Organic-acid transport in resealed haemoglobin-containing human erythrocyte 'ghosts'

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The transport of organic acids across the membrane of resealed haemoglobin-containing erythrocyte 'ghosts' prepared by a dialysis technique has been studied. The present work forms part of studies directed towards the use of erythrocyte cellular carriers in enzyme-replacement therapy of inherited metabolic diseases. Oxalic acid, glycollic acid and glyoxylic acid were taken as representative of aliphatic acids of low molecular mass and benzoic and cinnamic acids as representative of unsubstituted aromatic acids. These selected acids are important in the diseases with which the present work is concerned. Comparison of influx and efflux transport characteristics showed that erythrocyte 'ghosts' retain transport properties closely similar to those of normal erythrocytes. Rapid transport was observed with all organic acids studied and there was a linear relationship between initial amount of influx and external concentration of aliphatic acid. Saturation of the transport system was not observed up to 1 mM external concentration, and the presence of plasma in the external medium had no effect on transport characteristics. Transport in intact erythrocytes and prepared erythrocyte 'ghosts' from patients with hyperoxaluria was also studied.

The use of resealed erythrocyte 'ghosts' as carriers of therapeutic enzymes in the treatment of patients with inherited metabolic diseases has been suggested (Ihler *et al.*, 1973; Zimmermann *et al.*, 1976). Encapsulation of the enzyme inside erythrocyte 'ghosts' may protect against the rapid inactivation and immunological reactions that are observed when free enzyme or poorly protected enzyme is administered (Scriver, 1977; Desnick *et al.*, 1977). The erythrocyte 'ghost' is easily obtained, is biodegradable and is non-immunogenic if the patients' own erythrocytes are used.

We have been investigating the feasibility of using enzyme-loaded erythrocyte 'ghosts' as the actual site of degradation *in vivo* of potentially toxic small molecules in the circulation. This could be applied to the therapy of several inherited diseases of which primary hyperoxaluria type I (glycollicaciduria) is an example. This disease is characterized by an accumulation of glyoxylic acid and its oxidation and reduction products, oxalic acid and glycollic acid respectively, in tissues, blood and urine. Increased concentrations of oxalate in the tissues lead to the

deposition of its insoluble calcium salt in the kidneys and other organs (Watts, 1973). This results in progressive nephrocalcinosis and death from renal failure and heart block. Present methods of treatment are directed towards lowering the tendency of oxalate crystallization in the urine (Dent & Stamp, 1970) or the inhibition of oxalate biosynthesis (Watts *et al.*, 1979) and have met with only limited success in the majority of cases.

Therapy involving enzyme-loaded erythrocyte 'ghosts' could be directed towards decreasing concentrations of oxalic acid or its precursor glyoxylic acid in blood by encapsulation of oxalate decarboxylase (oxalate carboxy-lyase, EC 4.1.1.2) or glyoxylate reductase (glycollate-NAD⁺ oxidoreductase, EC 1.1.1.26).

Two important factors that must be considered with this form of therapy are the entrapment of sufficient active enzyme inside the erythrocyte 'ghosts' and the transport of the relevant substrates and products across the erythrocyte 'ghost' membrane (Ihler *et al.*, 1975).

We have shown previously that large amounts of ¹²⁵I-labelled human serum albumin could be entrapped inside erythrocyte 'ghosts' by using a dialysis technique. The results indicate that the

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entrapment of sufficient enzyme is not a limiting factor (Sprandel *et al.*, 1979a).

The present paper reports results of studies on the transport of organic acids across the membrane of haemoglobin-containing erythrocyte 'ghosts' and normal erythrocytes. Oxalic acid, glycollic acid and glyoxylic acid were selected as representative of aliphatic acids of low molecular mass and the transport of two aromatic acids, benzoic and cinnamic acids, was also examined as representative of that of unsubstituted aromatic acids of potential interest in other inherited metabolic diseases, e.g., hyperphenylalaninaemia (Sprandel *et al.*, 1979b).

Part of the present work has been published in abstract form (Hubbard *et al.*, 1979).

Experimental

Materials

Blood was collected freshly, and with informed consent, from human volunteers, by using EDTA as anticoagulant (1.5 mg/ml of blood). Radioactive ^{14}C -labelled organic acids ([U- ^{14}C]oxalic acid, [1- ^{14}C]glyoxylic acid, [1- ^{14}C]glycollic acid and [ring-U- ^{14}C]benzoic acid) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Labelled [2- ^{14}C]cinnamic acid was obtained from ICN Pharmaceuticals through Laboratory Impex, Twickenham, Middx., U.K.

Furosemide was obtained as Lasix in 2 ml ampoules (20 mg) from Hoechst U.K., Pharmaceutical Division, Hounslow, Middx., U.K.

PPO (2,5-diphenyloxazole) and POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene] were obtained from Nuclear Enterprises, Edinburgh, Scotland, U.K. Naphthalene and 1,4-dioxan were obtained from Koch-Light Laboratories, Colnbrook, Bucks., U.K. All other reagents were from BDH Chemicals, Poole, Dorset, U.K.

Preparation of erythrocyte 'ghosts'

The procedure was very similar to that described previously (Sprandel *et al.*, 1979a). Erythrocytes were washed twice (1100 g, 10 min) in phosphate-buffered saline (0.15 M-NaCl/5 mM- $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, pH 7.4) at 4°C. The packed erythrocytes obtained after the second wash were placed in a dialysis bag with phosphate-buffered saline to give a cell/buffer (v/v) ratio of 7:3.

Lysis of the erythrocytes was obtained by dialysing 5 ml of the resuspended erythrocytes against 150 ml of hypo-osmotic 5 mM- $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ solution, pH 7.4, for 105 min at 4°C. Resealing was obtained by subsequent dialysis against 150 ml of iso-osmotic phosphate-buffered saline, pH 7.4, for 30 min at room temperature.

The erythrocyte 'ghosts' were washed three times (1100 g, 10 min) in phosphate-buffered saline at 20°C.

Transport measurements

Experiments were carried out at 37°C for aliphatic organic acids and at 4°C for aromatic acids.

Substrate influx was monitored by incubating packed erythrocyte 'ghosts' mixed immediately with an equal volume of the relevant medium containing ^{14}C -labelled organic acid.

Aliphatic acid transport was studied by taking sequential samples (0.2 ml) from the same reaction mixture and adding these to 1 ml of ice-cold phosphate-buffered saline to terminate transport. This was followed by immediate centrifugation (15000 g/10 s) in an Eppendorf Microcentrifuge 3200. The extracellular buffer volume associated with the cell pellet obtained by this procedure is less than 5 μl . The pelleted cells were lysed with distilled water (0.1 ml) and protein was precipitated with 5% (w/v) trichloroacetic acid (0.4 ml). After centrifugation, the clear supernatant was sampled (0.25 ml), added to 10 ml of scintillation fluid and radioactivity was measured in a Nuclear-Chicago Mark-I liquid-scintillation counter to a minimum of 10^4 counts. Aromatic acid transport was studied similarly but by use of individual experiments for each datum point taken.

Scintillation fluid consisted of 100 g of naphthalene, 5 g of PPO and 0.05 g of POPOP in 1 litre of 1,4-dioxan.

Organic-acid efflux was monitored by firstly allowing the resealed erythrocyte 'ghosts' to become equilibrated with the ^{14}C -labelled organic acid in iso-osmotic phosphate-buffered saline, pH 7.4, separated by centrifugation and resuspended in an equal volume of phosphate-buffered saline containing no organic acid. Samples were taken and treated as before.

Control experiments were carried out by using normal washed erythrocytes throughout.

Initial amount of influx

This was measured by incubating a given volume of packed erythrocyte 'ghosts' with an equal volume of medium containing a constant amount of ^{14}C -labelled substrate together with various amounts of unlabelled organic acid to give a range of concentrations. After a timed incubation period (30 s for oxalic acid, 40 s for glyoxylic acid and glycollic acid) a sample was taken and treated as described above, except that the pelleted cells were washed twice in ice-cold phosphate-buffered saline before being lysed. This minimizes the contamination by external radioactivity trapped in the pelleted cells.

Results

Transport of aliphatic organic acids

The three acids (oxalic, glyoxylic and glycollic) showed very similar transport characteristics. In

each case, the transport appeared freely reversible, giving rise to the symmetrical influx and efflux curves (Fig. 1). The acids crossed the erythrocyte-'ghost' membrane rapidly, since equilibrium states

Fig. 1. Transport of aliphatic organic acids across the erythrocyte membrane

Influx (○, ●) and efflux (□, ■) of (a) oxalic acid, (b) glycollic acid (c) glyoxylic acid in resealed erythrocyte ghosts (●, ■) and normal erythrocytes (○, □) at 37°C. Transport was monitored by measuring intracellular radioactivity. Incubation media for influx experiments consisted of iso-osmotic phosphate-buffered saline with the relevant organic acid [oxalic acid ($38\mu\text{M}$ and $0.5\mu\text{Ci/ml}$), glycollic acid and glyoxylic acid (both $45\mu\text{M}$ and $0.25\mu\text{Ci/ml}$)].

Fig. 2. Effect of varying external concentrations on the initial amount of influx of aliphatic organic acids

Oxalic acid (a), glycollic acid (b), and glyoxylic acid (c) were studied with resealed erythrocyte ghosts (●) and normal erythrocytes (○) at 37°C. Incubation media contained a constant amount of labelled acid ($0.5\mu\text{Ci/ml}$) with various amounts of unlabelled acid.

were reached after about 90 s with oxalic acid ($38\mu\text{M}$) and after about 120 s with glyoxylic acid and glycollic acid (both at $45\mu\text{M}$). We found no differences in transport between normal erythrocytes and resealed erythrocyte 'ghosts', except for the difference in glyoxylate efflux (Fig. 1c).

Fig. 3. Effect of plasma and of furosemide on oxalic acid influx

Oxalic acid ($38\mu\text{M}$ and $2.5\mu\text{Ci/ml}$ of medium) influx into resealed erythrocyte ghosts were studied at 37°C with plasma as the incubation medium (■), with phosphate-buffered-saline medium (●) and with phosphate-buffered-saline medium in the presence of 2 mM-furosemide (▲).

Fig. 4. Effect of increasing the external concentrations of glyoxylic acid on the initial amount of influx of oxalic acid

Oxalic acid ($38\mu\text{M}$ and $2.5\mu\text{Ci/ml}$ of medium) influx into resealed erythrocyte ghosts (●) and normal erythrocytes (○) was examined at 37°C in the presence of increasing concentrations of glyoxylic acid (0–10 mM). The initial amount of influx was determined as the amount of radioactivity in the cells after 10 s incubation.

A linear relationship existed between the initial amount of influx and the external organic-acid concentration for all three acids (Fig. 2). This suggests that no saturation occurs, at least with external concentrations up to about 1 mM, which are much higher than the observed physiological and pathological ranges.

Blood plasma has no effect on oxalic acid influx, this being of importance in the therapeutic situation *in vivo*, where the surrounding medium will be plasma (Fig. 3). However, oxalic acid influx could be affected by the presence of furosemide (2 mM) in normal incubation medium (Fig. 3).

To determine whether the presence of a similar organic acid (glyoxylic acid) could affect the transport of oxalic acid, we examined the effects of increasing external concentrations of unlabelled glyoxylic acid on the initial amount of oxalic acid influx at a fixed concentration ($38\mu\text{M}$) (Fig. 4). We found that increasing external concentrations of glyoxylic acid resulted in an increased initial amount of influx of oxalic acid.

Since the use of the patients' own erythrocytes has been proposed, we examined the transport of oxalic acid and glycollic acid into erythrocyte 'ghosts' and normal erythrocytes derived from the blood of a patient with primary hyperoxaluria type I (glycollic aciduria). Transport was identical with that in cells from healthy donors.

Transport of aromatic acids

Benzoic and cinnamic acids crossed the erythrocyte-'ghost' membrane much more rapidly than did the three aliphatic acids. It was for this reason that transport was examined at 4°C . Equilibrium states for benzoic acid transport ($10\mu\text{M}$)

Fig. 5. Transport of benzoic acid across the erythrocyte membrane

Benzoic acid ($10\mu\text{M}$ and $1.0\mu\text{Ci/ml}$ of medium) influx into resealed erythrocyte 'ghosts' (●) and normal erythrocytes (○) at 4°C .

were reached after about 40 s with both normal erythrocytes and resealed erythrocyte 'ghosts' (Fig. 5).

Cinnamic acid transport was extremely rapid and could not be measured by the method used, since equilibrium states appeared to have been reached by about 5 s. Only by comparing the radioactivity of the cell pellet before and after efflux could we confirm that in fact transport had occurred.

Discussion

The present studies have shown that the erythrocyte 'ghosts' prepared by a dialysis technique possess transport characteristics identical with normal erythrocytes, at least with regard to the organic acids studied. This suggests that the preparative procedure did not affect the relevant membrane properties, not did it leave the membrane more 'leaky' to these organic acids. This is in agreement with other findings on the transport of D-glucose, Na⁺, L-phenylalanine and uric acid in identical erythrocyte 'ghosts' when compared with normal erythrocytes (Sprandel *et al.*, 1979b).

The transport of organic acids into normal bovine erythrocytes has been studied extensively by others (Giebel & Passow, 1960; Aubert & Motais, 1975). Similar studies have not been previously reported on human erythrocytes. The results reported in the present paper, obtained by using human erythrocytes and erythrocyte 'ghosts', showed comparative rates of transport between aliphatic and aromatic organic acids similar to those observed by Aubert & Motais (1975) in bovine erythrocytes.

It has been suggested (Aubert & Motais, 1975) that the rapid transport of the aromatic acids, e.g., benzoic and cinnamic acids, may be due to transport in the undissociated form, with passage through the lipid phase of the membrane at rates proportional to their lipid solubility. Transport of the three aliphatic acids was not as rapid as that of the aromatic acids and this may be due to transport in the dissociated anionic form when rapid passage through the membrane lipid phase alone is not possible. In such circumstances, Aubert & Motais (1975) have suggested that a strongly polar head is required for the transport of small organic anions, this allowing the anion to bind with a specific membrane component responsible for transport. Such a strongly polar head is provided by the presence of a carboxy group along with a suitable complementary group such as a second carboxy group (oxalate), an aldo group (glyoxylate) or a hydroxy group (glycollate).

The linear relationships observed between initial amount of influx and the external organic acid concentration under non-equilibrium conditions for oxalic acid, glycollic acid and glyoxylic acid demon-

strate that no saturation occurs over the concentration range used (up to 1 mM). This is important in the context of enzyme therapy of primary hyperoxaluria, since the concentration ranges studied exceed those found in patients with the disease. The results obtained here are in agreement with the observations of Deuticke (1973) who found a linear relationship between rate of efflux from pre-equilibrated bovine erythrocytes and internal concentration of lactate and glycollate, up to about 30 mM. Influx of ¹⁴C-labelled oxalic acid is apparently stimulated by increasing extracellular glyoxylic acid concentrations in a manner analogous to the stimulation observed by using increasing extracellular oxalic acid concentrations. This suggests that these organic anions share the same transport mechanism. The inhibitory action on oxalate transport in both 'ghosts' and normal erythrocytes of the unsubstituted sulphonamide furosemide, which acts as a specific inhibitor of anion transport in erythrocytes (Cousin & Motais, 1976) further confirms the retention of the same transport system in the prepared erythrocyte 'ghosts'.

Any binding of oxalate to plasma proteins could affect the transport into the erythrocyte 'ghost' and so lower the effectiveness of the enzyme-loaded erythrocyte 'ghosts' *in vivo*. However there appears to be little or no such binding, and this confirms the findings of others (Hodgkinson & Zaremski, 1968).

Effective therapy with enzyme-loaded erythrocytes in the present context depends not only on the rapid entry of substrates, but also on the rapid exit of degradation products in order to prevent any possible product inhibition of the encapsulated enzyme. We have demonstrated that organic acids satisfy these requirements. Moreover, the fact that resealed erythrocyte 'ghosts' prepared from the patients' own erythrocytes also allow rapid passage of these acids confirms their suitability for use as potential enzyme carriers *in vivo*.

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References

- Aubert, L. & Motais, R. (1975) *J. Physiol. (London)* **246**, 159–170
- Cousin, J. L. & Motais, R. (1976) *J. Physiol. (London)* **256**, 61–80

- Dale, G. L., Villacorte, D. G. & Beutler, E. (1977) *Biochem. Med.* **18**, 220–225.
- Dent, C. E. & Stamp, T. C. B. (1970) *Arch. Dis. Childhood* **45**, 735–745
- Desnick, R. J., Fiddler, M. B., Thorpe, S. R. & Steger, L. D. (1977) in *Biomedical Applications of Immobilised Enzymes and Proteins* (Chang, T. M. S., ed.), pp. 227–244, Plenum Press, New York
- Deuticke, B. (1973) in *Erythrocytes, Thrombocytes, Leukocytes* (Gerlach, E., Moser, K., Deutsch, E. & Wilmanns, W., eds.), pp. 81–87, Georg Thieme Publishers, Stuttgart
- Giebel, O. & Passow, H. (1960) *Pflügers Arch.* **271**, 378–388
- Hodgkinson, A. & Zarembski, P. M. (1968) *Calcif. Tissue Res.* **2**, 115–132
- Hubbard, A. R., Sprandel, U. & Chalmers, R. A. (1979) *Biochem. Soc. Trans.* **7**, 958–960
- Ihler, G. M., Glew, R. H. & Schnure, F. W. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 2663–2666
- Ihler, G. M., Lantzy, A., Purpura, J. & Glew, R. H. (1975) *J. Clin. Invest.* **56**, 595–602
- Scriver, C. R. (1977) in *Biomedical Applications of Immobilised Enzymes and Proteins* (Chang, T. M. S., ed.), pp. 121–146, Plenum Press, New York
- Sprandel, U., Hubbard, A. R. & Chalmers, R. A. (1979a) *Res. Exp. Med.* **175**, 239–245
- Sprandel, U., Hubbard, A. R. & Chalmers, R. A. (1979b) *Biochem. Biophys. Res. Commun.* **91**, 79–85
- Watts, R. W. E. (1973) *J. R. Coll. Physicians London.* **7**, 161–174
- Watts, R. W. E., Chalmers, R. A., Gibbs, D. A., Lawson, A. M., Purkiss, P. & Spellacy, E. (1979) *Q. J. Med.* **48**, 403–417
- Zimmermann, U., Riemann, F. & Pilwat, G. (1976) *Biochim. Biophys. Acta* **436**, 460–474