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Reconstitution of the L-lactate carrier from rat and rabbit erythrocyte plasma membranes

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1. Rat and rabbit erythrocyte plasma-membrane proteins were solubilized with decanoyl-*N*-methylglucamide and reconstituted into lipsomes. The procedure includes detergent removal by gel filtration, followed by a freeze–thaw step. 2. The rate of [1-^{14}C]pyruvate uptake into these vesicles was inhibited by approx. 70% by α-cyano-4-hydroxycinnamate and *p*-chloromercuribenzenesulphonate. The extent of uptake at equilibrium was not affected by the presence of these inhibitors, but was dependent on the osmolarity of the suspending medium. 3. Reconstituted bovine erythrocyte membranes, which have no lactate carrier, showed a much slower time course of pyruvate uptake, with no inhibitor-sensitive component. 4. L- but not D-lactate competed for α-cyano-4-hydroxycinnamate-sensitive [1-^{14}C]pyruvate uptake.

INTRODUCTION

The transport of L-lactate across the plasma membrane is of great physiological importance to many cells. The involvement of a carrier in this process was first demonstrated by Halestrap & Denton (1974), who found α-cyano-4-hydroxycinnamate to inhibit specifically L-lactate uptake into human erythrocytes. Although lactate can cross the erythrocyte membrane on the inorganic-anion exchanger (Halestrap, 1976; Deuticke, 1982), it appears that this represents only a minor contribution to total lactate flux at physiological concentrations (Deuticke *et al.*, 1982). Rather, the major route is via a specific carrier which transports L-lactate across the membrane with a proton (Halestrap, 1976; Dubinsky & Racker, 1978; Leeks & Halestrap, 1978). This carrier can catalyse both net transport and exchange, and is inhibited by α-cyanocinnamate derivatives, organomercurials and amino-group reagents (see Deuticke, 1982). There is evidence for the presence of a similar specific carrier in rat hepatocytes (Monson *et al.*, 1982; Fafournoux *et al.*, 1985; Edlund & Halestrap, 1988) and a number of other tissues (for references, see Edlund & Halestrap, 1988). In addition, there is a Na^+-dependent lactate carrier in both kidney and intestine brush borders (Barac-Nieto *et al.*, 1980; Hildmann *et al.*, 1980; Storelli *et al.*, 1980; Nord *et al.*, 1982; Mengual *et al.*, 1983; Jorgensen & Sheikh, 1984).

Although the kinetic properties of the human erythrocyte (Halestrap, 1976; Deuticke, 1982; De Bruijne *et al.*, 1983, 1985) and rat hepatocyte (Monson *et al.*, 1982; Fafournoux *et al.*, 1985; Edlund & Halestrap, 1988) carriers have been investigated in some detail, there have been few reported attempts to identify the transport protein(s) involved in these processes. Jennings & Adams-Lackey (1982) were able to demonstrate labelling of a protein of subunit molecular size 40–50 kDa with [^{3}H]-4,4′-di-isothiocyano-2,2′-stilbenedisulphonate ([^{3}H]H_2DIDS), paralleled by inhibition of L-lactate transport, in rabbit erythrocyte ghosts. Subsequently the transport inhibitors isobutylcarbonyl-lactyl anhydride and bis-(sulphosuccinimido)suberate were shown to decrease labelling of this band by [^{3}H]H_2DIDS (Donovan & Jennings, 1985, 1986). However, it is unlikely that these inhibitors are specific for the L-lactate carrier. Welch *et al.* (1984) have reported the surprising observation that [^{14}C]lactate and [^{14}C]pyruvate themselves bind to a protein of the rat hepatocyte plasma membrane, and remain bound during SDS/polyacrylamide-gel electrophoresis. The binding, which was to a protein of 40 kDa subunit molecular size, was inhibited by α-cyano-3-hydroxycinnamate. Welch *et al.* (1984) suggested that this polypeptide may be involved in lactate transport.

To identify positively the protein component(s) required for transport, it is necessary to purify the lactate carrier in an active form. However, to assay specific transport activity, solubilized and fractionated membrane proteins must be reconstituted into lipsomes. Many methods exist for the reconstitution of transport proteins, and these, along with the difficulties involved, have been reviewed in detail by Koepsell (1986). We have developed the method described by Lynch & McGivan (1987) to reconstitute the lactate carrier from rat and rabbit erythrocytes. Rat erythrocytes were chosen for these studies, since they are known to have a very high lactate-transport activity (Deuticke *et al.*, 1978). To our knowledge, functional reconstitution of the lactate carrier has not been reported previously, although Koepsell *et al.* (1984) have succeeded in reconstituting Na^+-dependent lactate transport activity from renal brush-border membranes.

EXPERIMENTAL

Materials

Sodium citrate was obtained from BDH Chemicals, Poole, Dorset, U.K. Sodium pyruvate was obtained from Boehringer, Lewes, U.K. All other chemicals and biochemicals were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Cellulose nitrate filters (pore size 0.45 μm) were obtained from Whatman, Maidstone,

Abbreviations used: pCMBS, *p*-chloromercuribenzenesulphonate; MEGA-10, decanoyl-*N*-methylglucamide; DIDS, 4,4′-di-isothiocyanostilbene-2,2′-disulphonate; H_2DIDS, 4,4′-di-isothiocyano-2,2′-dihydrostilbenedisulphonate.

Kent, U.K. The detergent MEGA-10 was prepared by the method of Hildreth (1982), and used after a single recrystallization from diethyl ether/methanol (9:1, v/v). Crude egg-yolk phospholipids were prepared by the method of Dawson (1963) and stored as small portions at −20 °C under N_2 in chloroform containing 0.1% (w/v) butylated hydroxytoluene. [1-^{14}C]Pyruvate (sp. radioactivity 30 mCi/mmol) was obtained from Amersham International, Amersham, Bucks., U.K., and was stored in freeze-dried portions of 2.5 μCi at −20 °C.

Methods

Preparation of membranes. Blood from freshly killed rats or rabbits was diluted in 4 vol. of sodium citrate buffer (84 mM-sodium citrate/1 mM-EGTA, adjusted to pH 7.4 with NaH_2PO_4) and centrifuged at 10000 *g* at 4 °C for 5 min, to sediment the cells. The erythrocytes were then resuspended in 5–10 vol. of the same buffer containing 15 μM-DIDS and incubated for 1 h at 37 °C. This concentration of DIDS is insufficient to inhibit lactate transport in rat erythrocytes (results not shown), but will irreversibly inhibit the inorganic-anion exchanger (Halestrap, 1976). After sedimentation of the erythrocytes, they were given a further wash in sodium citrate buffer to remove non-bound inhibitor. The cells were lysed in 10 vol. of ice-cold 5 mM-sodium phosphate/1 mM-benzamidine, pH 8.0, and the ghost membranes were sedimented by centrifugation at 20000 *g* for 10 min at 4 °C. The membranes were washed a further four or five times in this buffer before storage at −70 °C. This method was also used to prepare membranes from fresh bovine erythrocytes obtained from a slaughterhouse.

Reconstitution. The method used was based on that described by Lynch & McGivan (1987) for the reconstitution of Na^+-dependent neutral amino acid transport activity from bovine renal brush-border membranes. It was carried out at 4 °C in a buffer containing 20 mM-NaMops, 1 mM-EGTA, 1 mM-pyruvate, 0.1 mM-dithiothreitol, pH 7.4 (reconstitution buffer) throughout. Pyruvate was included in an attempt to protect the carrier during solubilization, as has been shown for substrates of other transport proteins (see, e.g., Koepsell *et al.*, 1983). DIDS-pretreated ghosts were solubilized at a protein concentration of 5 mg/ml in reconstitution buffer containing 1% (w/v) MEGA-10, 2 mM-benzamidine and 0.2 mM-phenylmethanesulphonyl fluoride. Non-solubilized protein was removed by centrifugation at 150000 *g* for 20 min at 4 °C, the supernatant being taken and assayed for protein content.

Cholesterol was added to the chloroform solution of phospholipids to 15% (mol/mol), and the solvent was evaporated under a stream of N_2. The lipid was resuspended in reconstitution buffer and sonicated on ice until translucent. Aggregated material was removed by centrifugation at 150000 *g* for 20 min at 4 °C. The supernatant was taken and assayed for lipid phosphate.

Solubilized protein and liposomes were mixed at a ratio of 15 μmol of lipid phosphate/mg of protein, and 2 ml portions applied to a Sephadex G-50 (coarse grade) column (30 cm × 1.5 cm) equilibrated in reconstitution buffer. The turbid fractions eluted in the void volume were collected, and diluted 3–6-fold in buffer before freezing in liquid N_2. This mixture was rapidly thawed in a shaking water bath at 37 °C, and the proteoliposomes were collected by centrifugation at 50000 *g* for 30 min at 4 °C. The fluffy white pellet was then resuspended to a protein concentration of approx. 1–1.5 mg/ml for the transport assay. Modifications to this reconstitution procedure are noted in the text or Figure legends.

Assay of reconstituted transport activity. Carrier-mediated transport was measured as inhibitor-sensitive uptake of [1-^{14}C]pyruvate in exchange for intravesicular pyruvate, by using a rapid filtration technique. Pyruvate was used since it has a higher affinity for the carrier than L-lactate (Edlund & Halestrap, 1988) and a lower pK_a value, which should decrease the contribution of free diffusion of the undissociated acid to uptake. All incubations were carried out at 20 °C. For each incubation, 10 μl of proteoliposomes (about 15 μg of protein) in reconstitution buffer was mixed with 30 μl of [1-^{14}C]-pyruvate (0.15 μCi) in 20 mM-NaMops/1 mM-EGTA/0.1 mM-dithiothreitol, pH 7.4 (assay buffer). This gave an external pyruvate concentration of 0.4 mM, with an initial intravesicular concentration of 1 mM. Uptake was stopped by addition of 0.6 ml of ice-cold reconstitution buffer containing 2 mM-α-cyano-4-hydroxycinnamate, and the mixture was applied to a 0.45 μm-pore-size nitrocellulose filter under reduced pressure. The filter was washed with 3 ml of the same buffer and then counted for radioactivity in 10 ml of scintillation fluid after being allowed to dissolve. Uptake at zero time, presumed to be due to binding, was determined by mixing the stopping solution with the proteoliposomes before addition of the [1-^{14}C]pyruvate solution, and rapidly filtering. Uptake of [1-^{14}C]pyruvate in the presence of α-cyano-4-hydroxycinnamate was measured by including the inhibitor at 2 mM concentration in both the proteoliposome suspension and radioactive pyruvate solution. Modifications to this protocol are noted in the text or Figure legends. Data shown are representative of at least two separate experiments, each point representing the mean of duplicate determinations agreeing within 10%.

Analytical procedures. SDS/polyacrylamide-gel electrophoresis was performed by the method of Laemmli (1970). Samples were precipitated with 10% (w/v) trichloroacetic acid, and washed with 0.2 M-Na_2HPO_4 before solubilization in 0.062 M-Tris/HCl, containing 20% (w/v) sucrose, 5% (w/v) SDS, 5% (v/v) mercaptoethanol and 0.02% Bromophenol Blue, pH 6.8. For reconstituted membrane vesicles, phospholipid was removed by washing the trichloroacetic acid precipitate with chloroform/methanol (2:1, v/v). Protein was measured by the method of Bradford (1976). Lipid phosphate was assayed as described by Chen *et al.* (1956), after digestion with H_2SO_4/$HClO_4$ (Lynch & McGivan, 1987).

RESULTS

The data of Fig. 1 demonstrate typical time courses for the uptake of pyruvate into proteoliposomes reconstituted from rat and bovine ghosts prepared from erythrocytes pretreated with a low concentration of DIDS. With reconstituted rat ghosts, there is a very rapid uptake of pyruvate which is greatly inhibited by the well-characterized transport inhibitors α-cyano-4-hydroxycinnamate and *p*-chloromercuribenzenesulphonate (pCMBS). Some variation in the absolute

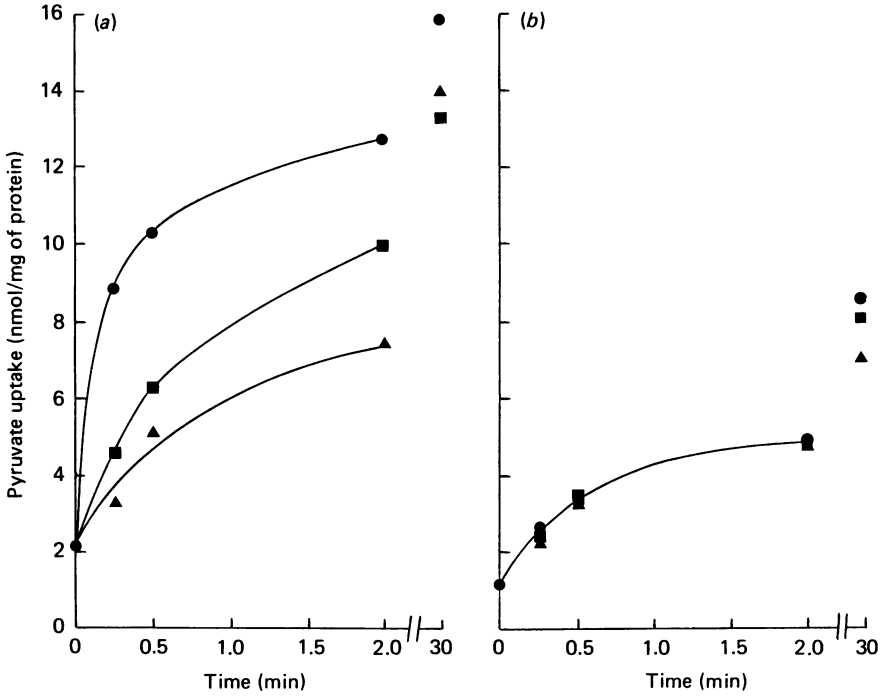

Fig. 1. Pyruvate transport into proteoliposomes reconstituted from rat and bovine erythrocyte ghost proteins

Reconstitution and assay of transport activity were performed as described under 'Methods'. For pCMBS, the proteoliposomes were preincubated with the inhibitor at 0.5 mM for 5 min at 20 °C before assay. The Figure shows time courses of pyruvate uptake for reconstituted rat (*a*) and bovine (*b*) erythrocyte ghost proteins. Assays were performed in the absence (●) or the presence of 2 mM-α-cyano-4-hydroxycinnamate (▲) or 0.5 mM-pCMBS (■).

values for inhibitor-sensitive pyruvate uptake was found between experiments. This probably reflects variation in both the intravesicular volume and the extent of incorporation of active carrier into the proteoliposomes. However, the proportion of uptake which was inhibited by 2 mM-α-cyano-4-hydroxycinnamate (after correction for binding at zero time) was quite consistent; in four separate experiments uptake at 10 s was inhibited by 65.2±2.5% (mean±S.E.M.). The data of Fig. 1 also show that the inhibitors have no significant effect on the extent of pyruvate uptake into the vesicles at equilibrium. Thus the uptake at 30 min in the presence of 2 mM-α-cyano-4-hydroxycinnamate, expressed as a percentage of the value in the absence of inhibitor, was 95.6±6.3% (mean±S.E.M., $n = 4$). We found that when a higher concentration of pyruvate was used in the assay the proportion of α-cyano-4-hydroxycinnamate-sensitive pyruvate transport was decreased. Thus at 30 s 2 mM-α-cyano-4-hydroxycinnamate inhibited uptake by 58± 2.5% at 0.4 mM-pyruvate, and by 38.4±2.3% at 1.4 mM-pyruvate (values expressed as means±S.E.M. for four separate experiments). This difference is largely due to an increase in the inhibitor-insensitive component of uptake, presumed to be due to diffusion of the free acid. The time course for pyruvate uptake into vesicles reconstituted from bovine ghosts (Fig. 1*b*) was similar to the inhibitor-insensitive component in rat, and was unaffected by the presence of inhibitors. Bovine erythrocytes have been shown to have no specific L-lactate carrier (Deuticke *et al.*, 1978; R. C. Poole, unpublished work).

The data of Fig. 2 show the effect of the addition of sucrose to increase the osmolarity of the assay buffer on [1-^{14}C]pyruvate uptake by proteoliposomes reconstituted from rat erythrocyte membranes. Such manipulations would be expected to cause the intravesicular volume to decrease and hence decrease pyruvate uptake at equilibrium. This was found to be the case, the uptake in the presence of 1 M-sucrose at 30 min (corrected for binding at zero time) being 1.63 nmol/mg of protein, as compared with a control value of 13.92 nmol/mg of protein for the experiment shown. This confirms that the radioactivity retained on the filter is largely contained within an osmotic space rather than bound to the membranes. Pyruvate uptake at 10 s in the presence of 50 mM-sucrose (about double the osmolarity of the intraliposomal milieu) was similar to that of the control. This is not surprising, since shrinkage of the vesicles will not change the surface area, and hence the initial rate of pyruvate uptake will be the same in all cases, whereas equilibration is more rapid the smaller the vesicle becomes.

We have investigated the effect of solubilization with different concentrations of MEGA-10 on α-cyano-4-hydroxycinnamate-sensitive pyruvate transport into proteoliposomes reconstituted from rat erythrocyte ghosts. Activity was similar when 0.5% or 1.0% MEGA-10 was used, but was consistently less if 1.5% detergent was used (results not shown). The solubilized protein concentrations were 1.0, 2.4 and 2.75 mg/ml respectively. Since there appeared to be no selective solubilization of the lactate carrier up to 1.0% MEGA-10, this concentration was routinely used to give the maximum yield of solubilized carrier. It was also found that inclusion of cholesterol with the phospholipid increased inhibitor-sensitive uptake, while decreasing the inhibitor-insensitive component, presumed to be due to diffusion of the free acid (Fig. 3). Mixing liposomes with solubilized protein at ratios increasing from 10 to 30 μmol of lipid phosphate/mg of protein caused no

consistent effect on transport activity (results not shown).

Time courses for uptake of 0.4 mM-pyruvate in the presence of either 10 mM-L- or D-lactate are shown in Fig. 4. α-Cyano-4-hydroxycinnamate-sensitive pyruvate transport was considerably greater in the presence of D-

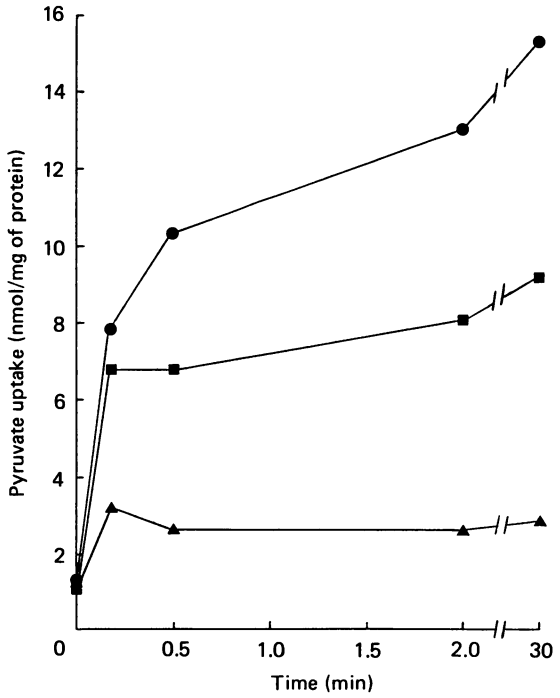

Fig. 2. Effect of the osmolarity of the assay medium on pyruvate uptake by reconstituted proteoliposomes

Rat erythrocyte membranes were solubilized and reconstituted as described in the Experimental section. Pyruvate uptake into the reconstituted vesicles was determined in the absence of α-cyano-4-hydroxycinnamate. The incubations were initiated by mixing 10 μl of proteoliposomes with 30 μl of [1-^{14}C]pyruvate in assay buffer without further additions (●) or in the presence of sucrose to give a final concentration of 50 mM (■) or 1 M (▲).

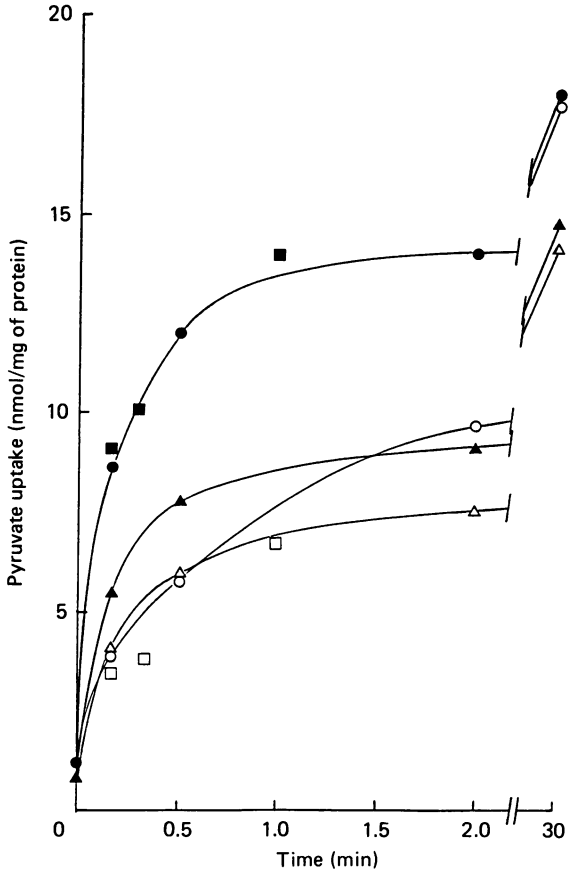

Fig. 4. Effect of L- and D-lactate on pyruvate uptake into proteoliposomes

The experimental protocol was as described under 'Methods', except that the [1-^{14}C]pyruvate buffer contained either L- (▲, △) or D- (●, ○) lactate to give a final concentration of 10 mM in the incubations. Assays were performed in the absence (▲, ●) or presence (△, ○) of 2 mM-α-cyano-4-hydroxycinnamate. Points are also shown for [1-^{14}C]pyruvate uptake with no added lactate present, in the absence (■) and the presence (□) of 2 mM-α-cyano-4-hydroxycinnamate, measured in a separate experiment on the same proteoliposome preparation.

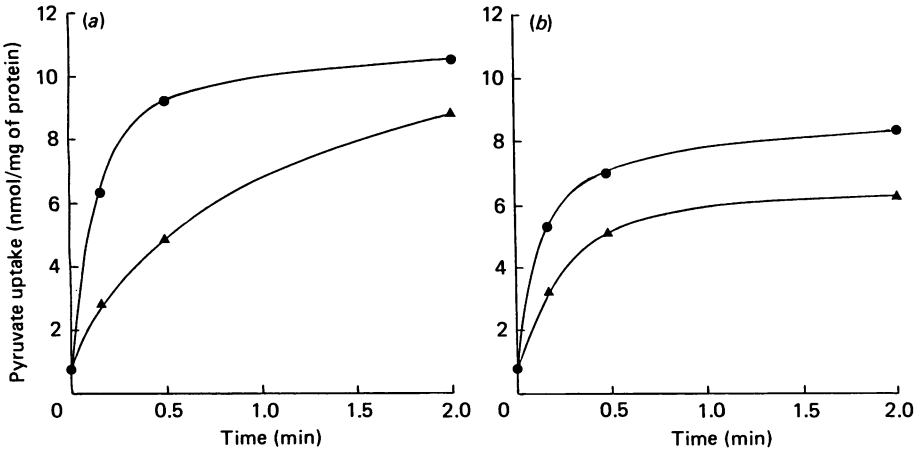

Fig. 3. Effects of cholesterol on pyruvate transport activity reconstituted from rat erythrocyte membranes

Reconstitution and assay of transport activity were performed as described in the text, except that liposomes were made with either phospholipid +15% (mol/mol) cholesterol (*a*) or phospholipid alone (*b*). Assays of reconstituted pyruvate transport activity were carried out in the absence (●) or the presence (▲) of 2 mM-α-cyano-4-hydroxycinnamate.

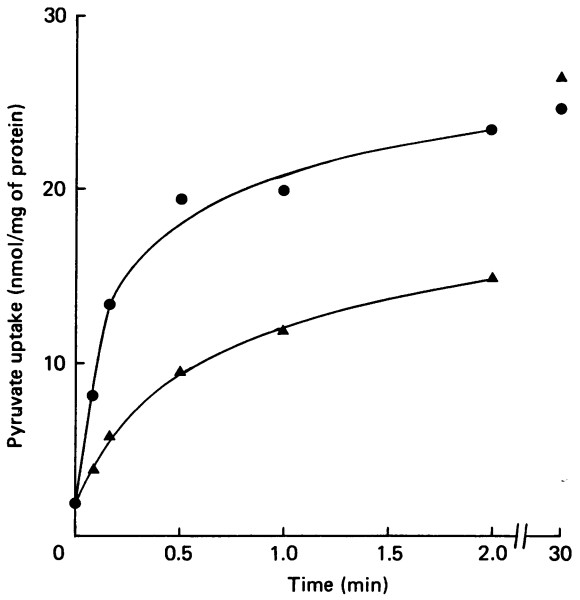

Fig. 5. Pyruvate transport into proteoliposomes reconstituted from rabbit erythrocyte ghosts

Reconstitution and assay of transport activity were performed as described under 'Methods'. Assays were performed in the absence (●) or the presence (▲) of 2 mM-α-cyano-4-hydroxycinnamate.

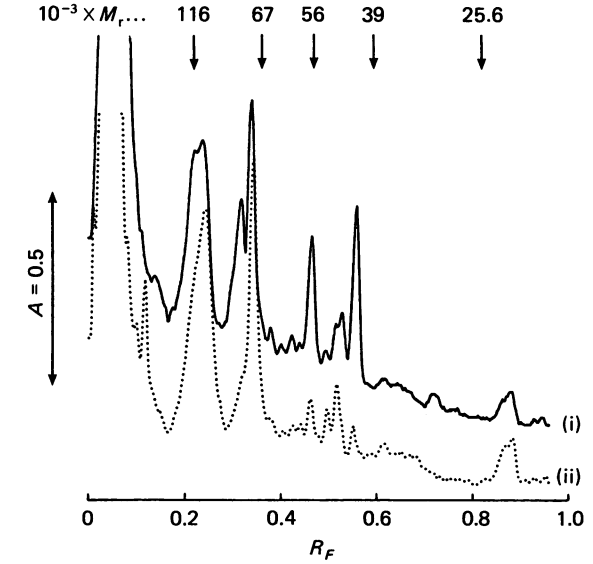

Fig. 6. SDS/polyacrylamide-gel electrophoresis of native and reconstituted membrane proteins from rat erythrocyte ghosts

Gel electrophoresis was performed by the method of Laemmli (1970), in 10% (w/v) polyacrylamide gels. After staining with Coomassie Brilliant Blue and drying down on to Cellophane, gels were scanned with a Joyce–Loebl Chromoscan instrument. The traces shown are: (i) native rat erythrocyte ghosts (continuous line) and (ii) rat ghost proteins reconstituted after solubilization with 1% MEGA-10 (dotted line). Arrows depict the R_F values of the molecular-mass markers used: β-galactosidase (116 kDa), bovine serum albumin (67 kDa), glutamate dehydrogenase (56 kDa), aldolase (39 kDa) and chymotrypsinogen A (25.6 kDa).

lactate than with L-lactate, the values being 4.95 and 1.5 nmol/mg of protein respectively at the 10 s time point. This compares with a value of 5.8 nmol/mg in the absence of added lactate, measured in a parallel experiment. There was no significant difference between the inhibitor-insensitive component of uptake in the presence of L- and D-lactate. These results show clearly that L-lactate stereospecifically competes with pyruvate for transport by the carrier.

A time course for pyruvate uptake into proteoliposomes reconstituted from rabbit erythrocyte membranes is shown in Fig. 5. Rabbit erythrocytes, like rat erythrocytes, have a very high L-lactate-transport activity (Deuticke *et al.*, 1978; R. C. Poole, unpublished work). As with rat erythrocytes, uptake was largely inhibited by α-cyano-4-hydroxycinnamate.

In Fig. 6 we show scans of the Coomassie Blue-stained proteins after SDS/polyacrylamide-gel electrophoresis of native rat erythrocyte ghosts and reconstituted proteoliposomes. As expected, there is little solubilization and reconstitution of the cytoskeletal components of the erythrocyte membrane under these conditions. The major solubilized and reconstituted proteins are band 3, the extrinsic membrane protein band 4.2 (nomenclature according to Fairbanks *et al.*, 1971), and a number of minor components in the 40–55 kDa range. In addition a small amount of spectrin was solubilized.

DISCUSSION

The data in the present paper demonstrate that the L-lactate carrier from both rat and rabbit erythrocytes can be reconstituted in a functional form, by the method of Lynch & McGivan (1987). Very few modifications have been made to the method, which has since been used to reconstitute Na^+-dependent amino acid-transport activity from rat liver plasma membranes (Quesada & McGivan, 1988). This procedure may therefore be of general use for the reconstitution of a variety of carriers. The criteria that we have used to assess the preservation of transport properties in this system are inhibitor-sensitivity and stereospecificity. Both α-cyano-4-hydroxycinnamate and pCMBS, at concentrations giving maximal inhibition of L-lactate fluxes in intact cells, have been shown to give considerable inhibition of pyruvate uptake into reconstituted rat erythrocyte membranes. It is of note that pCMBS, which does not permeate the intact erythrocyte, is almost as effective as α-cyano-4-hydroxycinnamate in the reconstituted system. This suggests that either the carrier is incorporated predominantly in its native orientation, or that the protein has susceptible thiol groups exposed on each side of the membrane. Non-specific effects of these inhibitors can be ruled out, since they have no effect on reconstituted bovine ghosts, which have no specific lactate carrier (Deuticke *et al.*, 1978).

An estimate of reconstituted transport activity can be made by using α-cyano-4-hydroxycinnamate-sensitive pyruvate uptake at 15 s; this was 23 nmol/min per mg for 0.4 mM-pyruvate in the experiment shown in Fig. 1(*a*). If the K_m, $V_{max.}$ and temperature-dependence of the rat erythrocyte carrier (Edlund & Halestrap, 1988) are taken into account, along with the effect of the large pH gradient in citrate buffer, this would compare with a value of about 160 nmol/min per mg of ghost protein for the native system. The difference between the two values

is likely to be an overestimate, since 10 or 15 s time points are probably not a good estimate of initial rates of transport in the proteoliposomes. It is well known that, on reconstitution, kinetic properties such as K_m values (Kohne *et al.*, 1981) and temperature-dependence (see, e.g., Wolosin, 1980) can be changed, and these factors, along with denaturation of transport proteins by detergents, cause low transport activities as compared with native systems for many reconstituted carriers (e.g. Kasahara & Hinkle, 1977; Fairclough *et al.*, 1979).

The cholesterol requirement for optimal reconstitution of transport activity is in agreement with Grunze *et al.* (1980), who found the K_m of the carrier for L-lactate to be decreased by cholesterol. We have shown reconstituted pyruvate transport activity from rat ghosts to be similar when 0.5% or 1.0% MEGA-10 is used for solubilization. It therefore appears that the lactate carrier cannot be selectively solubilized, unlike the erythrocyte membrane proteins glycophorin and the anion exchanger (Wolosin, 1980; Kohne *et al.*, 1981). With 1% MEGA-10 at a protein concentration of 5 mg/ml, about 2 mg of solubilized protein/ml is obtained. This corresponds to approx. 80% solubilization of intrinsic erythrocyte ghost proteins.

Our experimental protocol involves the preincubation of erythrocytes with 15 μM-DIDS to prevent any contribution of band 3 to reconstituted pyruvate-transport activity. There is no evidence for this protein being involved in stereospecific L-lactate transport, from kinetic data (Halestrap, 1976; Deuticke *et al.*, 1978). Furthermore, Kohne *et al.* (1981) were unable to demonstrate pCMBS-sensitive L-lactate fluxes in reconstituted band-3–phospholipid vesicles. As was noted in the Introduction, there is a certain amount of evidence from labelling experiments to suggest the involvement of a 40–50 kDa polypeptide in lactate transport (Jennings & Adams-Lackey, 1982; Welch *et al.*, 1984; Donovan & Jennings, 1985, 1986). Deuticke (1982) used pCMBS, under conditions where the carrier was selectively inhibited, to label human erythrocytes, and found that bands 4.5 and 7 (nomenclature according to Fairbanks *et al.*, 1971) bound most of the inhibitor. It should now be possible to follow the lactate carrier through purification with the reconstitution assay to confirm or otherwise these conclusions. Reconstitution has the advantage of being able to determine the identity of all the polypeptides required for transport, whereas labelling experiments can only detect those that bind a particular ligand.

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