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MEMBRANE TRANSPORT IN RESEALED HAEMOGLOBIN-CONTAINING HUMAN ERYTHROCYTE 'GHOSTS' PREPARED BY A DIALYSIS PROCEDURE

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SUMMARY: Resealed haemoglobin-containing erythrocyte 'ghosts' have been proposed as in vivo carriers for enzyme replacement therapy. Transport of substrates and metabolites into and out of the 'ghost' has been suggested to be a limiting factor in such therapy. Studies of the transport of L-phenylalanine and of uric acid in normal human erythrocytes and prepared 'ghosts', in which the transport of sodium ions and D-glucose was intact, have shown that transport characteristics of 'ghosts' are identical to those of normal erythrocytes with transport not being a quantitatively limiting factor.

In the treatment of metabolic diseases involving enzyme deficiencies repeated administration of exogenous enzyme is followed by immunological reactions (3). Other limiting factors are the low stability and the reduced half-life of free enzymes in the plasma (15). The patients' own erythrocyte 'ghosts' have been proposed as biodegradable in vivo carriers for the therapeutic enzyme (8, 16, 20). Recently we demonstrated the advantage of a dialysis method for protein loading of erythrocytes by reversible hypo-osmotic haemolysis (16). When studying this therapeutic approach to hyperuricaemia and hyperphenylalaninaemia, the former being a pilot model, it is important, that the transport of the relevant substrates across the erythrocyte 'ghost' membrane is not a limiting factor. Active and carrier mediated transport of sodium, D-glucose and phosphate were also examined in prepared erythrocyte 'ghosts' in comparison to transport in normal erythrocytes.

Some aspects of this work have been reported briefly elsewhere (17).

MATERIALS

Blood from healthy volunteers was collected into EDTA (1.5 mg/ml). Erythrocytes were used immediately after being washed (1100g/10 minutes) twice in cold (4°C) isotonic saline. Radiochemicals were purchased from the Radiochemical Centre (Amersham, U.K.). All chemicals were of reagent grade or better. The scintillation fluid contained naphthalene 100g, 2-(5 diphenyloxazole) 5g, p-bis 2-(5 phenyloxazolyl)-benzene 0.05g in 1l dioxan. Two different buffer solutions were used: Iso-osmotic buffer A: 150 mM NaCl, 5mM K_2HPO_4/KH_2PO_4, pH 7.4; Hypo-osmotic Buffer B: 5mM K_2HPO_4/KH_2PO_4, pH7.4.

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METHODS

Preparation of erythrocyte 'ghosts': The method of 'ghost' preparation followed the principles and details described elsewhere (4,16). A dialysis bag containing washed packed erythrocytes (7 volumes) and iso-osmotic buffer A (3 volumes) was dialysed against hypoosmotic buffer B with proper mixing at 4°C for 105 minutes. Resealing was brought about by dialysing against iso-osmotic buffer A at room temperature for 30 minutes. Finally the resealed erythrocyte 'ghosts' were washed (1100g 20°C) three times in iso-osmotic buffer A.

The lysis and resealing of the cells was followed by incorporation of [^{125}I]-HSA (human serum albumin) in separate control experiments. Haemoglobin, ATP and glucose-6-P dehydrogenase (G-6-P-DH) were also determined.

Influx measurements: Transport studies were carried out in both normal erythrocytes and resealed erythrocyte'ghosts' with the following substrates: D-glucose, sodium-ions, uric acid, phenylalanine and inorganic phosphate-ions. Influx was followed by measuring the uptake of radioactive substrate into the cells. Packed cells were incubated in an equal volume of medium and at a temperature as given in table 1. At certain time intervals 0.2ml of the incubation mixture was sampled and transport was terminated by diluting the cells in ten volumes of ice-cold iso-osmotic buffer A, containing, in the case of glucose, 3mM $HgCl_2$ as inhibitor. This was followed by immediate centrifugation at 15000g for 10 sec. in a microcentrifuge (Eppendorf 3200). The pellet was lysed with 0.1ml distilled water and precipitated with trichloracetic acid. D-glucose influx was studied using separate experiments at each individual time. Aliquots of 200µl were dissolved in 10ml of scintillation fluid and measured in a β-counter. Samples containing ^{22}Na-ions were determined directly in a γ-counter.

Efflux measurements: Packed cells were incubated in an equal volume of incubation medium. Details are given in table 1. Cells pre-equilibrated with substrates were washed in iso-osmotic buffer A before resuspension in the same medium. Samples were taken and treated as above.

Table 1: Composition and temperature of incubation media used in transport studies.

Transport studies of	Incubation medium		Temp °C
	Substrates	Radioactive tracers per mol of substrate	
D-glucose	0.5mM D-glucose 1mM D-galactose	1 µCi D-[U-^{14}C] glucose	10
Sodium	150mM NaCl 2mM ATP 5mM D-glucose	7 nCi[^{22}Na]Na^+	37
Phosphate	50mM K_2HPO_4/ KH_2PO_4	0.2 µCi [^{32}P] Pi	20
L-phenylalanine	1,2,4,8,16, or 20mM phenylalanine	0.1 µCi L-[U-^{14}C] phenylalanine	37
Uric acid	0.2,0.4,0.5,0.6,0.8, or 1.0 mM uric acid	1 µCi [2-^{14}C] uric acid	37

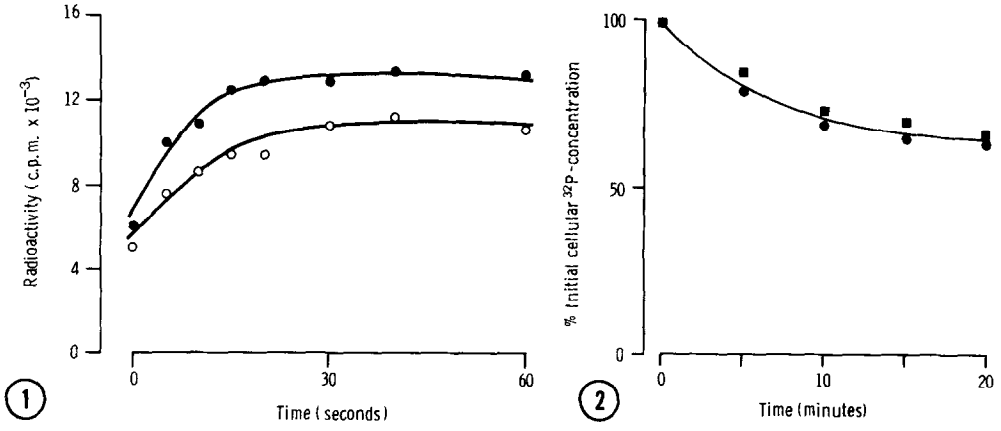

Fig. 1: D-Glucose influx into normal erythrocytes (open circles) and resealed erythrocyte 'ghosts' (closed circles). The ordinate represents the radioactivity of 0.1g packed cells.

Fig. 2: Phosphate-efflux from resealed erythrocyte 'ghosts' in absence (circles) and presence of 2mM phloridzin (squares). The ordinate represents the percentage of initial cellular [^{32}P] Pi-concentration.

RESULTS

Control experiments showed that the cells were lysed and resealed during preparation, since [^{125}I]-HSA could be entrapped in the erythrocyte 'ghosts' with no subsequent leakage. Haemoglobin and G-6-P-DH content were both 70% of the initial content of normal erythrocytes. ATP was found to be 0.34mM in resealed erythrocyte 'ghosts' compared to 0.52mM in normal washed erythrocytes.

Glucose transport was rapid in both normal erythrocytes and resealed erythrocyte 'ghosts' (figure 1). Transport was slightly faster and equilibrium was reached earlier with higher maximal values with erythrocyte 'ghosts' (figure 1). D-glucose influx was inhibited by phloridzin in both types of cells.

Phosphate efflux was not affected by addition of 2mM phloridzin in resealed 'ghosts' (figure 2).

Sodium transport was slow in both normal erythrocytes and resealed erythrocyte 'ghosts' (figure 3b). Sodium-ion influx reached maximum levels after 3 hours incubation with intact erythrocytes. However, continuous uptake was observed by the 'ghost' cells and equilibration had not been achieved by 4 hours, with much greater incorporation of sodium-ions into the 'ghosts' when compared to normal erythrocytes (figure 3b). Efflux of

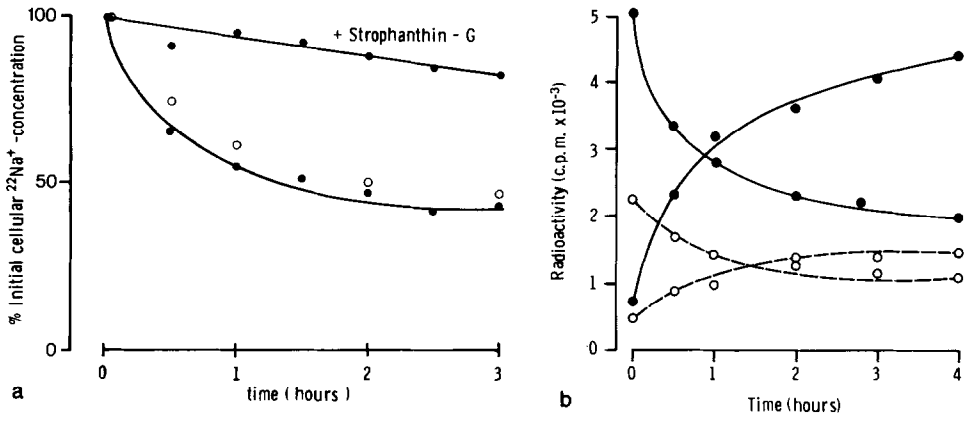

Fig. 3a: Efflux of sodium-ions from normal erythrocytes (open circles) and resealed erythrocyte 'ghosts' (closed circles) in absence and presence of 2mM strophanthin-G.

Fig. 3b: Transport of sodium-ions across the membranes of normal erythrocytes (open circles) and resealed erythrocyte 'ghosts' (closed circles).

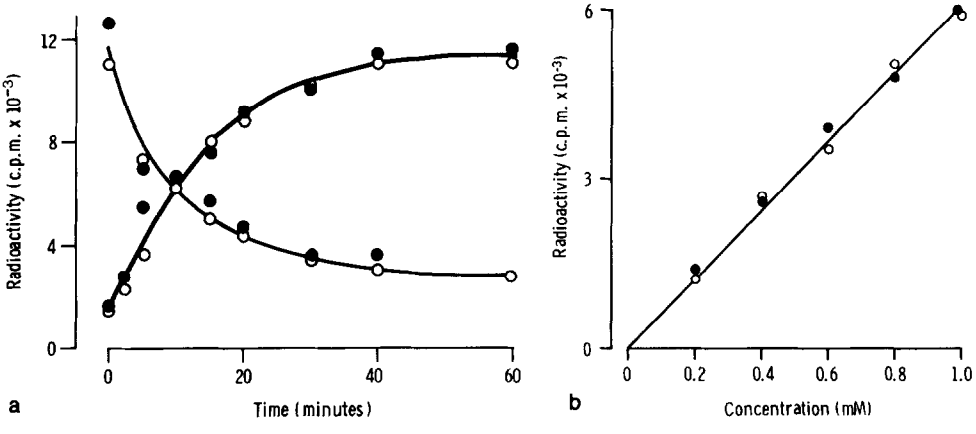

Fig. 4a: L-Phenylalanine (2mM) transport across the membranes of normal erythrocytes (open circles) and resealed erythrocyte 'ghosts' (closed circles).

Fig. 4b: Effect of increasing L-phenylalanine concentrations on initial rate (first minute) of influx of normal erythrocytes (open circles) and resealed erythrocyte 'ghosts' (closed circles).

sodium-ions was symmetrical to the influx. The percentages of net efflux of sodium-ions were in comparable range in both types of cells. Efflux of sodium-ions from resealed erythrocyte 'ghosts' could be inhibited by strophanthin G (figure 3a).

L-Phenylalanine transport was closely similar in intact erythrocytes and resealed 'ghosts' with symmetrical influx and efflux (figure 4a).

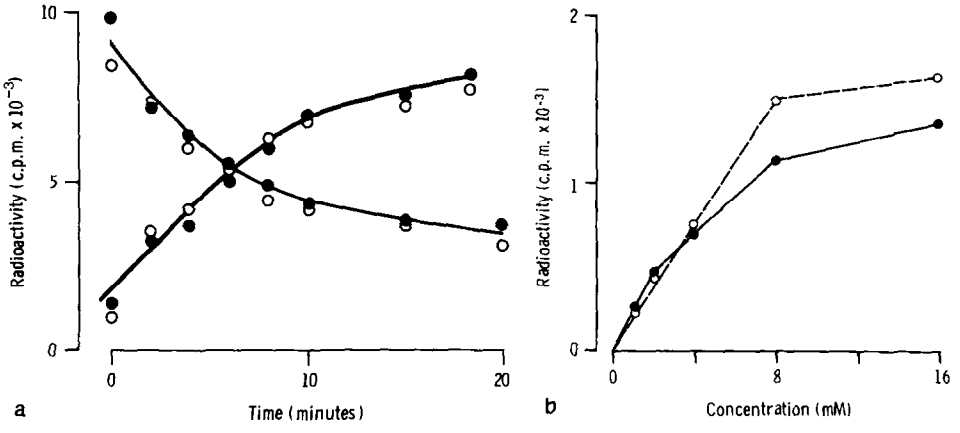

Fig. 5a: Uric acid (0.5mM) transport across the membranes of normal erythrocytes (open circles) and resealed erythrocyte 'ghosts' (closed circles).

Fig. 5b: Effect of increasing urate concentrations on initial rate (first minute) of influx of normal erythrocytes (open circles) and resealed erythrocyte 'ghosts' (closed circles).

Equilibrium was reached after 20 minutes with influx and efflux respectively. Initial transport rate measurements showed linear relationships with external concentration up to 8mM, but tended to plateau above this concentration (figure 4b). Erythrocytes or 'ghosts' of patients (n=2) with untreated phenylketonuria showed identical transport properties to cells from healthy volunteers.

Uric acid transport was identical in both normal erythrocytes and resealed 'ghosts' with symmetrical influx and efflux (figure 5a). Steady-state levels were reached after 40 minutes with both types of cels. Initial rate of influx increased with external concentrations in a linear manner (figure 5b).

DISCUSSION

It has been suggested that the effectiveness of enzyme-loaded erythrocyte 'ghosts' in the degradation of elevated circulatory molecules could be limited by the transport of the relevant substrates across the erythrocyte 'ghosts' membranes (9). There are definite differences between the 'ghost' preparation used in the present studies and white 'ghosts' obtained by the other methods (5,9). These differences may be critical because components in the haemolysate are thought to be responsible for the

stabilization of the membrane and structural alterations in the membranes were found to be a function of the number of washing steps (13, 18).

Carrier-mediated D-glucose transport survived the hypo-osmotic dialysis procedure relatively well and inhibition by phloridzin could be demonstrated. Similarly, haemoglobin-free erythrocyte 'ghosts' prepared by hypo-osmotic as well as iso-osmotic procedures have been reported to compare rather well with normal erythrocytes (2, 10, 13). Removal of the internal contents of the red cell had a marked effect on the symmetry of the sugar transport system and it was deduced that haemoglobin may affect the sugar transport system (14).

Sodium transport across normal erythrocyte membranes followed the same kinetics and magnitudes which are well established (7). Sodium efflux in haemoglobin-containing erythrocyte 'ghosts', correspond favourably with the findings in 'ghosts' prepared by direct dilution haemolysis and also with those in normal erythrocytes (7, 9). The efflux of sodium-ions can be divided into three components: active and passive transport and exchange diffusion (7) and the results of our efflux experiments suggest that these mechanisms are unaltered in our 'ghost' preparations. The differences in sodium influx between normal erythrocytes and resealed erythrocyte 'ghosts' may be connected with the disturbance in cation balances. Phosphate transport could not be inhibited by phloridzin in these erythrocyte 'ghost' preparations and this is in accord with studies on haemoglobin-free erythrocyte 'ghosts' (13).

L-Phenylalanine transport into normal erythrocytes was similar to results reported by others (6, 19). Results on L-phenylalanine efflux showed symmetrical kinetics, but comparable data on this subject and on transport in resealed erythrocyte 'ghosts' were not found in the literature. The saturation effect seen with high external concentrations indicated that transport of L-phenylalanine may be carrier mediated.

Results on uric acid transport into normal erythrocytes were in agreement with the findings of others (9, 11,12). It is already established that the urate influx is temperature dependent, linearly proportional to plasma

concentrations and reversible. We confirmed that the initial rate of influx increases linearly with external concentration and that efflux and influx show symmetrical characteristics. Whereas we could not observe any differences in influx and efflux between normal erythrocytes and resealed erythrocyte 'ghosts', a two-fold increase in influx has been obtained with 'ghosts' prepared by direct dilution in comparison to normal red cells (9).

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