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KINETIC PARAMETERS OF GLUCOSE EFFLUX FROM HUMAN RED BLOOD CELLS UNDER ZERO-*TRANS* CONDITIONS

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SUMMARY

1. Efflux of glucose from human red blood cells under zero-*trans* conditions has been measured at 20°.

2. The values of the K_m and v_{max} were found to be 25 ± 3 mM and 139 ± 11 mmoles/min per l isotonic cell water, respectively.

3. These observations indicate that the conventional, symmetric carrier is not a valid model for glucose transport in human red blood cells.

4. Our results are consistent, however, with a recently proposed model in which transport occurs by the internal transfer of glucose molecules across protein tetramers embedded in the plasma membrane.

INTRODUCTION

Recent studies of glucose transport across the human red cell membrane have suggested that the classical carrier models cannot account for all of the experimental data in a consistent fashion[1,2]. The available evidence involves a comparison of the kinetic parameters describing, on the one hand, exchange of labeled glucose at chemical equilibrium, and, on the other hand, net efflux of glucose. In view of possible complications in the interpretation of exchange data[3], we have developed a simpler test[4] of the validity of symmetric carrier models, a test which involves only measurements of net fluxes. The test is to compare the Michaelis constants for the net efflux of glucose under the following two conditions:

(i) The zero-*trans* procedure. Here the concentration of glucose at the *trans* (outer) face of the membrane is maintained at or near zero, while the concentration of sugar at the *cis* (inner) face is varied. The efflux is measured as a function of the *cis* concentration, and the Michaelis constant K_m is defined as that concentration which reduces the rate of net outward movement to one-half the maximum value.

(ii) The Sen–Widdas procedure. Here the concentration of glucose at the *cis* face is maintained at a value greatly in excess of the half-saturation concentration K_m operative in a zero-*trans* experiment. The *trans* concentration of sugar is varied and the net efflux measured for different values of this external concentration. The

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Michaelis constant here is defined as that concentration of glucose at the *trans* face which reduces the net efflux to one-half of the maximal value.

Our analysis[4] shows that the value of the zero-*trans* Michaelis constant cannot be greater than that for the Sen–Widdas procedure if a general symmetric carrier model is to hold.

There have been a number of estimates for the Michaelis constant for the Sen–Widdas procedure. At 20° these range from 1.7 to 1.86 mM[5,6,2]. To our knowledge, no published estimate of the zero-*trans* K_m is available. The present paper reports values of this K_m, which we show to be an order of magnitude greater than the Sen–Widdas K_m.

We have recently presented a model for glucose transport based on internal transfer across protein tetramers embedded within the cell membrane[7,8], a model which reconciles many of the paradoxical observations mentioned above[1,2]. It is of interest that this model predicts, for the case of glucose transport, a far greater value for the zero-*trans* K_m than for the Sen–Widdas K_m. Our present measurements are consistent with this internal transfer model.

METHODOLOGICAL CONSIDERATIONS

A major technical difficulty in the design of these experiments arose from the fact that the K_m we wished to measure (the zero-*trans* K_m) was possibly an order of magnitude greater than that concentration (the Sen–Widdas K_m) of external glucose which diminishes the net efflux by one-half. But in order to obtain an accurate estimate of the zero-*trans* K_m it was necessary to preload the cells with sugar to a concentration well above this zero-*trans* Michaelis constant. To keep the external sugar concentration sufficiently low, we were thus forced to dilute the preloaded cells with a very large volume of sugar-free solution. By diluting 0.2 ml of a suspension of cells equilibrated at 80 mM into 100 ml of saline buffer, we were able to ensure that the external concentration of glucose never rose above one-tenth of the lowest reported value of the Sen–Widdas K_m.

The most direct procedure for the determination of the zero-*trans* K_m would be to measure the initial rates of outward glucose movement from cells preloaded to different concentrations of sugar. We used this procedure in preliminary experiments, until it became clear that errors inherent in this approach (as we proceed to discuss) made it difficult to obtain a precise estimate of the desired parameters. To obtain the initial rate of loss, it is necessary to establish the amount of glucose present within the cells at zero time and to subtract from this the amount of glucose present after a very short period of efflux (in practice, some 5 sec). Since this difference is between two relatively much larger numbers, each subject to experimental error, it suffers from proportionately much larger errors. In addition to these errors arising from the determination of glucose within the cells, there is also the error associated with imprecise timing of such very short intervals. Both of these problems can be overcome if one uses an integrated rate equation for the time course of glucose loss, since longer time intervals can be used and the cellular concentration of glucose at any time is now a substantial fraction of the concentration at time zero.

The zero-*trans* efflux of glucose follows essentially the same kinetics as that of

a simple enzyme reaction. For the latter, the appropriate integrated rate equation is given by:

$$K_m \ln \frac{S_0}{S_t} + (S_0 - S_t) = v_{max} t \tag{1}$$

where K_m is the Michaelis constant, v_{max} is the maximum velocity of the reaction, S_t is the concentration of substrate at time t, and S_0 is the concentration at time zero[9].

In our experiments, the cells are preloaded with glucose to an initial concentration S_0. At time zero they are rapidly mixed into a very large volume of solution containing isotonic saline buffer to which NaCl, osmotically equivalent to S_0, has been added. The cells can be assumed to be in osmotic equilibrium at all times, so that the efflux of glucose is followed by contraction of the cells. The integrated rate equation for glucose efflux which takes into account these volume changes has the same form as Eqn. 1 and can readily be shown to be:

$$\frac{310\, K_m}{310 + S_0} \ln \frac{N_0}{N_t} + \frac{(310 + S_0 + K_m)}{310 + S_0}(N_0 - N_t) = v_{max} t \tag{2}$$

where N_0 is the absolute amount (mmoles) of glucose present at time zero in that number of cells whose solvent water volume is 1 l under isotonic conditions (see ref. 2), N_t is the absolute amount of glucose present at time t in the same number of cells, and v_{max} is now the maximum velocity of net glucose loss (mmoles/min per l isotonic cell water) (compare ref. 6).

A convenient transformation of Eqn. 2 for purposes of plotting experimental data is:

$$\frac{1 - f}{\log_{10} f} - \frac{V_{max}(310 + S_0)}{S_0(310 + S_0 + K_m)} \cdot \frac{t}{\log_{10} f} = \frac{714 K_m}{S_0(310 + S_0 + K_m)} \tag{3}$$

where we write $f = N_t/N_0$. If one plots $(1-f)/\log_{10} f$ against $t/\log_{10} f$, one obtains a straight line, K_m and v_{max} being derived from the intercept on the ordinate and the slope.

As we describe below, we measure the net efflux of glucose by a radioactive tracer method. Since the specific activity of the [^{14}C]glucose is the same everywhere, the absolute amount of glucose present in the cells at any time is directly proportional to the measured radioactivity (counts/min); therefore f is given as the ratio of the counts/min present in unit quantity of cells at a given time to the counts/min present in the same number of cells at time zero.

One might consider how the presence of an unstirred layer outside the cell[12] could affect the observed value of the parameter K_m. After the first few msec the sugar concentration changes in such an unstirred layer would parallel the changes in the intracellular concentration. Thus initially, when the intracellular concentration is high, efflux would be retarded more by an unstirred layer effect than later, when both concentrations are lower. For this reason, that intracellular concentration (the apparent K_m) which gives one-half the maximal rate of loss would be lower than the true K_m. Our measured value of K_m would thus be a minimum estimate for the true K_m. However, unstirred layer effects do not seem to be important in our system, since we have shown (see EXPERIMENTAL METHODS) that provided the initial mixing

is vigorous, the time course of measured glucose loss is independent of the rate of subsequent stirring. This is consistent with the relatively long half-time of glucose loss.

EXPERIMENTAL METHODS

The following solutions were used:

(1) SP buffer. This consisted of NaCl, 147 mM; Na_2HPO_4, 20 mM; the pH was adjusted to 7.4 with HCl. The total osmolarity was 310 mosmoles/l.

(2) Zero-*trans* washout medium. This consisted of SP buffer with the addition of sufficient NaCl to maintain isotonicity with the glucose-loaded cells at the instant of mixing. For cells preloaded to 80 mM with glucose, we added NaCl to 43.5 mM.

(3) Stopper solution. This consisted of NaCl, 1 % (w/v); $HgCl_2$, 10^{-6} M; KI, 1.25 mM; phloretin, dissolved in ethanol, was added to give a final concentration of 10^{-4} M phloretin and 1 % ethanol. The stopper was kept at 0°.

The red blood cells were obtained from out-dated transfusion blood by washing three times in SP buffer, followed by centrifugation at 2100 × *g* for 20 min. The cells were preloaded with glucose by suspension in twenty volumes of a solution of glucose in SP buffer (final glucose concentration 80 mM) and incubated at 37° for 1 h. The cells were then centrifuged as above and the supernatant removed. About 1 ml of the cells were resuspended in SP buffer *plus* glucose at 80 mM to a hematocrit of approx. 30 % and incubated a further 5 min at 37°, following which radioactive glucose in a negligible volume of water was added and the exchange of glucose allowed to proceed (to completion) for a 5-min period. The cell suspension was thereafter kept at 0° until required for use.

To follow the efflux of glucose, 0.2 ml of the suspension of cells preloaded with radioactive glucose was blown rapidly with vigorous magnetic stirring into 100 ml of zero-*trans* washout medium, maintained at 20 ± 0.1°. Mrs. B. Hankin in our laboratory has shown that provided the initial mixing is vigorous, subsequent stirring has no detectable effect on the time course of glucose loss and is therefore unnecessary. 10-ml aliquots of this reaction mixture were transferred at given times to 30 ml of ice-cold stopper solution, using an automatic syringe, the whole then being transferred to the ice bath. After completion of a run (some 1 to 2 min), the cell suspensions were centrifuged at 2100 × *g* for 12 min at 0°. The supernatants were removed by thorough aspiration and the cell pellet treated as described below.

The red cell pellet was suspended in 0.5 ml of SP buffer with vigorous mixing. For the determination of the radioactivity remaining in the cells, 0.1 ml of this suspension was added to an equal volume of 20 % trichloroacetic acid solution and vigorously mixed. The cell debris was removed by centrifugation, and 0.1 ml of the clear supernatant was added to 10 ml of scintillation fluid (68 % (v/v) of a solution of 0.4 % (w/v) 2,5-diphenyloxazole, 0.04 % (w/v) 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene in toluene; 32 % (v/v) ethanol). Radioactive counting was performed using the Packard liquid scintillation spectrometer. The relative number of cells present in each pellet was determined by measurement of the hemoglobin content using the standard Drabkin procedure[10]; 0.25-ml aliquots of the cell pellet suspension were added with rapid mixing to 3 ml of the Drabkin solution and the resulting absorbance read at 540 nm in a Gilford spectrophotometer. This measurement of the hemoglobin content provided an internal control for possible variations in the recovery of cells.

For the interpretation of the experimental results we required also the value of the counts/min present in the cells at zero time and the counts/min in the supernatant fluid trapped with the cell pellet. To obtain the former, we added 0.02 ml of the cells preloaded with radioactive glucose directly to a mixture of 30 ml of stopper solution and 10 ml of SP buffer, at 0°. For the latter, we allowed the efflux of glucose to proceed for at least 10 half-lives, *i.e.* to effective completion, before removal of a 10-ml aliquot of cell suspension into the stopper solution. This latter correction was important only for the longer efflux times.

Materials used were Analar; phloretin was from Fluka A.G.; scintillation reagents were from Packard Instrument Co., Inc.; and D-[^{14}C]glucose was from the Radiochemical Centre.

RESULTS

Stopper solutions used routinely in the past for experiments on glucose transport in human red blood cells have contained high concentrations of $HgCl_2$ (see, for example, refs. 1 and 11). We have observed (unpublished experiments) a rapid and extensive destruction in these solutions, as judged by the release of cellular glucose and hemoglobin. In order to overcome this problem, we have tested the effectiveness of stopper solutions containing low concentrations of $HgCl_2$. In the present stopper solution, which contains a low concentration of $HgCl_2$ (1 μM) and phloretin (0.1 mM), the cellular glucose and hemoglobin contents are maintained constant during centrifugation and for at least 20 min when the cells are allowed to stand at 0° before centrifugation. This solution, which is both effective as a stopper of glucose efflux and nondestructive to the cells, has been adopted for routine use.

The time course of glucose loss in a representative experiment is shown in Fig. 1a. The fraction of counts/min remaining in the cells is plotted against the time at which the sample was taken. The half-time of efflux in this case when the cells

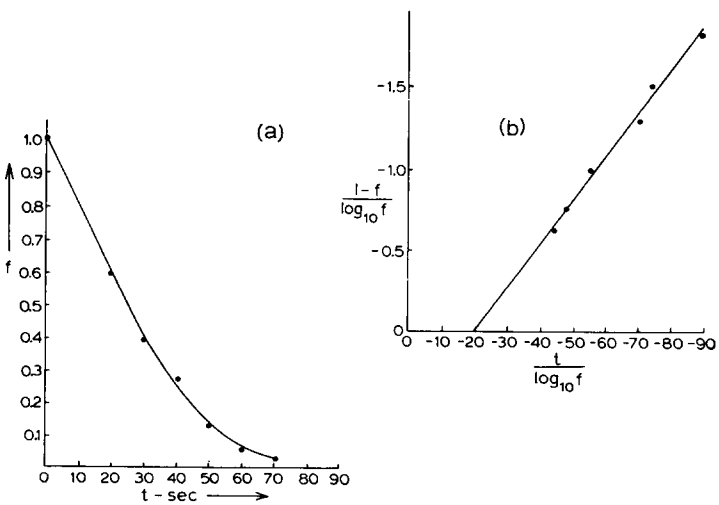

Fig. 1. Glucose efflux from human red blood cells under zero-*trans* conditions. (a) Time course of glucose loss from cells, in Expt. 2 of Table I. (b) The data of (a) plotted according to Eqn. 3. Details are given in the text.

were equilibrated initially in 80 mM glucose was 25 sec. Each point is the mean of four separate observations, the average standard error here being 3 % and not varying significantly at different points of the time course. Experiments which involved the repeated determination of hemoglobin and isotope content of aliquots of the same sample established that the standard deviation here was 2 % for each type of determination. Hence the isotope content per unit number of cells, which involves both of these determinations, will have a standard deviation of some 3 % arising from these sources. In the experiment of Fig. 1a, where the standard deviation of each point was 6 % (hence standard error of 3 %, in the mean), the remaining 3 % of variation must arise from timing errors, minor variations in temperature, and other such factors. A consideration of errors is particularly important in the present work, since, even using the integrated rate equation treatment, it is still necessary to subtract the counts/min remaining in the cell at any time from those present initially, a step which magnifies the effect of error in any individual observation.

In Fig. 1b, the data of Fig. 1a have been plotted according to Eqn. 3. The line drawn through the points has been fitted by a least squares analysis in order to make it nonsubjective. From this line the value of K_m can be calculated to be 25 mM, while the value of v_{max} is found to be 135 mmoles/min per l isotonic cell water. In Table I we collect the results of six such experiments. The average value of K_m from these six experiments is 25.4 mM (with a standard error of 3.0 mM), while that of v_{max} is 139 (S.E. = 11) mmoles/min per l isotonic cell water.

TABLE I

KINETIC PARAMETERS OF GLUCOSE EFFLUX FROM HUMAN RED BLOOD CELLS UNDER ZERO-*trans* CONDITIONS

Experiment	K_m *(mM)*	v_{max} *(mmoles/min per l isotonic cell water)*
1	26.5	137
2	25.0	135
3	25.8	106
4	14.8	118
5	23.0	159
6	37.5	181
Mean ± S.E.	25.4 ± 3.0	139 ± 11

DISCUSSION

It is clear from our observations that the value of K_m in the zero-*trans* procedure is more than ten times greater than that reported from three independent laboratories[5,6,2] for the Sen–Widdas procedure at the same temperature. Unstirred layers do not seem to be important under our experimental conditions and in any case would cause us to underestimate the true zero-*trans* K_m. But a very general analysis of the symmetric carrier[4] shows that this model requires the zero-*trans* K_m to be less than or at most equal to the Sen–Widdas K_m. Since this prediction conflicts with the experimental observations, it is clear that the conventional, symmetric carrier model

cannot describe the facilitated diffusion of glucose across the membrane of the human red blood cell. On the other hand, the value obtained for the K_m of the zero-*trans* procedure is very close to that expected on the basis of the internal-transfer model for glucose transport, which we have recently proposed[7,8].

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