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A NEW METHOD FOR MEASURING GLUCOSE TRANSLOCATION THROUGH BIOLOGICAL MEMBRANES AND ITS APPLICATION TO HUMAN ERYTHROCYTE GHOSTS

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SUMMARY

A convenient new method for investigating glucose flux through human erythrocyte membranes has been devised. Glucose oxidase and catalase were introduced into the interior spaces of erythrocyte pink ghosts; the rate of glucose entry into these ghosts was studied under a variety of conditions by measuring with an oxygen electrode the rate of enzyme-catalyzed oxygen consumption.

The values obtained for the half-saturation constant (K_T) and the maximal transport rate (V_T) agreed well with the values obtained by independent methods. In addition, the values for V_{TP}, defined as the number of μmoles of glucose traversing the membrane per min per μmole of membrane lipid P when flux is maximal, were similar for intact erythrocytes and pink erythrocyte ghosts.

It is suggested that the new method may be generally applied to the study of glucose translocation through any membrane from which vesicles containing glucose oxidase can be formed.

INTRODUCTION

Kinetic studies of the translocation of glucose and certain other monosaccharides through the human erythrocyte membrane have been widely interpreted to mean the process is mediated by a membrane-bound "carrier"[1] which is probably protein in nature. Evidence for a saturable glucose carrier in the intact red cell membrane has been obtained from studies of the rates or translocation of isotopically-labeled monosaccharides[2], and by the light-scattering method originally divised by Orskov[3]. However, neither of these methods lends itself conveniently to studies of monosaccharide translocation by erythrocyte ghosts which have been partially[4] or completely[5] depleted of cell contents by hypotonic lysis and washing. The classical method of Orskov[3] depends upon a substantial refractive index increment between cells and suspending medium which, in intact cells, is provided by the high internal hemoglobin concentration; although the method has been utilized for studying carrier-mediated transport in partially depleted ghosts[6] it is unsuitable for use with erythrocyte ghosts which have been completely depleted of internal contents and membrane-associated hemoglobin. The use of isotopically-labelled sugars, although

it has been successfully applied[6], requires fast-sampling methods and the employment of some unequivocal means of instantaneously stopping all sugar movements after sampling. Furthermore, a discontinuous record of translocation is obtained.

We now wish to report results obtained with a new, convenient method for measuring glucose translocation across the membranes of both "pink" and "white" erythrocyte ghosts. We have incorporated glucose oxidase and catalase into the interior spaces of these cells and have measured the rates of glucose entry into them by following oxygen uptake with an oxygen electrode. In this and a subsequent paper[7] we demonstrate retention of carrier-mediated glucose transport in "pink" ghosts, "white" ghosts, and in vesicles[7] derived from the membranes of "white" ghosts.

MATERIALS AND METHODS

Materials

Glucose oxidase Type II was purchased from Sigma Chemical Company. Crystalline catalase was obtained from Worthington Biochemical Corporation. Anhydrous D-glucose was a product of Pfanstiehl Laboratories. DEAE-cellulose was purchased from Schleicher and Schuell Company. Phloretin was purchased from Nutritional Biochemicals Corporation. Prior to use, it was recrystallized twice from hot water. For use, a concentrated solution in absolute alcohol was prepared; its concentration was 21 mM as determined from its absorbancy at 288 nm using an extinction coefficient of $25.5 \cdot 10^3$ $cm^2 \cdot mole^{-1}$ (ref. 10). Measured small aliquots of this concentrated solution were introduced into ghost suspensions to inhibit glucose transport.

Methods

The oxygen electrode assembly and its calibration. The fast response oxygen electrode apparatus depicted in Fig. 1 was constructed from Pyrex capillary tubing. A 0.5-mm diameter platinum cathode was sealed in glass with a minute portion of

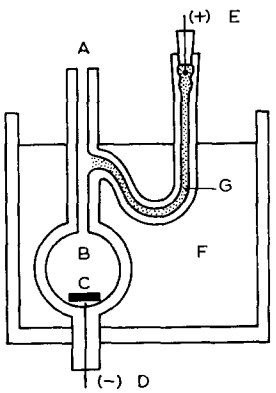


Fig. 1. Cross sectional diagram of the oxygen electrode assembly. Samples were introduced by syringe through capillary tube (A) into chamber (B) and mixed by magnetic stirring bar (C). A polarizing voltage of 0.5 V was applied between the platinum cathode (D) and the Ag–AgCl anode (E). The assembly was immersed in the constant-temperature water bath (F). (G) was a saturated KCl salt bridge.

the cathode projecting into the reaction chamber. The volume of the chamber was 0.65 ml at 15.5 °C. Within the reaction chamber a magnetic stirring bar provided efficient mixing of its contents. This chamber was connected by a saturated KCl bridge to a Ag–AgCl anode. A polarizing voltage of 0.5 V was maintained across the electrodes. The long capillary outlet above the reaction chamber, through which additions were made to the chamber by insertion of small-bore polyethylene tubing attached to syringes, very effectively prevented access of atmospheric O_2 to the chamber. Current flowing between the electrodes was recorded potentiometrically on a Varian Model G-10 recorder. The entire electrode assembly was made isothermal by immersion in a constant-temperature bath; unless otherwise specified, measurements were carried out at 15.5 °C. Before use, all solutions were saturated with air at the temperature of the reaction vessel.

Calibration of the electrode was achieved by measuring total oxygen uptake after inserting with a Hamilton microliter syringe 25-μl aliquots of standard glucose solutions into the chamber, which contained 1.625 mg of purified glucose oxidase and 65 units of catalase in 155 mM phosphate buffer, pH 7.4. To ensure complete thermal equilibration, the enzyme solution was allowed to remain in the chamber with stirring for several minutes prior to the addition of glucose. The concentrated stock standard solution of glucose had been allowed to attain mutarotational equilibrium prior to its use. Total oxygen uptake was linearly related to the quantity of glucose introduced into the chamber, and full scale deflection of the recorder was produced by 500 nmoles of glucose. It was ascertained that there was no alteration of electrode sensitivity when erythrocyte ghosts were also present in the chamber.

Glucose oxidase purification and kinetics. Crude glucose oxidase Type II was purchased from the Sigma Chemical Company. It was purified by repeated precipitation with $(NH_4)_2SO_4$ followed by chromatography on DEAE-cellulose[11]. Concentration of the enzyme protein was measured both spectrophotometrically[12] and colorimetrically[13]. The activity of the enzyme and its Michaelis constant were assayed by measuring the initial rates of oxygen consumption when 3.85–77.0 μmoles of D-glucose in mutarotational equilibrium was introduced into the oxygen electrode reaction chamber which contained a measured quantity of enzyme protein and 2000 units of catalase in 155 mM phosphate buffer (pH 7.4) which had been equilibrated with air at 15.5 °C. In the presence of this large excess of catalase the stoichiometry of the enzyme-catalyzed reaction was

$$\beta\text{-D-Glucose} + \tfrac{1}{2}O_2 \rightarrow \text{gluconic acid}$$

One unit of enzyme activity is defined as that quantity of glucose oxidase which catalyzes the uptake of one μatom of oxygen per min at saturating substrate concentration under the conditions specified. The purified glucose oxidase used in our experiments had a specific activity of 120 units per mg, which represented an eightfold purification of the starting material. The K_m of the enzyme for glucose under these conditions was 23.6 ± 1.6 mM. This value is higher than the value of 15 mM reported by Bentley[11], whose studies were performed at 30 °C and at pH 5.6, conditions which are optimal for the enzyme.

Preparation from human erythrocytes of "pink ghosts" containing glucose oxidase and catalase. Fresh whole blood from healthy donors was drawn into 7.0-ml tubes containing 7 mg of sodium ethylenediamine tetraacetate (EDTA). All samples were

refrigerated and washed repeatedly by centrifugation in 310 mosM phosphate buffer, pH 7.4. Leukocytes were eliminated by aspiration of the buffy layer.

Pink ghosts containing glucose oxidase and catalase were prepared by lysing 2.0-ml aliquots of packed erythrocytes in 10 ml of ice-cold 31 mosM phosphate buffer (pH 7.4) containing, per ml, 2.5 mg of purified glucose oxidase and 2000 units of catalase. After lysis was complete, sufficient 1.0 M sodium phosphate buffer (pH 7.4) was added to restore isotonicity (310 mosM). The suspensions were incubated at 37 °C for 5 min, which resulted in resealing or annealing of the membranes[6,24]. They were then cooled by immerson in an ice bath. After centrifugation at $12000 \times g$ for 30 min at 3 °C, the supernatant layers were carefully removed by aspiration, and the ghost pellets were resuspended in fresh 310 mosM phosphate buffer. The pellets were washed repeatedly by resuspension in 310 mosM buffer and sedimentation until glucose oxidase could no longer be detected in the supernatant wash fluid. The pellets were finally suspended in 20 ml of cold isotonic buffer and were kept at 0 °C until used in transport experiments. Although some variation was noted, the enzyme-containing pink ghosts were usually stable for at least 24 h at 0 °C. Pink ghosts prepared by this method retained approximately 20% of the hemoglobin originally present in the red cell suspension.

It was established by experiment that no glucose oxidase was taken up by intact erythrocytes incubated in a glucose oxidase solution in isotonic buffer and subsequently washed by centrifugation. Furthermore, pink ghosts which had been reannealed prior to exposure to glucose oxidase incorporated none of the enzyme when incubated with it in isotonic buffer.

Determination of membrane phospholipid and calculation of V_{TP}. Lipids were extracted from pink ghost membranes by the procedure of Folch *et al.*[14] and total phosphorus was measured by the micro method of Bartlett[15]. The value V_{TP} is defined as μmoles of glucose flux per μmole of membrane lipid phosphorus per min when flux is maximal.

RESULTS

Fig. 2 illustrates the rates of oxygen consumption when increasing amounts of D-glucose were injected into the oxygen electrode chamber containing pink ghosts into which glucose oxidase and catalase had been incorporated. It is evident that,

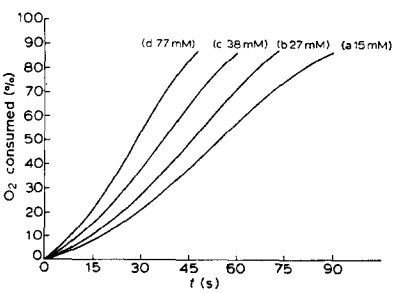


Fig. 2. Oxygen consumption by glucose oxidase-containing pink ghosts as functions of time and external glucose concentration. Oxygen consumption with time was recorded at the different glucose concentrations indicated.

after an initial period of acceleration, the rate in each case became linear until most of the O_2 had been consumed. The central linear portion of each curve was assumed to represent glucose net flux in a steady state. That is, where J's represent fluxes:

$$v_{\text{enzyme}} = J_{\text{net}} = J_{\text{in}} - J_{\text{out}} \tag{1}$$

$$\frac{dJ_{\text{net}}}{dt} = 0 \tag{2}$$

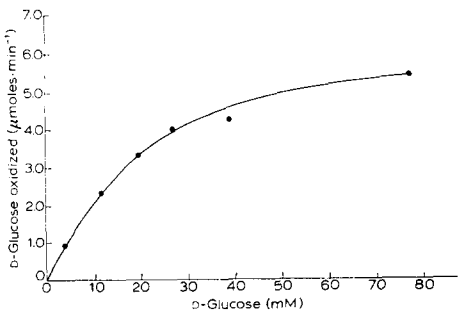
It is well established[16] that glucose entry into or efflux from intact erythrocytes seems, as a good approximation, to obey Michaelis–Menten kinetics. Moreover, the processes of entry and exit appear to be essentially symmetrical[17]. Therefore, one may rewrite Eqn 1 as:

$$v_{\text{enzyme}} = \frac{V_T[G]_{\text{ext}}}{K_T + [G]_{\text{ext}}} - \frac{V_T[G]_{\text{int}}}{K_T + [G]_{\text{int}}} \tag{3}$$

where V_T is the maximal unidirectional flux; $[G]_{\text{ext}}$ and $[G]_{\text{int}}$ are the external and internal glucose concentrations, respectively; K_T is the glucose concentration giving half-maximal flux; v_{enzyme} is the measured rate of enzyme-catalyzed oxygen consumption. Addition of digitonin to the enzyme-containing ghosts resulted in their lysis and release of their contained glucose oxidase and catalase into solution; the V of the released enzyme could then be measured as illustrated in Fig. 3. From this and the previously determined K_m of the enzyme for glucose ($K_m = 23.6$ mM) it was possible to calculate the glucose concentration within the ghosts in each experiment from the equation:

$$[G]_{\text{int}} = \frac{v_E K_m}{V_E - v_E} \tag{4}$$

where v_E represents the rate measured in the ghosts, V_E is the maximal rate of the incorporated enzyme, and K_m is the Michaelis constant of glucose oxidase for glucose. So long as the internal glucose concentration remained very low the second term of Eqn 3 could be regarded as negligible, net flux closely approximated influx,



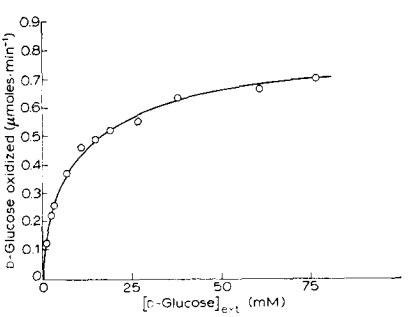


Fig. 3. Dependence upon glucose concentration of the rate of oxygen consumption by glucose oxidase *plus* catalase liberated from pink ghosts by digitonin treatment.

Fig. 4. Dependence upon external glucose concentration of the steady-state rates of oxygen consumption by pink ghosts containing glucose oxidase and catalase.

and influx was essentially equal to the measured rate of oxygen consumption. The high concentration of glucose oxidase employed in these experiments achieved this condition. Therefore, the measured rates of glucose oxidation in the steady state could be plotted against external glucose concentrations to obtain K_T and V_T. In Fig. 4 the data from Fig. 2 are presented in this manner.

In Fig. 4 it is evident that the curve is hyperbolic and reaches a limiting value. For actual calculation of V_T and K_T, the data were treated by a non-linear, least squares regression analysis devised by Wilkinson[18]. These results are presented in Table I. Comparison of the values obtained with this method with similar values obtained by other methods using intact erythrocytes[19] reveals that they agree well. It should be noted that K_m and K_T are quite different.

TABLE I

COMPARISON OF K_T, V_T AND V_{TP} FOR GLUCOSE TRANSLOCATION ACROSS THE MEMBRANES OF NATIVE HUMAN ERYTHROCYTES AND OF PINK GHOSTS

The values for pink ghosts were calculated from results obtained by the methods described in the text. The values for intact erythrocytes were calculated from the data of Sen and Widdas[20], who used the method of Orskov[3]. The experiments with pink ghosts were carried out at 15.5 °C, while those of Sen and Widdas were performed at 37 °C.

Preparation	K_T (*mM*)	V_T (*μmoles·min⁻¹·l⁻¹ packed cells*)	V_{TP} (*μmoles·min⁻¹·μmole⁻¹ lipid P*)
Pink ghosts	9.0±1.2	56.0±2.4	12.0±2.4
Erythrocytes	4.0	50.0	11.0

Although the preceding experiments provided strong evidence that the rate-determining step in the measured glucose oxidation was carrier-mediated diffusion of glucose from the exterior solution to the interior, glucose oxidase-containing compartment, additional evidence concerning this was sought. Sen and Widdas[20], and more recently Bolis and co-workers[21], observed a rather high Arrhenius activation energy for glucose translocation through the erythrocyte membrane. On the other hand, as shown by the data in Fig. 5a, glucose oxidase has a rather low E_a. Therefore the temperature dependence of the rate of glucose oxidation by pink ghosts containing glucose oxidase and catalase was measured. The results, shown in Fig. 5b, closely resemble those obtained by others using intact erythrocytes, and are clearly different from those obtained with glucose oxidase in solution. This provides additional evidence that glucose translocation through the ghost membrane was rate determining for glucose oxidation by the enzyme-containing pink ghosts.

As an additional test, the influence of phloretin on the rate of glucose oxidation by enzyme-containing ghosts was investigated. Phloretin is a potent competitive inhibitor of glucose translocation in intact erythrocytes[22], but we have found that it has no influence on the rate of glucose oxidation by free glucose oxidase in solution. Therefore, phloretin should profoundly affect the observed oxidative rate if carrier-mediated translocation were rate-determining, while it should have little influence

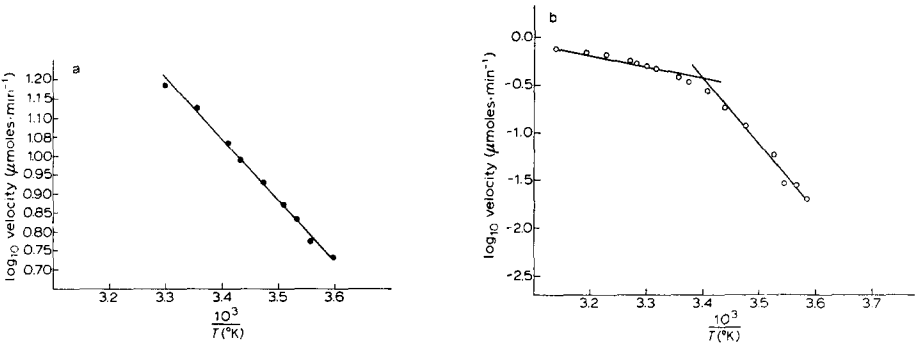


Fig. 5. Temperature dependence of the rates of glucose oxidation by pink ghosts containing glucose oxidase and by free glucose oxidase. The rates of oxygen consumption by enzyme-containing pink ghosts (○—○) and by free glucose oxidase (●—●) in the presence of 40 mM glucose were measured at different temperatures. E_a for oxygen consumption by pink ghosts was 41.0 kcal·mole^{-1} below 21 °C and 5.25 kcal·mole^{-1} above this temperature. E_a for oxygen consumption by free glucose oxidase was 7.6 kcal·mole^{-1} over the entire range of temperatures studied.

if glucose oxidase were rate-limiting or if glucose were traversing the membrane by noncarrier-mediated diffusion. The results, illustrated in Fig. 6 show clearly that phloretin is a potent competitive inhibitor of glucose oxidation by pink ghosts containing glucose oxidase.

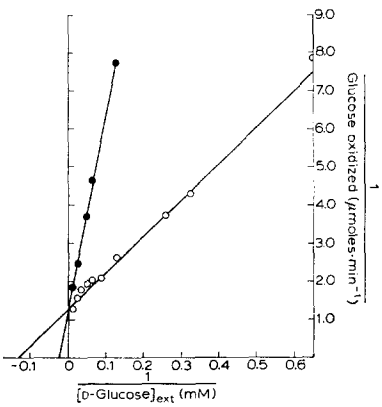


Fig. 6. Competitive inhibition by phloretin of glucose oxidation by pink ghosts containing glucose oxidase and catalase. The rate of glucose oxidation by control ghosts (○—○) is compared with the rate of glucose oxidation in the presence of 0.5 mM phloretin (●—●).

Additional evidence which supports the conclusion that glucose translocation across the ghost membrane was indeed rate determining in the observed oxygen uptake of ghosts containing glucose oxidase is provided by the data in Table II. In the preparation of ghost sample B, the quantity of glucose oxidase added during the lysis and reannealing steps was deliberately made one-half the routinely employed amount used in the preparation of sample A, and the amounts of enzyme incorporated in the two samples reflect this difference. It is evident, however, that the measured rates of glucose oxidation by the two preparations of enzyme-con-

TABLE II

RATES OF GLUCOSE OXIDATION BY PINK GHOSTS CONTAINING TWO DIFFERENT CONCENTRATIONS OF GLUCOSE OXIDASE

A preparation of erythrocytes was divided into separate aliquots which were lysed and reannealed in the presence of (A) the routine and (B) one-half the routine concentrations of glucose oxidase. Catalase concentrations were the same in each. V_T is the maximal rate of glucose oxidation by the pink ghosts. V_E is the maximal rate of glucose oxidation by the liberated free enzyme following lysis of the ghosts by digitonin.

D-*Glucose* (*mM*)	*Glucose oxidized* (*nmoles·min*$^{-1}$)	
	A	*B*
1.5	134	120
3.1	230	231
3.8	261	268
11.5	438	502
77.0	708	850
V_T	721	918
V_E	6887	3040

taining ghosts were virtually identical at all external glucose concentrations. Had the contained enzyme been rate limiting, it would have been anticipated that sample A would have consumed O_2 at twice the rate of sample B.

DISCUSSION

It had been demonstrated earlier that during lysis the erythrocyte membrane becomes permeable to molecules as large as ferritin[23], but that the membrane subsequently annealed in warm isotonic buffer recovered its characteristic impermeability to both small[24] and large molecules. We have utilized this to incorporate glucose oxidase into the interior spaces of erythrocyte ghosts, and measure unidirectional glucose flux across these membranes. This ability to enzymatically monitor unidirectional glucose flux obviates some limitations inherent in other methods of following transport. As will be shown in the subsequent paper[7], it is particularly useful with membranes which retain facilitated diffusion but are also "leaky" and allow non-carrier mediated diffusion to occur simultaneously.

It was first important to establish that the process being measured was indeed facilitated diffusion, and that the results obtained were not due to externally absorbed glucose oxidase or some similar artifact. Several types of evidence bear on this point. It has been found that glucose oxidase is not bound to or incorporated into intact erythrocytes or ghosts reannealed in isotonic buffer prior to exposure to glucose oxidase. The data in Table II show that the rate of glucose-dependent oxygen uptake by enzyme-containing ghosts was independent of the glucose oxidase concentration when the enzyme concentration was high; clearly the rate-determining step was not that catalyzed by the enzyme. However, the rate was dependent upon the external

glucose concentration, and the half-saturation constant for glucose transport was quite similar to that obtained by other methods. Moreover, the maximal velocity was very close to that calculated from data obtained by other investigations using different methods for measuring glucose transport. In addition, the temperature dependence of the process being measured was similar to that of glucose transport as determined by independent methods. Finally, glucose oxidation, by ghosts containing glucose oxidase was strongly inhibited by phloretin, a classical inhibitor of erythrocyte glucose transport which has no influence on the activity of glucose oxidase. The weight of the cumulative evidence argues strongly that the method indeed measures unidirectional glucose flux across the erythrocyte membrane.

It seems quite possible that the method may be rather generally employed to measure glucose translocation across any membrane from which vesicles containing glucose oxidase can be formed. In the succeeding paper we report its use to measure glucose translocation across the membranes of "leaky", hemoglobin-free erythrocyte ghosts and in membrane vesicles derived from white erythrocyte ghosts. Furthermore, in experiments to be reported subsequently, we have used a variation of the method to measure glucose diffusion across synthetic lipid bilayer membranes. It also seems feasible to employ the technique to measure recovery of membrane function after deletions of membrane components which allow "leakiness" of small molecules; preliminary application of this type are also reported in the next paper[7]. The technique thus seems to be simple and versatile.

ACKNOWLEDGEMENTS

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