*J. Physiol.* (1962), **160**, *pp.* 392–403
*With 6 text-figures*
*Printed in Great Britain*

# DETERMINATION OF THE TEMPERATURE AND pH DEPENDENCE OF GLUCOSE TRANSFER ACROSS THE HUMAN ERYTHROCYTE MEMBRANE MEASURED BY GLUCOSE EXIT

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(*Received* 12 *June* 1961)

Glucose is generally considered to be transferred across the erythrocyte membrane by facilitated diffusion (Lefevre, 1948; Widdas, 1954; Wilbrandt, 1954). There is strong evidence that an essential step in the process is a reaction between glucose and a component of the membrane to form a complex. The process of transfer is presumed to depend on the amount of this complex, but the exact sequence of molecular reactions is still unknown.

From a practical point of view the mechanism can be characterized by two parameters: (i) the capacity for transfer if the membrane component is fully saturated with glucose, and (ii) the affinity of the component for glucose. The former parameter is analogous to the $V_{max.}$ for an enzymic reaction and the latter to the Michaelis constant for the enzyme-substrate reaction. Movement through the membrane and other factors may influence the apparent affinity and formal identification with the Michaelis constant is unjustified; the term half-saturation constant is therefore preferable.

Attempts to determine the half-saturation constant were made by Widdas (1953, 1954) using sorbose–glucose competition and by Lefevre (1954) using competition between phloretin and glucose. The values obtained were in the region of 10 mM. In rabbit red cells, where the transfer is much slower, Morgan, Kalman, Post & Park (1955) used more direct measurements and obtained a value of 4 mM. In human red cells Reinwein, Kalman & Park (1957) reported a value of 5 mM, but the method was not published.

In the present work the problem has been tackled by observing the exit of glucose from cells previously equilibrated with glucose into a number of saline media containing very low but different concentrations of glucose. The advantages of this method are, first, that the cells are suspended in so large a volume of saline that glucose lost from the cells during an experiment does not sensibly alter the outside concentration; secondly, the exit

process is rapid and measurable at very low temperatures where determinations based on entry are technically impossible; thirdly, only glucose is used in the experiments and consequently assumptions as to competitive mechanisms are not involved.

This method of study has been used to investigate the influence of pH and temperature on the two parameters of transfer. A preliminary account was presented to the Physiological Society (Sen & Widdas, 1960).

## METHODS

All the experiments were carried out on human erythrocytes. Blood was obtained by venepuncture and dry heparin was used to prevent clotting. The cells were washed three times in saline buffered with phosphate to the required pH. The saline contained 0·8 % NaCl but the addition of sodium phosphate increased the tonicity to that of 1 % NaCl (342 milliosmolar).

Washed red cells were resuspended in a saline medium containing glucose to give a concentration of 76 mM, and after incubation for 30–60 min at 37° C the cells were packed by centrifugation for 4 min. Tests of 'exits' were carried out as follows:

3 $mm^3$ of packed cells were taken up in a micropipette and transferred to 0·2 ml. of the sugar-containing medium in a small beaker supported at an angle of about 30°. The cells were uniformly suspended by drawing up the suspension and discharging it into the beaker several times before finally pipetting the suspension into the cuvette.

The cuvette contained 21 ml. of saline buffer which was either glucose-free or contained low concentrations of glucose. The contents were continually stirred to prevent sedimentation of the cells and the cuvette was surrounded by a water jacket for temperature control.

Volume changes were followed by a photo-electric apparatus previously described (Widdas, 1953). This employed a chopped double light-beam system with an a.c. amplifier and phase-sensitive rectifier. The output operated a pen recorder, and over the limited range of cell volume changes (maximally 23 %) the excursion of the pen recorder was proportional to the volume change.

On adding the cell suspension to the saline in the cuvette it was usually necessary to make a rapid initial adjustment of the shutter in the reference light beam to compensate for small variations in the density of the suspensions; thereafter the cell volume changes were traced by the pen recorder. General procedures have been described by Widdas (1954) and Bowyer & Widdas (1958). In the present work de-ionized distilled water was used in making up the solutions.

## RESULTS

### *Analysis of results*

A tracing of records of a typical 'exit' experiment is shown in Fig. 1. The volume changes, which are proportional to the pen deflexion, show considerable initial portions which are linear with time in all the records. This is characteristic of exit records and contrasts with those from entry experiments. The linearity can readily be explained on the basis of a nearly complete saturation of the sites or carriers on the inside of the cell membrane and a low saturation of those on the outside. This low outside saturation is maintained because the outside concentration is not sensibly

changed by glucose lost from the cells. So long as the inside of the membrane is nearly saturated the process is proceeding at a constant and (at low outside concentrations) nearly maximal rate. When the inside glucose concentration falls, so that saturation is reduced, the linear part of the record is not maintained. It curves towards equilibrium asymptotically.

Where the outside concentration is higher the exit is still linear over most of its course, but the rate of volume change is less. This is interpreted as being due to the efflux of glucose being opposed by an increased rate of

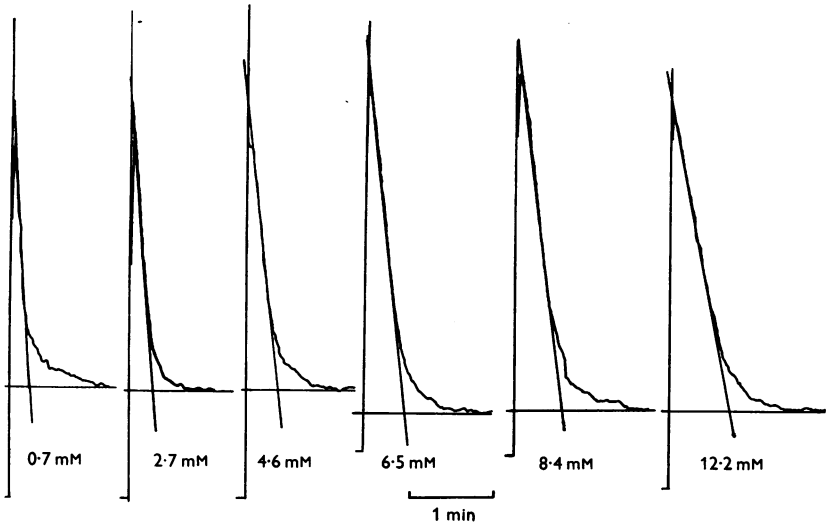


Fig. 1. Tracings of a series of records from the photo-electric apparatus during 'exit' experiments at 37° C and pH 7·4. Cells equilibrated in 76 mM glucose were losing glucose into media containing glucose at the concentrations shown. The linear part of each record has been produced to cut the base line, and the time from injection of the cells to this intersection was measured for analysis of the results.

influx. In principle the maximal rate of exit (i.e. that into a glucose-free medium) would correspond to the rate of glucose efflux and a rate of exit exactly half maximal would correspond to the same efflux opposed by a half-maximal influx. The concentration of glucose in the outside medium which satisfied this condition would be the half-saturation concentration.

In practice 3 $mm^3$ of packed cells previously equilibrated with 76 mM glucose were resuspended in 0·2 ml. glucose saline before injecting into 21 ml. of solution in the cuvette; thus the minimal concentration of glucose outside was that solely due to the dilution of the cells and their suspending medium by 21 ml. glucose-free saline. This concentration was 0·72 mM and

was largely due to the medium. Glucose lost from the cells could only change the value by 0·01 mM.

The total cell volume change was slightly less when the outside concentration was raised. This was because there was not so much osmotic swelling and the cells attained equilibrium before losing so much glucose. To minimize the influence of such factors in the analysis of the results, the linear part of each exit record was produced to cut the base line (representing the final equilibrium volume) and the time from injection of the cells to this point of intersection was measured. The times for the various experiments were then plotted against the outside concentrations as shown in Fig. 2. The theoretical justification of this simple treatment follows.

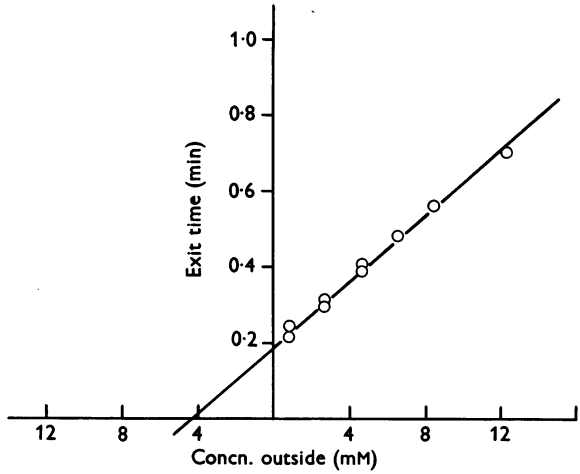


Fig. 2. 'Exit' times obtained from the records described in Fig. 1 plotted against the concentration of glucose in the outside media. The line (drawn by eye) gives two intercepts; that on the ordinate represents the time ($t_0$) which would have been taken for exit into a glucose-free medium and that on the abscissa gives the concentration of glucose into which the exit time would be twice $t_0$.

### *Theoretical treatment*

It was assumed that hexose transfer depended on glucose concentrations according to the following equation (Widdas, 1954)

$$\frac{dS}{dt} = K\left\{\frac{C}{C+\phi_g} - \frac{S/V}{(S/V)+\phi_g}\right\}, \qquad (1)$$

where $C$ is the concentration of glucose in the outside medium,
$S$ is the amount of sugar inside the cell,
$V$ is the volume of the cell,
$\phi_g$ is the half-saturation concentration, and
$K$ is a constant.

Isotonic units are used; concentrations and volumes are expressed in fractions of the isotonic concentration and isotonic volume respectively. The amount of sugar ($S$) is expressed as a fraction of that amount which would be contained in an isotonic cell volume ($V = 1$) of solution at isotonic concentration.

When $C$ and $\phi_g$ are small the integrated equation for exit takes the form

$$(C+\phi_g)(S_i-S)+(C+\phi_g)^2 \ln \frac{S_i-C}{S-C} = \phi_g K t, \tag{2}$$

where $S_i$ is the amount of glucose in the cells at the beginning of the exit experiment, and $S$ is the amount in the cells at any time $t$.

Since the record of an exit is apparently linear for at least two-thirds of the volume change, the magnitude of $(S_i-C)/(S-C)$ will, at that point, be about 3 and $\ln (S_i-C)/(S-C)$ will be close to unity. If this value is inserted as the contribution of the logarithmic term to the time measured (by producing the linear part of the record to the base line) we have

$$(C+\phi_g)(S_i-S+C+\phi_g) = \phi K t. \tag{3}$$

When the sugar concentration is the same inside and outside the cell, $S = CV$, but, at final equilibrium, the cell volume returns to its isotonic volume ($V = 1$) and so $S = C$. Using this relation, we get, by rearranging,

$$t = \frac{(S_i+\phi_g)}{\phi_g K}(C+\phi_g). \tag{4}$$

This suggests that the time as measured should be a linear function of $C$ (so long as $C$ is small) and should give an intercept $t_0$ when $C = 0$. Further the concentration which gives a value of $t = 2t_0$ must equal $\phi_g$, the half-saturation constant. From geometry this is numerically the same as the intercept of the line on the abscissa.

The errors introduced by the approximations in this treatment are estimated at less than 5% and are small relative to uncertainties in the technique, due, for example, to possible mixing delays. The errors of this simple method of analysis are less than those based on a more laborious technique of measuring initial rates of volume change during the linear part of the records without regard to the logarithmic contribution. The plot of measured times against outside concentration, shown in Fig. 2, is reasonably linear, as predicted by the above treatment, and the two intercepts are used to obtain the two parameters required. The intercept with the ordinate gives the time $t_0$ (0·2 min) and that with the abscissa (4 mM) gives the half-saturation concentration. The constant $K$ is proportional to the reciprocal of $t_0$ and can be estimated therefrom.

### *Effect of pH*

The effect of pH was studied at 37° C between the limits of pH 5·4 and pH 8·4. The effect on the half-saturation constant ($\phi_g$) and maximal transfer ($K$) are shown in Fig. 3. The effect on $\phi_g$ was not large but was statistically significant ($P < 0{\cdot}05$), but the change in the maximal transfer rate was greater ($P < 0{\cdot}001$) and could be represented by the regression equation

$$\text{Maximal rate } (K) = 0{\cdot}17\ (\text{pH} - 0{\cdot}87) \text{ isotonic units/min.}$$

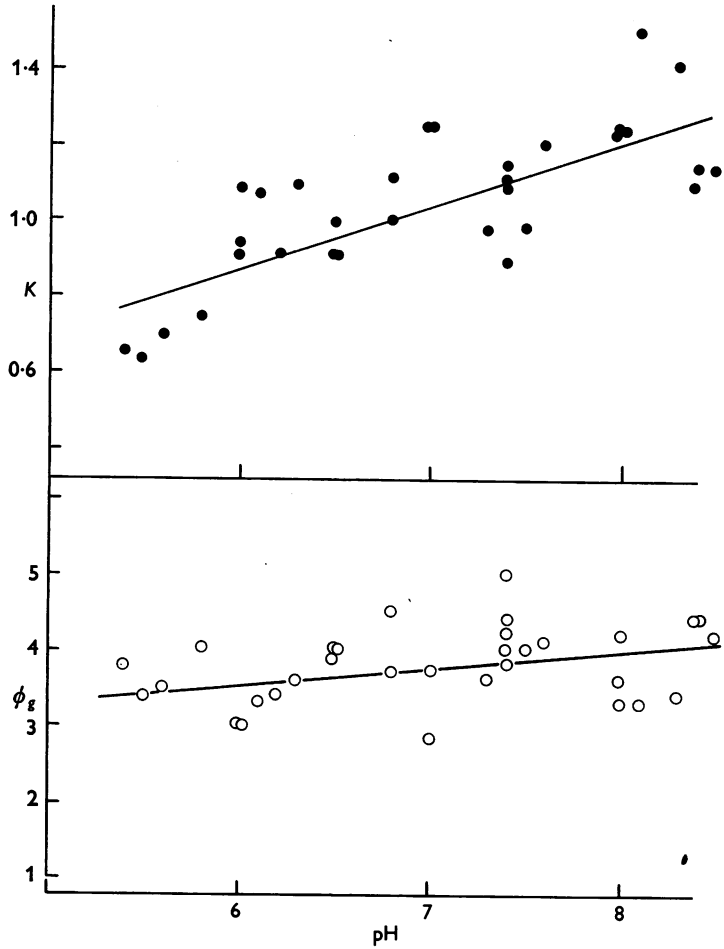


Fig. 3. The effect of pH on the two parameters of hexose transfer at 37° C. The variation of $K$ (●) is greatest and the line is drawn to the regression equation given in the text.

In the range studied there were no discontinuities or maxima which might have been expected if the transfer system was closely dependent upon ionizable groups with pK values in this range.

*Effect of temperature*

The effect of temperature was studied at pH 7·4 and covered the range 7–47° C. The half-saturation constant ($\phi_g$) was found to decrease at low temperatures and varied from 0·58 ± 0·06 mM at 7° C to 6·3 ± 0·35 mM at 47° C, that is, by a factor of 10. Over the same range the maximal rate of

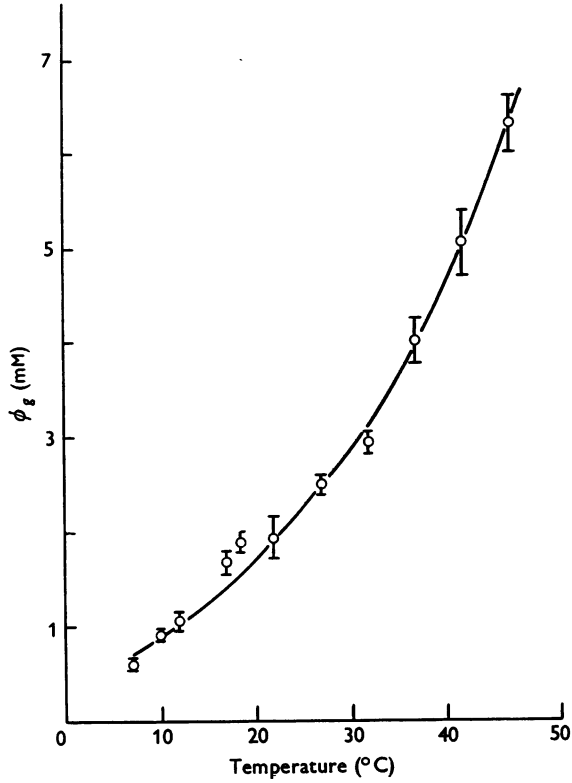


Fig. 4. The effect of temperature on the half-saturation constant at pH 7·4. Vertical lines indicate standard errors of means.

transfer ($K$) varied from 0·048 ± 0·005 to 1·87 ± 0·07 isotonic units/min, a factor of 39. The effect of temperature is shown graphically in Figs. 4 and 5 and it will be seen that neither of the parameters varied linearly with temperature.

Since the half-saturation concentration is nearly analogous to the

Michaelis constant of an enzyme reaction, it was decided to see if a plot of the logarithm of this constant against the reciprocal of the absolute temperature was linear. The result is shown in Fig. 6.

Such plots, first suggested by Van't Hoff, have been extensively used in enzyme studies (Dixon & Webb, 1958). The results plotted in this way are

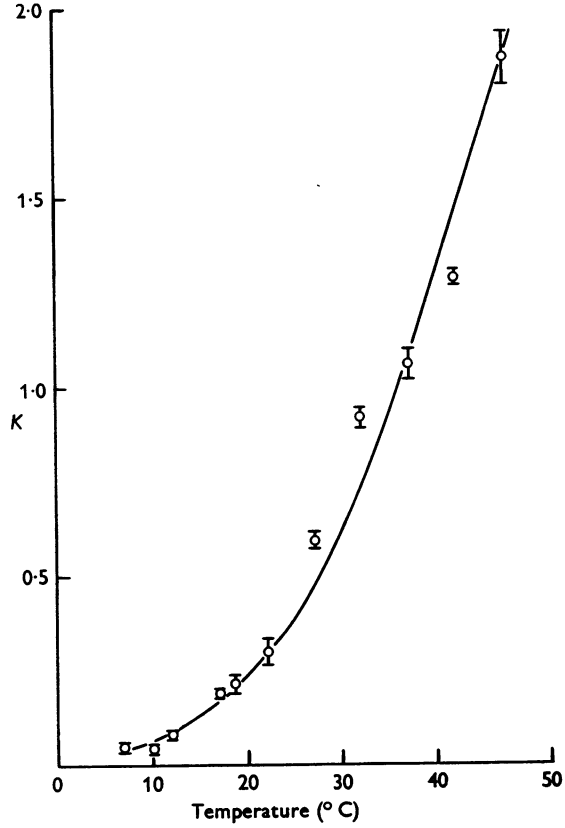


Fig. 5. The effect of temperature on the maximal transfer rate at pH 7·4. Vertical lines indicate standard errors of means.

reasonably linear, and from the slope of the line it is possible to calculate that the energy of dissociation of the complex, which glucose is postulated to form with the membrane component, is about 10,000 cal/mole.

On the other hand, a similar plot of the values for the maximal transfer rate was not linear but was convex upwards.

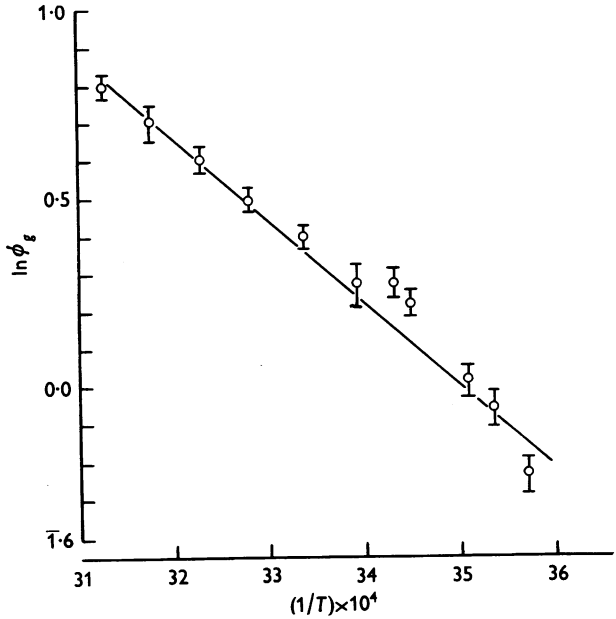


Fig. 6. The Van't Hoff plot of data for the half-saturation constant. The slope of the continuous line indicates that an energy of 10,000 cal/mole is required for dissociation of the complex which glucose is postulated to form.

## DISCUSSION

Three aspects of these results warrant further comment: (*a*) the value for the half-saturation constant at 37° C, (*b*) its dependence on temperature and pH, and (*c*) the dependence on temperature and pH of the maximal transfer rate.

### *Half-saturation constant at* 37° C

The value of $\phi_g$ (4 mM) at 37° C is lower by a factor of two when compared with results obtained by sorbose–glucose competition (Widdas, 1954); it is also lower by nearly the same factor than the value of 7 mM obtained by Lefevre (1954) using competition between phloretin and glucose. One of the disadvantages of the sorbose method was the long time course of the experiments. In the absence of glucose, sorbose entry into red cells would (at the concentrations used) take 10 min, and when inhibited by glucose in the medium may take up to 100 min. Over such long time courses the cell volumes may not remain stable and this could introduce an error into the evaluation. The long time courses prohibit the use of this method at low temperatures. A further consideration is that the penetration of sorbose could induce a loss of glucose by a process of uphill

transport by counter-flow, so that the reduced glucose concentration inside would thus exert a lower inhibitory effect. Approximations in the kinetic treatment of competitive experiments, which are discussed in a further paper, may have introduced errors (Sen & Widdas, 1962).

In the simple treatment the possibility of an exchange reaction when a competitive sugar molecule collides with a carrier or site already complexed to another sugar molecule is not provided for. There are grounds for entertaining this possibility, since isotopic estimations of the maximal transfer rate tend to be greater than those based on net transfer measurements (Britton, 1956; Lefevre & McGuinniss, 1960).

The same exit techniques as described in this paper have been employed in the presence of various inhibitors, and these and other experiments to be reported support the conclusion that the half-saturation constant is of the order of 4 mM at 37° C (Sen & Widdas, 1962).

This value fits in well with the value obtained by Morgan *et al.* (1955) for rabbit erythrocytes, where, because of the greatly reduced rates, the determination was made by more direct means. A further lowering of the value cannot be completely ruled out on the present results, as there may be a small systematic error from mixing delays. Again, information on inhibited human cells (Sen & Widdas, 1962) would suggest that any lowering of the value from this cause will not be more than 0·5 mM. The value of 4 mM corresponds to 72 mg/100 ml., and if this is common to several *in vivo* sites it is clear that, at normal levels of blood sugar, the glucose transfer systems on the outside of cells will be working at and above half-saturation.

It is worthy of note that the normal physiological range for the functioning of haemoglobin as an oxygen carrier is at and above half-saturation, where the shape of the dissociation curve adds to the efficiency of the system. It can be shown that a sugar transfer system following the kinetics described in this and earlier papers would work optimally if $\phi_g = \sqrt{(C_1 C_2)}$, where $C_1$ and $C_2$ are the concentrations at the two sides of the cell membrane. This means that for the optimal working of such a system the half-saturation constant should be less than the higher concentration and consequently, at the side in equilibrium with the higher concentration, the system should be more than half-saturated. On these criteria the value of 4 mM cannot be far from optimal.

*Dependence of the half-saturation constant on temperature and pH*

The pH dependence calls for comment only because of the absence of any change which might suggest an ionizable group. The temperature effect is in a direction which suggests that the glucose complex forms spontaneously and readily even at low temperatures and that breaking

the complex is the reaction which requires energy. The value of this energy of dissociation (10 kcal/mole) would suggest that several hydrogen bonds may be involved in complexing the glucose.

*The maximal transfer rate*

The maximal transfer rate as obtained in these experiments has a $Q_{10}$ between 37 and 27° C of 1·8, but, since an entry or exit experiment takes a time $\alpha(1/K\phi_g)$ (see eqn. 2) and since $\phi_g$ has a $Q_{10}$ of 1·6, the over-all $Q_{10}$ is 2·9, which agrees well with the value of 2·8 reported by Faust & Parpart (1961). The dependence on pH, however, had no maximum at pH 7 as reported by Faust (1960). The greater pH effect on this constant may indicate that it is a non-specific effect on the membrane and this would be supported by the observation by Faust & Parpart (1961) of a similar effect on amino-acid transfer. Treating the maximal transfer rate as if it were the velocity of a chemical reaction, the energy of activation was not constant with temperature but was of the order of 20 kcal/mole at 20° C and 7–10 kcal/mole at 37° C. This non-linearity of the Van't Hoff plot has been interpreted by Stearn (1949) as indicating different rate-limiting steps at different temperatures. Thus it could be that mobility of the carrier complexes in the membrane was rate-limiting at low temperatures, but some other process (possibly the break-down of complexes) becomes rate-limiting at higher temperatures. A full interpretation of these effects must await more definite information as to the underlying mechanisms of transfer and also the state of the cell membranes over this temperature range.

SUMMARY

1. A method for determining the parameters of glucose transfer in red cells is described. This is based on following the rates of loss of glucose from equilibrated cells into saline media of very low glucose concentrations.

2. By this method the half-saturation constant for glucose for human erythrocytes was found to be $4{\cdot}0 \pm 0{\cdot}24$ mM at 37° C, pH 7·4. The value was found to decrease at lower temperatures and varied from $0{\cdot}58 \pm 0{\cdot}06$ mM at 7° C to $6{\cdot}3 \pm 0{\cdot}35$ mM at 47° C.

3. From the temperature dependence it was calculated that 10,000 cal/mole are required for the dissociation of the complex which glucose is presumed to form with a component of the membrane.

4. The maximal transfer rate of glucose varied by a factor of 39 over the temperature range 7–47° C but only had a $Q_{10}$ of 1·8 between 37 and 27° C. It had a greater $Q_{10}$ as the mean temperature of the interval was lowered.

## REFERENCES


BOWYER, F. & WIDDAS, W. F. (1958). The action of inhibitors on the facilitated hexose transfer system of erythrocytes. *J. Physiol.* **141**, 219–232.

BRITTON, H. G. (1956). The permeability of the human red cell to labelled glucose. *J. Physiol.* **135**, 61–62*P*.

DIXON, M. & WEBB, E. C. (1958). *Enzymes*, pp. 8–34, 62–181. London: Longmans Green and Co.

FAUST, R. G. (1960). Monosaccharide penetration into human red blood cells by an altered diffusion mechanism. *J. cell. comp. Physiol.* **56**, 103–122.

FAUST, R. G. & PARPART, A. K. (1961). Permeability studies on the red blood cell of the ground-hog, *Marmota monax*. *J. cell. comp. Physiol.* **57**, 1–3.

LEFEVRE, P. G. (1948). Evidence of active transfers of certain non-electrolytes across the human red cell membrane. *J. gen. Physiol.* **31**, 505–527.

LEFEVRE, P. G. (1954). The evidence for active transport of monosaccharides across the red cell membrane. *Symp. Soc. exp. Biol.* **8**, 118–135.

LEFEVRE, P. G. & MCGUINNISS, G. F. (1960). Tracer exchange vs. net uptake of glucose through human red cell surface: new evidence for carrier-mediated diffusion. *J. gen. Physiol.* **44**, 87–103.

MORGAN, H. E., KALMAN, C. F., POST, R. L. & PARK, C. R. (1955). Kinetics of glucose transport across cell membranes. *Fed. Proc.* **14**, 103–104.

REINWEIN, D., KALMAN, C. F. & PARK, C. R. (1957). Transport of glucose and other sugars across the cell membrane of the human erythrocytes. *Fed. Proc.* **16**, 237.

SEN, A. K. & WIDDAS, W. F. (1960). The effect of temperature and pH on the facilitated transfer of glucose across the human erythrocyte membrane. *J. Physiol.* **152**, 64–65*P*.

SEN, A. K. & WIDDAS, W. F. (1962). Variations of the parameters of glucose transfer across the human erythrocyte in the presence of inhibitors of transfer. *J. Physiol.* **160**, 404–416.

STEARN, A. E. (1949). Kinetics of biological reactions with special reference to enzymic processes. *Advanc. Enzymol.* **9**, 25–74.

WIDDAS, W. F. (1953). *Permeability studies on membranes*. Ph.D. Thesis, University of London.

WIDDAS, W. F. (1954). Facilitated transfer of hexose across the human erythrocyte membrane. *J. Physiol.* **125**, 163–180.

WILBRANDT, W. (1954). Secretion and transport of non-electrolytes. *Symp. Soc. exp. Biol.* **8**, 136–162.