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# GLUCOSE TRANSPORT IN WHITE ERYTHROCYTE GHOSTS AND MEMBRANE-DERIVED VESICLES

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## SUMMARY

Glucose oxidase was incorporated into the interior spaces of hemoglobin-free erythrocyte ghosts and into small vesicles derived from their membranes. By employing the oxygen uptake method described previously (Taverna, R. D. and Langdon, R. G. (1972) *Biochim. Biophys. Acta* 412–421), kinetic parameters of glucose translocation across the membranes of these preparations were investigated.

It was found that the facilitated diffusion system for glucose was completely retained in the membranes of both hemoglobin-free ghosts and in the membrane-derived vesicles. In addition to its entry by carrier-mediated diffusion, glucose also entered white ghosts through a "leak" which, although permeable to glucose, did not allow the passage of glucose oxidase.

The kinetics of entry of glucose into the membrane-derived vesicles, which were predominantly "inside-out", were identical to those of intact erythrocytes. This provides additional evidence for symmetry of the facilitated diffusion process.

## INTRODUCTION

The rates of translocation of glucose and other monosaccharides across the membranes of intact human erythrocytes have been extensively investigated and characterized, and it is generally accepted that glucose enters these cells by carrier-mediated diffusion. However, attempts to demonstrate retention of this process in hemoglobin-free erythrocyte membranes ("white ghosts") have led to conflicting results.

Bobinsky and Stein[2] and Bonsall and Hunt[3] originally reported that protein components of pink and white ghost membranes could be dissolved in 2.0 M NaI or 5% Triton X-100; following dialysis and adsorption of these "soluble" components on celite or DEAE-cellulose, it was reported that glucose was preferentially bound to the adsorbed components. However, LeFevre and Masiak[4] demonstrated that the supposed binding of glucose actually resulted from distribution of the sugar into the internal spaces of small, membrane-derived vesicles which retained the carrier-mediated diffusion system; although vesicles derived from pink ghosts were demonstrated to retain glucose transport activity, this appeared to be essentially absent in white ghosts[3]. On the other hand, Jung *et al.*[5] have presented evidence

which argues strongly that the glucose-facilitated diffusion system is fully retained in hemoglobin-free erythrocyte ghosts. Very recently, Kahlenberg and co-workers[6] have reported that glucose is specifically bound to white ghost membranes, which they presumed to lack the ability to transport or to trap glucose.

In the preceding paper[1] we described a new enzymatic method for examining the kinetics of entry of glucose into pink erythrocyte ghosts. We now wish to report that application of this method to white ghosts[7] and small vesicles[8] derived from these membranes has revealed that carrier-mediated translocation of glucose persists in both.

## METHODS

### *Preparation of hemoglobin-free erythrocyte ghosts containing glucose oxidase and catalase*

Hemoglobin-free, or white, ghosts were prepared by a method very similar to that described by Dodge *et al.*[7]. Fresh, whole human blood containing 1 mg/ml of disodium ethylenediaminetetraacetate (EDTA) as an anticoagulant was centrifuged for 15 min at $600 \times g$, and both the plasma and buffy coats were carefully removed by aspiration from the sedimented erythrocytes. The erythrocytes were washed several times by resuspending them in isotonic (310 mosM) sodium phosphate buffer, pH 7.4, and recovering them by centrifugation; any residual buffy layer was carefully removed after each wash. The washed red cells were then hemolyzed by resuspending them in 20 vol. of 20 mosM sodium phosphate buffer, pH 7.4, at 4 °C. The resulting ghosts were recovered by centrifugation at $15000 \times g$ for 30 min at 4 °C. Resuspension of the ghosts in fresh 20 mosM phosphate buffer and resedimentation by centrifugation resulted in a membrane pellet which was free of detectable hemoglobin. This pellet was suspended in 5 vol. of cold 20 mosM phosphate buffer containing 2.5 mg of purified glucose oxidase[1,9] and 2000 units of catalase per ml of buffer. Phase-contrast microscopy revealed that the ghosts were spherical in shape. To this optically-transparent suspension of spherical ghosts was added sufficient molar sodium phosphate buffer to increase the ideal osmolarity of the solution to isotonicity (310 mosM), and the suspension was incubated at 37 °C for 5 min; the suspension was gently agitated at frequent intervals. During this time, the suspension became birefringent when stirred, and phase-contrast microscopy revealed that the reannealed ghosts had regained the discoid shape characteristic of native erythrocytes. After the suspension had been cooled to 4 °C, the ghosts were sedimented by centrifugation at $15000 \times g$ for 15 min. The ghosts were washed repeatedly by suspension in fresh, cold isotonic buffer until glucose oxidase could no longer be detected in the wash fluid. The final pellet was suspended in cold isotonic buffer and the suspension was kept in an ice bath until the enzyme-containing ghosts were examined for glucose transport activity.

### *Preparation of membrane-derived vesicles containing glucose oxidase and catalase*

The procedure for preparation of membrane vesicles which we employed was derived from that described by Steck and co-workers[8]. The erythrocytes from 7 ml of fresh whole human blood containing 1 mg/ml of EDTA as an anticoagulant

were collected by centrifugation at $600 \times g$ for 15 min, and the supernatant plasma and buffy coats were removed by aspiration. The red cells were washed twice by suspension in and sedimentation from 5 ml of cold 0.15 M NaCl containing 10 mM EDTA. The cells were washed three additional times by suspending them in 10 ml of 0.15 M NaCl containing 5 mM sodium phosphate (pH 8.0) and recovering them by centrifugation at $600 \times g$ for 10 min. The washed erythrocytes were lysed by mixing 2.0-ml aliquots of packed cells with 80 ml of cold 5 mM phosphate buffer, pH 8.0. The resulting ghosts were sedimented by centrifugation at $20000 \times g$ for 15 min at 4 °C. After they had been washed twice by resuspension in fresh 5 mM phosphate buffer, the ghosts were white and devoid of hemoglobin.

Aliquots containing 2 ml of packed white ghosts were suspended in 25 ml of cold 0.5 mM sodium phosphate, pH 8.0, containing 50 mg of purified[1,9] glucose oxidase and 125000 units of catalase. Examination under a phase-contrast microscope initially revealed invaginations of the ghost membranes. After endocytosis had continued for 4 h at 4 °C, ghosts were no longer present but had been replaced by much smaller, spherical vesicles. These were stabilized[8] by adding 100 $\mu$moles of $MgSO_4$, and the buffer was made isotonic by addition of 1.0 M sodium phosphate, pH 7.4. The suspension was incubated at 37 °C for 5 min. It was then cooled to 4 °C and passed quickly through a 27-gauge hypodermic needle, which sheared the filamentous connections between aggregated vesicles. The vesicle suspension was centrifuged at $80000 \times g$ for 30 min at 4 °C. The resulting pellet was resuspended in 15 ml of cold 310 mosM sodium phosphate, pH 7.4, and the vesicles were sedimented by centrifugation at $50000 \times g$ for 15 min. This washing procedure was repeated until glucose oxidase was no longer detected in the supernatant fluid. The vesicles were finally suspended in 20 ml of cold 310 mosM sodium phosphate, pH 7.4, and they were stored at 4 °C until they were examined for glucose transport activity.

*Analytical methods*

Phospholipids were extracted from white ghosts and vesicles by the procedure of Folch *et al.*[10]. Inorganic phosphate liberated from the extracted lipids by sulfuric acid digestion was determined by the method of Bartlett[11]. The sensitive pyridine hemochromogen method described by Rimington[12] was used to measure hemoglobin, which was undetectable in either white ghost or vesicle preparations used here. The methods previously described[1] were used to study glucose entry into white ghosts and vesicles; the temperature of the oxygen electrode vessel was maintained at 15.5 °C throughout the experiments described here.

## RESULTS

Investigation of the rate at which glucose enters into membrane-derived vesicles at various external glucose concentrations yielded the results depicted in Fig. 1. It is evident that the rate of glucose entry exhibited a hyperbolic dependence upon the external glucose concentration and approached a limiting value. Calculation of $K_T$ and $V_{TP}$ by nonlinear regression analysis[13] of the data in Fig. 1 yielded the values shown in Table I. Also shown for comparison in Table I are the results of similar studies conducted with pink erythrocyte ghosts[1]. It is evident that the

half-saturation constants, $K_T$, for pink ghosts and membrane-derived vesicles are very similar. Moreover, the values for $V_{TP}$, the maximal rate of glucose permeation per $\mu$mole of membrane lipid phosphorus, are virtually identical for pink ghosts and vesicles.

Because phloretin has been shown to be an inhibitor of carrier-mediated glucose translocation in numerous studies on intact erythrocytes[14] and pink ghosts[1],

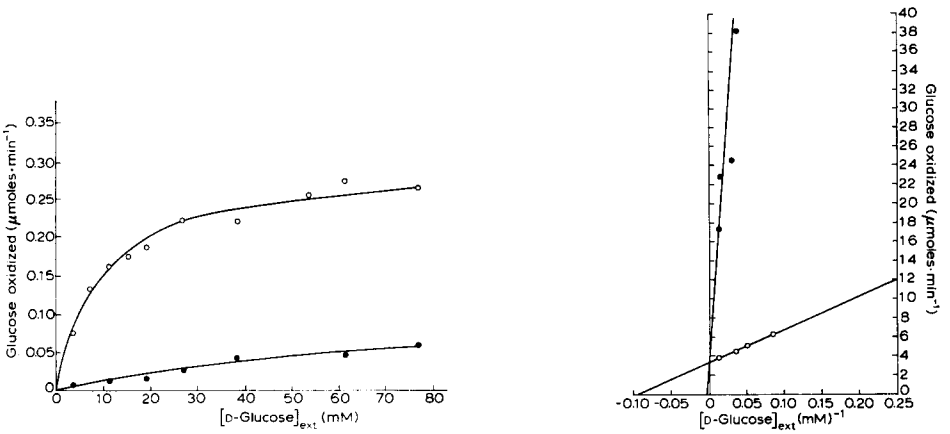


Fig. 1. Oxygen consumption by glucose oxidase-containing vesicles as a function of external glucose concentration. (○—○), illustrated the rates of glucose permeation in the absence of phloretin; (●—●), rates of permeation in the presence of 0.15 mM phloretin.

Fig. 2. Double reciprocal representation of uninhibited (○—○) and phloretin-inhibited (●—●) glucose translocation across the vesicle membrane. (Values of the ordinate and abcissa intercepts were calculated from resulting kinetic parameters obtained from nonlinear regression analysis.)

TABLE I

COMPARISON OF $K_T$, $V_T$ AND $V_{TP}$ FOR GLUCOSE TRANSLOCATION ACROSS THE MEMBRANES OF ERYTHROCYTE, PINK GHOST, WHITE GHOST AND MEMBRANE-DERIVED VESICLE PREPARATIONS

For calculation of each $K_T$, $V_T$, and $V_{TP}$ value for pink and white ghosts, the data obtained from four separate, complete experiments of the type illustrated in Fig. 3 using preparations derived from four batches of erythrocytes were pooled. Each composite curve was then analyzed by the procedure of Wilkinson[13]. The standard errors (S.E.) were calculated as described by Wilkinson[13]. For calculation of the corresponding values for membrane vesicles, the data from two complete, separate experiments were pooled in a similar manner. The values for intact erythrocytes were obtained from the literature[18].

| *Preparation* | $K_T$ ($mM \pm S.E.$) | $V_T$ (*mmoles·min*$^{-1}$·*l*$^{-1}$ *packed cells*) | $V_{TP}$ (*μmoles·min*$^{-1}$· *μmole*$^{-1}$ *lipid P*) |
|---|---|---|---|
| Erythrocytes | 4.0 | 50.0 | 11.0 |
| Pink ghosts (4) | 9.0±1.2 | 56.0±2.4 | 12.0±2.4 |
| White ghosts (4) | 10.0±1.1 | 42.0±1.61 | 9.0±1.61 |
| Membrane vesicles (2) | 10.9±1.1 | 49.0±1.49 | 11.0±1.49 |

TABLE II

COMPARISON OF $K_i$ FOR PHLORETIN INHIBITION OF GLUCOSE TRANSLOCATION ACROSS THE MEMBRANES OF ERYTHROCYTES[16], PINK GHOSTS, WHITE GHOSTS AND MEMBRANE-DERIVED VESICLES

Values for $K_i$ were calculated from the relationship[17]:

$$\frac{v}{v_i} = 1 + \frac{K_T}{K_i}(I/K_T + S)$$

where: $v$= velocity; $v_i$= velocity in the presence of phloretin; $K_T$= half-saturation constant for D-glucose transport; $K_i$= apparent dissociation constant for transport-site–inhibitor complex; $I$ = concentration of inhibitor; $S$= concentration of substrate.

| *Preparation* | *$K_i$ phloretin ($\mu M$)* |
|---|---|
| Human erythrocytes | 2.5 |
| Pink ghosts | 41.8 |
| White ghosts | 39.0 |
| Membrane-derived vesicles | 5.97 |

the effect of this compound on glucose penetration into the membrane-derived vesicles was investigated; the results are depicted in Figs 1 and 2. It is clear that phloretin acted as a competitive inhibitor of glucose translocation across the vesicle membrane. From the data, an apparent $K_i$ value of 5.97 $\mu$M was calculated. (Table II).

Investigation of the rates of glucose entry into white ghosts at various external glucose concentrations yielded the results depicted in Curve A, Fig. 3. It may be seen that the glucose entry rate did not approach a limiting value at high glucose concentrations but instead seemed to become linearly dependent upon external

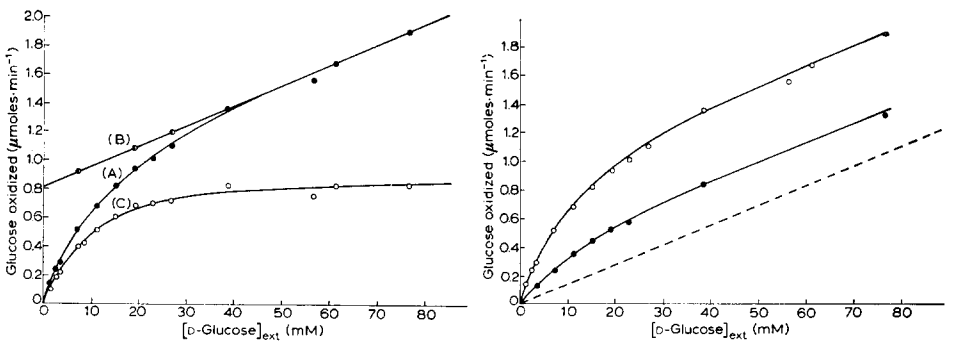


Fig. 3. Oxygen consumption by glucose oxidase-containing white ghosts as a function of external glucose concentration. Curve A (●—●), which represents the experimental data, was resolved into a linear and a nonlinear component. Curve C (○—○), the nonlinear component, resulted from the subtraction of Curve B (◐—◐) from Curve A. See text.

Fig. 4. The influence of phloretin on glucose entry into white erythrocyte ghosts. Glucose entry into white ghosts was measured at various glucose concentrations in the presence (○—○) and absence (●—●) of 0.6 mM phloretin. Curve (- - -) represents the linear component, which was identical in both.

glucose concentration. This suggested that glucose might be entering these cells by two simultaneous, additive processes: carrier-mediated diffusion and free diffusion through a "leak". Based upon the assumptions that both processes were operative at all glucose concentrations, but that the rate of glucose entry through the "leak" was linearly dependent upon glucose concentration, Curve A of Fig. 3 was resolved into two components, a linear component (Curve B) and a nonlinear component (Curve C) which resulted from the subtraction of B from A. It is apparent that Curve C is hyperbolic in form and approaches a limiting value at high glucose concentrations. Nonlinear regression analysis of Curve C yielded values for $K_T$ of 10.0 mM and for $V_{TP}$ of 9.0 $\mu$moles of glucose per min per $\mu$mole of membrane lipid phosphorus (Table I). These values correspond closely to similar values for pink ghosts and membrane vesicles.

If the assumptions made above were correct, it would be anticipated that phloretin, a competitive inhibitor of carrier-mediated glucose translocation would have little effect upon glucose entry through the postulated leak giving rise to Curve B of Fig. 3. Depicted in Fig. 4 are the results obtained when glucose translocation was investigated in the presence of 0.6 mM phloretin and in its absence. It is evident that phloretin had no influence on the linear component, but inhibited only the nonlinear portion. It is apparent that this prediction was completely verified. By calculation, the $K_i$ for phloretin was 39 $\mu$M.

## DISCUSSION

The results presented here show clearly that carrier-mediated diffusion of glucose is fully retained by vesicles derived from the membranes of human erythrocytes. Not only are the kinetic parameters $K_T$ and $V_{TP}$ quite similar for vesicles, pink ghosts and intact erythrocytes, but vesicles retain their sensitivity to phloretin, a classical inhibitor of glucose transport in red cells[14].

The apparent $K_i$ for phloretin is evidently somewhat higher for pink ghosts and white ghosts than it is for either erythrocytes or membrane vesicles (Table II); an explanation for this difference, which was reproducibly observed in a number of experiments, is not currently available, but is under investigation.

It is of interest that the vesicles employed here were prepared by a method which leads predominantly to a population in which the normally interior membrane surface has become the exterior surface[8]; inasmuch as the kinetic parameters of glucose transport remained unchanged by this inversion of membrane polarity, it seems quite probable that the transport process itself possesses some type of symmetry. It is also of interest that the rates of glucose translocation across the vesicle membrane and the pink ghost membrane were essentially the same; this strongly suggests that the glucose carrier is tightly bound to the membrane, unlike the pericytoplasmic monosaccharide binding proteins of bacteria[15] which are readily released by osmotic shock. Jung *et al.*[5] using an isotopic tracer method, have reached essentially the same conclusion.

The evident discrepancy between our results and those of other investigators who have reported that white erythrocyte ghosts and vesicles derived from them are devoid of glucose transport activity cannot be decisively resolved. However, it seems possible that previous workers may not have obtained sealed vesicles,

since they did not add a divalent cation to stabilize[8] the membrane-derived vesicles. Alternatively, their vesicles may have been damaged or rendered "leaky" by adsorption to the solid supports employed.

The results which we have obtained with white ghosts show that the total rate of permeation into these objects is actually greater than that observed with either pink ghosts[1] or membrane-derived vesicles. This apparently results from the additive contributions of carrier-mediated diffusion and a "leak" which is dependent only upon the concentration gradient. This suggests the possibility that during the preparation of white ghosts, areas of their membranes are rendered abnormally permeable to small molecules such as glucose, while retaining their impermeability to large molecules such as glucose oxidase. This "leak" apparently disappears during the preparation of membrane-derived vesicles. Although a number of speculative possibilities exists to explain this "leak", it is evident that further work will be necessary in order to clarify its actual basis. In preliminary experiments it has been possible to partially "seal" the "leak" by addition to white ghosts of a protein fraction derived from the soluble proteins present in a crude hemolysate and by addition of $Mg^{2+}$.

Kahlenberg and his co-workers[6] have recently reported results which they have interpreted as direct evidence for binding of glucose to white ghost membranes. However, their interpretation was dependent upon the assumption that white ghosts or membrane-derived vesicles did not retain the capacity for facilitated diffusion or retention of glucose. Inasmuch as we have clearly demonstrated that these properties are retained by white ghosts and vesicles derived from them, it may be necessary to reinterpret the results obtained by Kahlenberg *et al.*[6].

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REFERENCES

1 Taverna, R. D. and Langdon, R. G. (1973) *Biochim. Biophys. Acta* 298, 412–421
2 Bobinsky, H. and Stein, W. D. (1966) *Nature* 211, 1366–1368
3 Bonsall, R. W. and Hunt, S. (1966) *Nature* 211, 1368–1370
4 LeFevre, P. G. and Masiak, S. J. (1970) *J. Membrane Biol.* 3, 387–399
5 Jung, C. Y., Carlson, L. M. and Whaley, D. A. (1971) *Biochim. Biophys. Acta* 241, 613–627
6 Kahlenberg, A., Urman, B. and Dolansky, D. (1971) *Biochem. J.* 10, 3154–3162
7 Dodge, J. T., Mitchell, C. and Hanahan, D. J. (1963) *Arch. Biochem. Biophys.* 100, 119–130
8 Steck, T., Weinstein, R. S., Straus, J. H. and Wallach, D. F. H. (1970) *Science* 10, 255–257
9 Bently, R. (1963) in *The Enzymes* (Boyer, P. D., Lardy, H. and Myrback, K., eds), Vol. 7, pp. 567–586, Academic Press, New York
10 Folch, J., Lees, M. and Sloane-Stanley, G. H. (1957) *J. Biol. Chem.* 226, 497–509
11 Bartlett, G. R. (1959) *J. Biol. Chem.* 234, 466–468
12 Rimington, C. (1942) *Br. Med. J.* 1, 177–178
13 Wilkinson, G. N. (1961) *Biochem. J.* 80, 324–332
14 LeFevre, P. G. (1959) *Science* 130, 104–105
15 Anraku, Y. (1967) *J. Biol. Chem.* 242, 793–800
16 LeFevre, P. G. and Marshall, J. K. (1959) *J. Biol. Chem.* 234, 3022–3026
17 Wilson, P. W. (1949) *Respiratory Enzymes*, pp. 16–57, Burgess, Minneapolis
18 Sen, A. K. and Widdas, W. F. (1962) *J. Physiol. London* 160, 392–403