2 ± 0.2	(2.1 ± 0.2)‡	L
	Galactose	0.064 ± 0.009		2.1 ± 0.1		L
20	Mannose	0.019 ± 0.003	(0.021 ± 0.002)‡	0.40 ± 0.05		L
	Mannose	0.023 ± 0.003		0.35 ± 0.01	(0.37 ± 0.04)‡	I
	Galactose	0.040 ± 0.004	(0.037 ± 0.006)‡	0.35 ± 0.03		L
	Galactose	0.034 ± 0.008		0.37 ± 0.06		I

* L is light scattering, I is isotope.
‡ Pooled estimates.

results for glucose and mannose in Table I agree reasonably well with those of 0.012 and 0.042 isotones/minute respectively as found at 37°C by Sen and Widdas (1952*a* and *b*). There is also fair agreement with LeFevre's value of K for mannose, but in the case of galactose, the present value is less than half his.

According to the present theory, k is assumed to be the same for all sugars carried by the same transport system. LeFevre (1962) has shown this to be true for six different sugars having a wide range of K values, for which he obtained a k of approximately 2 isotones minute^{-1} at 37°C. The same results were obtained in the present work for the three sugars tested. Sen and Widdas, however, obtained a value of only one isotone minute^{-1} for both mannose and glucose at this temperature. At 20°C Sen and Widdas (1962*a*) obtained a value of 0.25 isotones minute^{-1} and Harris (1964) a value of 0.67 isotones minute^{-1} for glucose, neither of which agree too well with the value listed in Table I. As will be shown in the next paper in this series however, the results obtained in the present work are consistent with quantitative data on induced uphill transport for mannose and galactose at 20°C.

Finally it should be noted that the results obtained by both the light scattering and the isotope methods are in good agreement for measurements made on both mannose and galactose (Fig. 2 and Table I), thus increasing the confidence to be placed in either method for the measurement of net sugar movements.

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