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# Identification and partial purification of the erythrocyte L-lactate transporter

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1. Intact erythrocytes were incubated with 100 μM-4,4′-di-isothiocyanostilbene-2,2′-disulphonate (DIDS), a concentration sufficient to inhibit lactate transport irreversibly by 65%. DIDS-labelled proteins were detected by immunoblotting of erythrocyte membrane proteins with an anti-DIDS antibody. Labelled polypeptides of 35–45 kDa in rat erythrocytes, and of 40–50 kDa in rabbit and guinea pig erythrocytes, were detected by this technique. In human erythrocytes, which have 10-fold less transport activity, no labelled polypeptide in this molecular mass range was detected. 2. Labelling of these 35–50 kDa polypeptides was decreased markedly in the presence of the specific inhibitors of lactate transport α-cyano-4-hydroxycinnamate and 4,4′-dibenzamidostilbene-2,2′-disulphonate (DBDS), which compete with DIDS for binding to the transporter. However, the weakly bound inhibitor 4,4′-dinitrostilbene-2,2′-disulphonate (DNDS) afforded little protection against labelling by DIDS. 3. The lactate transporter from rat erythrocytes was solubilized with decanoyl-*N*-methyl glucamide (MEGA-10) and partially purified by Mono-Q anion-exchange chromatography, with transport activity eluting at 0.1–0.15 M-NaCl. The 35–45 kDa DIDS-labelled polypeptide from rat erythrocytes was eluted in the same peak of protein as lactate transporter activity during Mono-Q chromatography. 4. These observations provide strong evidence that the lactate transporter is a polypeptide of 35–45 kDa in rat erythrocytes and of 40–50 kDa in rabbit and guinea pig erythrocytes.

## INTRODUCTION

In most mammalian cells, the physiologically important process of plasma membrane lactate transport is mediated by specific $H^+$/monocarboxylate cotransporters (for reviews, see Deuticke, 1982, 1989; Halestrap & Poole, 1989; Halestrap *et al.*, 1990). The carrier present in erythrocytes has been characterized most extensively, and transports a range of aliphatic monocarboxylates. It is inhibited by α-cyanocinnamate derivatives, some stilbene disulphonates, and amino and thiol-reactive compounds (Halestrap, 1976; Deuticke *et al.*, 1978; Poole *et al.*, 1990; Poole & Halestrap, 1991). Although most cells appear to possess monocarboxylate transporters similar to that of the erythrocyte, cardiac myocytes (Poole *et al.*, 1989, 1990) and tumour cells (Spencer & Lehninger, 1976) have transporters with differences both in substrate affinity and in stereoselectivity for L- over D-lactate. This has led to the suggestion that there is a family of monocarboxylate transport proteins (Halestrap *et al.*, 1990).

Whereas the functional properties of lactate transport in mammalian cells have been studied extensively, the carrier protein(s) responsible have not been identified. Deuticke (1979, 1982) incubated human erythrocyte membranes with the organomercurial thiol reagent *p*-chloromercuribenzoate (*p*CMB) at a concentration which caused selective inhibition of lactate transport, and demonstrated labelling in the regions of molecular mass 45–60 kDa and 30 kDa on SDS/PAGE. However, in human erythrocytes it is likely that the lactate transporter was only a very minor component of the *p*CMB-labelled protein, since the monocarboxylate transport activity in these cells is very low (see Deuticke, 1989). Erythrocytes from rat, rabbit and guinea pig are more suited for labelling the carrier protein, since they have a much higher capacity for monocarboxylate transport (Deuticke *et al.*, 1978; Halestrap & Poole, 1989). Indeed, the capacity of the transport system in these cells suggests that the transporter may be an abundant protein, perhaps 1–5% of erythrocyte membrane protein. Jennings & Adams-Lackey (1982) found that incubation of rabbit erythrocytes with [$^3$H]4,4′-di-isothiocyanodihydrostilbene-2,2′-disulphonate ([$^3$H]$H_2$DIDS) labelled a membrane protein of 40–50 kDa on SDS/PAGE, in parallel with inhibition of lactate transport. Other reagents which, like $H_2$DIDS, are capable of reacting with exofacial amino groups, afforded protection against labelling of this band by [$^3$H]$H_2$DIDS (Donovan & Jennings, 1985, 1986). However, this evidence for the identity of the transporter is circumstantial, and attempts to label a similar protein in rat erythrocytes, which have a similarly active monocarboxylate transporter to those from rabbit, have met with failure (see Halestrap & Poole, 1989; Poole & Halestrap, 1990). In addition, there are no reports of specific reversible inhibitors or substrates of the transporter having effects on labelling of this band.

Inhibition of lactate transport by stilbene disulphonate derivatives has recently been investigated in some detail (Poole & Halestrap, 1991; Poole *et al.*, 1991). These compounds bind reversibly to the transporter, in competition with substrates, and cause instantaneous inhibition. Inhibition by the isothiocyanate derivatives 4,4′-di-isothiocyanostilbene-2,2′-disulphonate (DIDS) and $H_2$DIDS eventually becomes irreversible, reflecting covalent modification of the transporter. The unreactive 4,4′-dibenzamidostilbene-2,2′-disulphonate (DBDS) protects against this inhibition, by competing for a common binding site.

In the present paper we use anti-DIDS antibodies to detect DIDS-labelled erythrocyte membrane proteins on Western blots and to assess the protection afforded by specific reversibly binding inhibitors and substrates of the monocarboxylate transporter. We also correlate reconstituted lactate transporter activity (see Poole & Halestrap, 1988) with the presence of DIDS-labelled proteins during ion-exchange fractionation. These experiments provide strong evidence that the lactate transporter is a poly-

Abbreviations used: DIDS, 4,4′-di-isothiocyanostilbene-2,2′-disulphonate; $H_2$DIDS, 4,4′-di-isothiocyanodihydrostilbene-2,2′-disulphonate; DBDS, 4,4′-dibenzamidostilbene-2,2′-disulphonate; DNDS, 4,4′-dinitrostilbene-2,2′-disulphonate; MEGA-10, decanoyl-*N*-methyl glucamide; *p*CMB *p*-chloromercuribenzoate; KLH, keyhole limpet haemocyanin; PMSF, phenylmethanesulphonyl fluoride; PBS, phosphate-buffered saline; SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulphonic acid.

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peptide of 35–45 kDa in rat erythrocytes and 40–50 kDa in rabbit and guinea pig erythrocytes.

## EXPERIMENTAL

### Materials

Chemicals, biochemicals and radiochemicals were obtained from the sources given previously (Poole & Halestrap, 1988, 1991), with the following additions. Keyhole limpet haemocyanin (KLH), Q-Sepharose, alkaline phosphatase conjugated to avidin, and biotinylated standards for SDS/PAGE were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Acrylamide and piperazine diacrylamide were obtained from Bio-Rad Laboratories, Hemel Hempstead, Herts., U.K. Horseradish peroxidase-conjugated antibody to rabbit immunoglobulins (raised in the donkey) and the ECL (enhanced chemiluminescence) Western blotting detection system were obtained from Amersham International, Amersham, Bucks., U.K.

### Labelling of erythrocyte membrane proteins with DIDS

Suspensions of erythrocytes, at 10% haematocrit in 84 mM-$Na^+$-citrate/15 mM-Hepes/1 mM-EGTA, pH 7.4, were prepared as described previously (Poole & Halestrap, 1991). For maximal inhibition and labelling of the band 3 protein, the cells were incubated for 1 h at 37 °C with 5 $\mu$M-DIDS. For labelling of the erythrocyte lactate transporter, the cells were incubated with 100 $\mu$M-DIDS for 1 h at 37 °C; under these conditions the carrier is inhibited irreversibly by approx. 65% (Poole & Halestrap, 1991). At the end of the incubations the cells were sedimented by centrifugation (2000 *g*, 5 min). The cells were washed once with citrate buffer containing 0.5% (w/v) BSA to remove excess DIDS (Cabantchik & Rothstein, 1972) and once with citrate buffer alone, prior to preparation of erythrocyte membranes.

When protection against labelling of the lactate transporter by 100 $\mu$M-DIDS was investigated, the cells were first preincubated with 5 $\mu$M-DIDS for 30 min at 37 °C to allow complete (> 99%) labelling of the stilbene disulphonate binding site on band 3. The protecting agents were then preincubated with the cells at 37 °C for 5 min prior to addition of 100 $\mu$M-DIDS, and the incubations were continued for 1 h at 37 °C, before washing and lysis of the cells.

### Preparation of erythrocyte membranes (ghosts)

Erythrocyte membrane ghosts were prepared from control and DIDS-pretreated erythrocytes by hypo-osmotic lysis as described previously (Poole & Halestrap, 1988). For the initial lysis step, the buffer (5 mM-sodium phosphate, pH 8.0) also contained 0.4 mM-phenylmethanesulphonyl fluoride (PMSF). Erythrocyte membranes, prepared as above, were stripped of peripheral proteins by addition of > 10 vol. of ice-cold reconstitution buffer (see below) containing 1 M-KI (Bennet, 1983), and the membranes were collected by centrifugation at 30000 *g* for 15 min at 4 °C. The pellet was washed once with reconstitution buffer prior to further treatment.

### Anti-DIDS antibodies

Anti-DIDS antibodies were raised in New Zealand White rabbits essentially as described by Jentsch *et al.* (1989) and Garcia & Lodish (1989). DIDS was conjugated to KLH as follows. KLH was dialysed against several changes of 30 mM-sodium phosphate/100 mM-NaCl/1 mM-EDTA to remove any $Cu^{2+}$ ions present, and then diluted to 2 mg/ml with phosphate-buffered saline (PBS; 10 mM-sodium phosphate/150 mM-NaCl/1 mM-$MgCl_2$, pH 7.6). DIDS was added to a final concentration of 10 mg/ml, and the mixture was incubated for 2 h at 37 °C before dialysis against several changes of PBS over 24 h at 4 °C. The conjugate was stored in small aliquots at −20 °C. Rabbits were injected subcutaneously with 50 $\mu$g quantities of the KLH–DIDS conjugate. The presence of anti-DIDS antibodies in the sera was confirmed by demonstrating (with e.l.i.s.a.) reactivity towards a BSA–DIDS conjugate (prepared essentially as described for KLH–DIDS, above), but not against BSA alone. Immunoreactivity against BSA–DIDS could be detected at dilutions of serum greater than 1:50000. For Western blotting applications, a crude immunoglobulin fraction was prepared by ammonium sulphate fractionation (Hudson & Hay, 1976).

### Immunoblotting

Immunoblots of SDS/polyacrylamide gels were performed using the method of Towbin *et al.* (1979), except that separated proteins were transferred to polyvinylidene difluoride membranes (Millipore). The membranes were blocked with 10% (w/v) dried milk powder in Tris-buffered saline: 10 mM-Tris/HCl, 150 mM-NaCl, 1 mM-EDTA and 0.2% (v/v) Triton X-100, pH 7.5. All further steps were performed in this buffer, but without the milk powder. For the immunoblots with anti-DIDS antibodies, a peroxidase-conjugated anti-rabbit secondary antibody was used, and the blots were developed using the Amersham enhanced chemiluminescence detection system, according to the manufacturers' instructions. Labelling was quantified by densitometric scanning of the exposed film, and calculation of peak areas was carried out using a Joyce Loebl Chromoscan connected to a microcomputer. The molecular mass of each of the DIDS-labelled proteins was estimated with the aid of the following biotinylated molecular mass markers: phosphorylase (97 kDa), catalase (58 kDa), alcohol dehydrogenase (40 kDa) and carbonic anhydrase (29 kDa). Following immunodetection of DIDS-labelled proteins, the standards were stained using avidin–alkaline phosphatase and the chromogenic substrates 5-bromo-4-chloro-3-indoyl phosphate and NitroBlue tetrazolium (Blake *et al.*, 1984).

### Reconstitution

Functional reconstitution of lactate transporter activity from rat erythrocyte membranes was performed as described by Poole & Halestrap (1988), with the following modifications. A buffer containing 20 mM-Mops, 1 mM-EGTA, 1 mM-pyruvate and 0.1 mM-dithiothreitol, adjusted to pH 7.4 at 4 °C with NaOH (reconstitution buffer) was used throughout. Erythrocyte membranes stripped of peripheral proteins (see above) were solubilized at 2.5 mg/ml with 1% (w/v) decanoyl-*N*-methyl glucamide (MEGA-10) in reconstitution buffer containing the following proteinase inhibitors: benzamidine (2 mM), PMSF (0.5 mM), pepstatin (1 $\mu$g/ml), antipain (1 $\mu$g/ml) and leupeptin (1 $\mu$g/ml). We have shown previously that preparation of liposomes containing cholesterol in addition to phospholipid results in improved transport activity and lower non-specific permeability in the final proteoliposome preparation (Poole & Halestrap, 1988). In the experiments reported here, the ratio of cholesterol/phospholipid was increased, to 1:2 (mol/mol). This caused a further improvement in the transport activity of the final reconstituted preparation (results not shown). Finally, instead of mixing liposomes with solubilized protein at a ratio of 15 $\mu$mol of lipid phosphate/mg of protein, the ratio was increased to 30 $\mu$mol/mg; the lower density of protein in the final membranes facilitated the estimation of initial rates of transport in reconstituted fractions derived from purified extracts. Uptake of 0.4 mM-[1-$^{14}$C]pyruvate into the proteoliposomes were measured at 20 °C by a rapid filtration assay as described previously (Poole & Halestrap, 1988). Pyruvate was used as the substrate because it diffuses across membranes more slowly than does L-lactate,

whilst being a better substrate for the transporter (Halestrap, 1976; Poole *et al.*, 1990). Estimation of the activity of the monocarboxylate transporter was achieved by measuring the fraction of pyruvate uptake at 10 s which was sensitive to the transport inhibitor α-cyano-4-hydroxycinnamate (5 mM).

### Ion-exchange chromatography

Rat erythrocyte membrane proteins were fractionated by anion-exchange chromatography on a 1 ml Mono-Q column (HR 5/5) attached to a Pharmacia f.p.l.c. system. All buffers were filtered through nitrocellulose filters (0.4 μm pore size) before use, and the chromatography was performed at a temperature of 7–10 °C to avoid precipitation of MEGA-10. Solubilized membrane proteins were loaded on to the column, which was equilibrated with reconstitution buffer containing 0.5% (w/v) MEGA-10, 2 mM-benzamidine, and 1 μg/ml of each of pepstatin, antipain and leupeptin. After removal of non-bound protein, the column was eluted by raising the NaCl concentration in this buffer from 0 to 500 mM, as shown in the Figures. Samples from ion-exchange fractionation of erythrocyte membrane proteins were assayed for protein and reconstituted into liposomes (30 μmol of lipid phosphate/mg of protein) within 120 min of completion of the fractionation.

### Analytical procedures

Protein was assayed by the dye-binding method of Bradford (1976), using BSA as a standard. Correction was made for any interference by the turbidity caused by lipid in the reconstituted samples by measuring the absorbance of parallel samples with Bradford solution lacking the Coomassie Blue G-250 dye. This reading was subtracted from that obtained with the dye present to calculate the protein concentration. SDS/PAGE was performed essentially by the method of Laemmli (1970). However, piperazine diacrylamide was substituted for *NN'*-methylenebisacrylamide as the cross-linker on an equal mass basis; this results in a lower background staining in silver-stained gels (Hochstrasser *et al.*, 1988). Protein samples were solubilized in 62 mM-Tris/HCl, pH 6.8, containing 20% (w/v) sucrose, 7% (w/v) SDS, 200 mM-dithiothreitol and 1 mM-PMSF. In experiments where immunoblotting was performed with anti-DIDS antibodies, all solutions involved in SDS/PAGE were prefiltered through nitrocellulose filters (0.4 μm pore size) and the glass plates were washed with chromic acid. This decreased considerably background staining on immunoblots with anti-DIDS antibodies. Protein staining of SDS/PAGE gels was performed using the silver staining method of Nielsen & Brown (1984).

## RESULTS AND DISCUSSION

### Labelling of rabbit erythrocyte membrane proteins with DIDS

Covalent labelling of erythrocyte membrane proteins by DIDS was assessed by immunoblotting with anti-DIDS antibodies, as has been done with anti-4-acetamido-4'-isothiocyanostilbene-2,2'-disulphonate (SITS) antibodies for SITS-labelled proteins (Jentsch *et al.*, 1989). We found that the polyclonal anti-DIDS antibodies recognized BSA–DIDS, but not BSA alone, on both e.l.i.s.a. and immunoblots. In Fig. 1 we show the use of anti-DIDS antibodies to detect membrane proteins labelled upon incubation of rabbit erythrocytes with DIDS under various conditions. Rabbit erythrocytes were used, since Jennings and coworkers (Jennings & Adams-Lackey, 1982; Donovan & Jennings, 1985) demonstrated labelling of a 40–50 kDa polypeptide in these cells, in parallel with inhibition of L-lactate transport (see the Introduction section). No band was detected on immunoblots if erythrocytes were incubated in the absence of DIDS (lane i). As expected, incubation with 5 μM-DIDS resulted in labelling of the band 3 protein (lane ii). The anti-DIDS antibodies also recognized the band 3 protein when labelled by $H_2$DIDS and SITS (results not shown). Upon increasing the concentration of DIDS to 100 μM, an additional polypeptide of approx. 40–50 kDa was labelled (lane iii). This is likely to be the same polypeptide as is labelled by similar concentrations of $^3H_2$DIDS (Jennings & Adams-Lackey, 1982; Poole & Halestrap, 1990). The appearance of this band on the immunoblots might be taken to imply the presence of both low- and high-molecular-mass components, separated by a sharp, clear band. However, this is an artifact, caused by a major protein that is clearly visible on gels of erythrocyte membranes from different species. In all species this causes a decrease in immunostaining at exactly the same position, regardless of the precise migration of the DIDS-labelled protein (see also Fig. 2). It would be predicted from the kinetic data obtained previously (Poole & Halestrap, 1991; see the Introduction section) that if the 40–50 kDa polypeptide is the lactate transporter, then DBDS ($K_i$ approx. 25 μM) and α-cyano-4-hydroxycinnamate ($K_i$ approx. 30 μM) should protect against its labelling by DIDS. Lanes iv and vi in Fig. 1 show this to be the case. 4,4'-Dinitrostilbene-2,2'-disulphonate (DNDS), which has a $K_i$ value approx. 30-fold higher than that of DBDS for inhibition of L-lactate transport in rat erythrocytes (Poole & Halestrap, 1991), affords much less protection against labelling of the 40–50 kDa polypeptide (Fig. 1, lane v).

Table 1 summarizes the data from a number of experiments on separate erythrocyte preparations, and provides a quantitative analysis of the protection against DIDS labelling afforded by the inhibitors. The data show that DBDS, DNDS and α-cyano-4-hydroxycinnamate each decreased labelling of the band 3 protein by 20–30%. This unexpected finding is unlikely to be due to incomplete occupation of the specific binding site for stilbene disulphonates during the preincubation with 5 μM-DIDS, carried out in the absence of the protecting agents. Rather, it is more probable that some labelling of additional site(s) occurred upon prolonged incubation with 100 μM-DIDS. The data show clearly

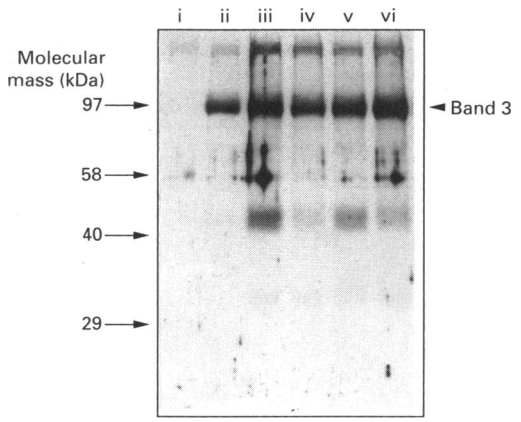


**Fig. 1. Detection of DIDS-labelled proteins in rabbit erythrocyte membranes by immunoblotting with anti-DIDS antibodies, and protection against labelling by inhibitors of lactate transport**

Rabbit erythrocytes were incubated in the absence of DIDS (lane i), with 5 μM-DIDS for 1 h at 37 °C (lane ii) or with 5 μM-DIDS for 30 min at 37 °C and then 100 μM-DIDS for 1 h (lanes iii–vi; see the Experimental section). The incubations with 100 μM-DIDS were carried out in the absence of inhibitor (iii), or in the presence of 1 mM-DBDS (iv), 1 mM-DNDS (v) or 5 mM-α-cyano-4-hydroxycinnamate (vi). Samples of erythrocyte membranes, stripped of peripheral proteins (2 μg/track), were subjected to immunoblotting with an anti-DIDS antibody as described in the Experimental section. The migration of molecular mass standards is indicated with arrows, and that of the band 3 protein is indicated with an arrowhead.

**Table 1. Labelling by 100 $\mu$M-DIDS of the 40–50 kDa polypeptide in rabbit erythrocyte membranes in the presence of inhibitors and substrates of the erythrocyte monocarboxylate transporter**

Rabbit erythrocytes at 10% haematocrit were incubated with DIDS under various conditions as described in the Experimental section and the legend to Fig. 1. Labelling of band 3 and of the 40–50 kDa polypeptide was quantified by densitometric scanning of exposed film from the ECL-developed immunoblots. Results are expressed as percentages of the labelling in the control incubation (100 $\mu$M-DIDS in the absence of other inhibitors or substrates), as mean values ± S.E.M. for four experiments on different preparations of erythrocytes. Labelling of the 40–50 kDa polypeptide is also presented after normalization to the labelling of band 3, assuming that any effects on labelling of band 3 reflected a general effect on nonspecific labelling (see text). This was calculated as follows: normalized labelling of 40–50 kDa polypeptide ($C$) = $B/A$

| | Labelling (% of control) | | |
|---|---|---|---|
| | Band 3 ($A$) | 40–50 kDa polypeptide ($B$) | 40–50 kDa polypeptide (normalized to band 3) ($C$) |
| ($a$) Inhibitors | | | |
| DBDS (1 mM) | 69±5 | 22±8 | 35±7 |
| DNDS (1 mM) | 76±1 | 69±3 | 91±4 |
| $\alpha$-Cyano-4-hydroxycinnamate (5 mM) | 80±10 | 32±3 | 42±2 |
| ($b$) Substrates | | | |
| Pyruvate (40 mM) | 108±16 | 94±11 | 90±10 |
| L-Lactate (40 mM) | 115±25 | 126±32 | 108±14 |
| D-Lactate (40 mM) | 112±8 | 140±13 | 124±10 |

that DBDS and $\alpha$-cyano-4-hydroxycinnamate protect against labelling of the 40–50 kDa polypeptide to a much greater extent than for band 3, whereas DNDS causes a similar level of protection for both proteins. If the protection against labelling of band 3 is a reflection of a non-specific reaction of DIDS and applies to proteins in general, then the data for protection of the 40–50 kDa protein may be normalized with reference to labelling of the band 3 protein, as shown in Table 1. When treated in this way, the data show more clearly that, relative to band 3, DNDS gives very little (approx. 10%) protection of the 40–50 kDa polypeptide, whereas DBDS and $\alpha$-cyano-4-hydroxycinnamate each resulted in more than 50% protection.

Table 1 also includes results obtained by labelling rabbit erythrocytes with 100 $\mu$M-DIDS in the presence of the transported substrate pyruvate and L-lactate, and the poorly transported D-lactate. A high concentration (40 mM) of the monocarboxylates was used for protection, since the affinity of the carrier for substrates is much lower than for the inhibitors DBDS and $\alpha$-cyano-4-hydroxycinnamate (Halestrap, 1976; Deuticke, 1982; Poole *et al.*, 1990). In these experiments, an increase in the labelling of the band 3 protein was usually observed, but its extent was quite variable between experiments, as indicated by the large statistical errors. The most likely cause of this increase in labelling is the use of high concentrations of these substrates. During the preincubation, the monocarboxylate will enter the erythrocyte, accompanied by a proton, and so cause a small extracellular alkalinization, which was observed to be approx. 0.1 pH unit. This would increase the reactivity of lysine residues towards the isothiocyanate groups of DIDS. For technical reasons we did not attempt to adjust the pH of individual incubations back to 7.4. However, each of the substrates caused the same pH change and had the same effect on labelling of band 3. This allowed comparisons to be made of the relative protective effects of different monocarboxylates.

As with labelling of the band 3 protein, the monocarboxylate substrates tended to increase the labelling of the 40–50 kDa polypeptide, presumably for the reasons given above, possibly masking any specific protective effects of these compounds. Although errors were quite large, there was a trend for labelling of this polypeptide to increase in the order: pyruvate < L-lactate < D-lactate, which would be expected on the basis of the $K_m$ values of the monocarboxylate transporter for these compounds (see for example Poole *et al.*, 1990). If the differences in the extent of the general increase in labelling observed in the presence of the substrates were decreased by normalizing the data to the labelling of band 3 (which was similar in the presence of each of these compounds), then the errors were decreased considerably. Indeed, analysis of the data using a paired $t$ test revealed significantly lower labelling of the 40–50 kDa polypeptide in the presence of pyruvate than of the weakly bound substrate D-lactate ($P < 0.05$); presumably this reflects specific protection by pyruvate. The difference between labelling in the presence of pyruvate and L-lactate was not significant ($P > 0.05$). Taken together, these data lend further support for the involvement of the 40–50 kDa polypeptide in lactate transport.

### DIDS-labelled membrane proteins in erythrocytes from various mammalian species

There are large differences in the activities of the monocarboxylate transporter in erythrocytes from various mammalian species (Deuticke *et al.*, 1978; Halestrap & Poole, 1989), which probably reflect the relative abundances of the transport protein. The ratio of lactate transport activity in human, rat, rabbit and guinea pig erythrocytes is approx. 1:10:10:25. It was therefore of interest to see if a DIDS-labelled polypeptide similar to that of 40–50 kDa in rabbit erythrocytes was present in other species which have a high capacity for lactate transport. In Fig. 2 we show labelling of human, rat, rabbit and guinea pig erythrocytes by 100 $\mu$M-DIDS, and the protective effect of the substrate analogue and inhibitor of lactate transport, $\alpha$-cyano-4-hydroxycinnamate. In each of these species the band 3 protein was the major labelled protein, as expected. In human erythrocytes, which have a much lower capacity for lactate transport than the other species, a polypeptide of approx. 65 kDa was labelled by 100 $\mu$M-DIDS, but this labelling was not protected by $\alpha$-cyano-4-hydroxycinnamate. This polypeptide is of similar molecular mass to the DIDS-labelled fragment derived from chymotryptic cleavage of the band 3 protein (Jennings & Passow, 1979), and so could be due to proteolysis of a small fraction of this protein. Rat, rabbit and guinea pig erythrocytes each possessed a broad band in the region 35–50 kDa that was labelled by 100 $\mu$M-DIDS, and in each case $\alpha$-cyano-4-hydroxycinnamate afforded considerable protection against this labelling. This suggests that these proteins are related, and strongly supports their involvement in lactate transport. There were no other proteins whose labelling was decreased dramatically in the presence of $\alpha$-cyano-4-hydroxycinnamate. It is of note that labelling of the 35–45 kDa polypeptide from rat erythrocytes was less than that of the slightly larger proteins of 40–50 kDa in rabbit and guinea pig erythrocytes. This agrees with a comparison of proteins labelled by $^3H_2$DIDS in these species, where no labelled protein was detected in rat (Halestrap & Poole, 1989; Poole & Halestrap, 1990). The observation of the 35–45 kDa band from rat erythrocytes in the present experiments reflects a higher sensitivity of detection by our current immunoblotting technique, which could also be used to detect the same polypeptide when rat erythrocytes were labelled with 100 $\mu$M-$H_2$DIDS (results not shown).

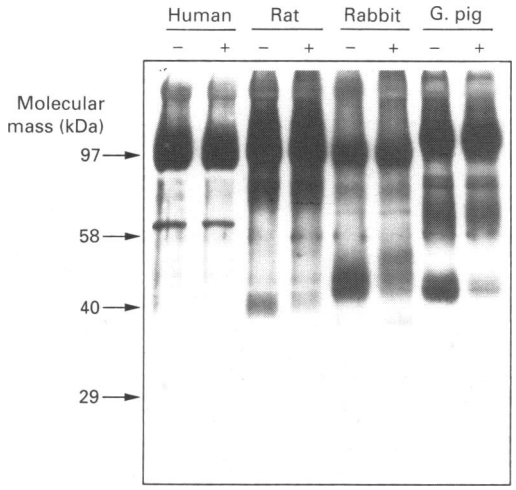


**Fig. 2. Labelling of erythrocyte membrane proteins from different mammalian species by 100 $\mu$M-DIDS and protection by $\alpha$-cyano-4-hydroxycinnamate**

Erythrocytes were incubated first with 5 $\mu$M-DIDS for 30 min at 37 °C, and then for 1 h with 100 $\mu$M-DIDS in the absence (−) or presence (+) of 5 mM-$\alpha$-cyano-4-hydroxycinnamate. Ghost membranes, stripped of peripheral proteins, were analysed by immunoblotting of 4 $\mu$g samples of protein, separated on 10% (w/v) polyacrylamide gels, with an anti-DIDS antibody. Results are shown for human, rat, rabbit and guinea pig (G. pig) erythrocytes as indicated. The migration of molecular mass standards is indicated with arrows.

## Purification and reconstitution of the rat erythrocyte monocarboxylate transporter

Attempts to investigate further the role of rabbit erythrocyte 40–50 kDa polypeptides in monocarboxylate transport by ion-exchange fractionation and reconstitution of transport activity were unsuccessful, since in five such experiments transport activity was lost upon fractionation (R. C. Poole, unpublished work). Therefore for these studies we used membranes from rat erythrocytes, which have a similar capacity for lactate transport (see above). Typical results for a Mono-Q anion-exchange fractionation of rat erythrocyte membrane proteins are shown in Fig. 3. Fig. 3(*a*) shows that there were two major peaks of protein ($A_{280}$) eluted from the anion-exchange column under these conditions: one at approx. 0.1–0.15 M-NaCl and the other at > 0.25 M-NaCl, with the latter containing approx. 3-fold more protein. The apparent rise in the baseline of the trace observed at approx. 50 mM-NaCl probably reflects a phase transition in the detergent solution induced by a change in ionic strength, since it did not correlate with a change in protein concentration, as measured by the method of Bradford (1976). The elution profile was highly reproducible, with only small shifts in the elution of the first peak in the shallow salt gradient. Fig. 3(*b*) shows that the fractions eluting in the first peak of protein (0.1–0.15 M-NaCl) contained almost all of the reconstituted transport activity. For the experiment shown, the carrier-mediated pyruvate transport activity over the 10 s assay period was 6.8 nmol/mg of protein for unfractionated proteins, and 28 nmol/mg in the most active fraction (Fig. 3*b*). This implies that the fractionation represents approx. 4-fold purification of the transporter. However, this is likely to be an underestimate of the true purification, since the active fraction contained only 0.4 mg of protein, as compared with 18 mg of protein applied to the column. This recovery of activity (approx. 10%) implies that the transport function is labile, through either denaturation of the protein or perhaps some loss of essential lipids or other factors during fractionation;

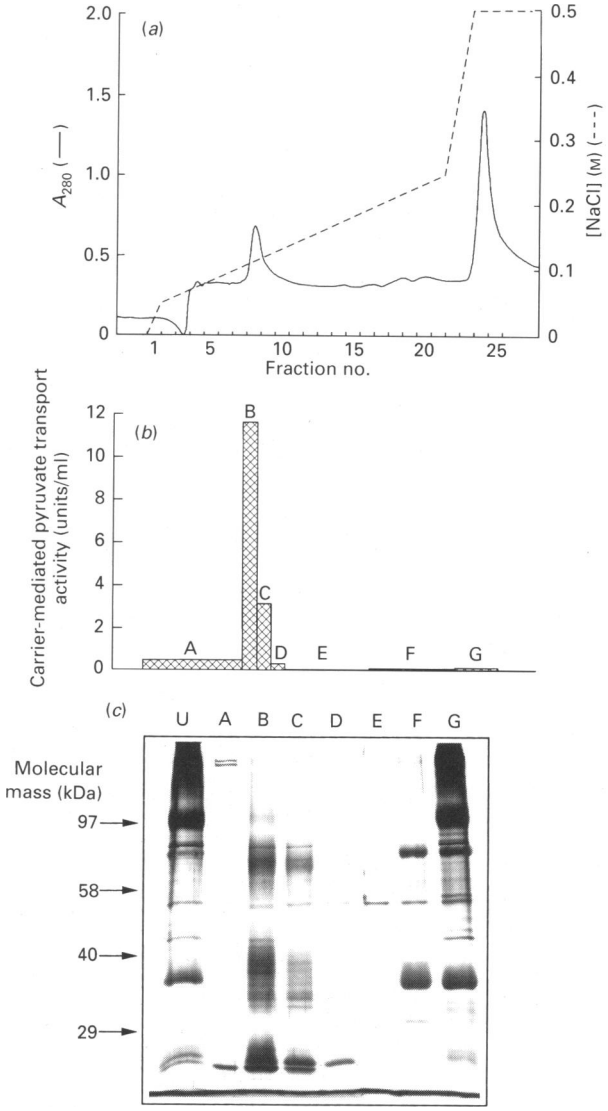


**Fig. 3. Fractionation of the rat erythrocyte lactate transporter by anion-exchange chromatography (Mono-Q)**

Mono-Q chromatography of MEGA-10-solubilized rat erythrocyte membrane proteins and reconstitution of monocarboxylate transporter activity were performed as described in the Experimental section. Approx. 18 mg of protein was applied to the Mono-Q column, and following removal of any non-bound protein, fractions of 1 ml were collected during the application of a 0–0.5 M-NaCl gradient. Fractions were pooled and reconstituted into liposomes, and reconstituted transport activity was determined as described in the Experimental section. The pooled samples were derived from column fractions as follows: A, 1–7; B, 8; C, 9; D, 10; E, 11–16; F, 17–22; G, 23–25. In (*a*), the elution profile for protein, as judged by $A_{280}$, is shown, and in (*b*) the reconstituted pyruvate transport activities for the pooled fractions are shown. Results for the measurement of reconstituted transport activity were calculated in units/ml of pooled fraction, where a unit is defined as the activity required for the uptake of 1 nmol of pyruvate in 10 s at 20 °C under the conditions used for the assay. In (*c*) a silver-stained SDS/PAGE separation of the fractions is shown. The tracks were loaded with 2 $\mu$g of unfractionated solubilized protein (lane U) or with 5 $\mu$l of each of the pooled column fractions (lanes A–G). The migration of molecular mass standards is indicated with arrows.

presumably the failure to recover activity upon fractionation of rabbit erythrocyte membranes is a more emphatic reflection of the same phenomenon. Fig. 3(*c*) shows SDS/PAGE separations

of the proteins present in the fractions reconstituted for measurement of transport activity. The polypeptides which correlated best with transport activity were in a broad region of 30–50 kDa, another diffuse area at 65–80 kDa, and a protein of < 25 kDa.

### Fractionation of DIDS-labelled rat erythrocyte membrane proteins

The data presented in Fig. 3 indicate that the functional rat erythrocyte lactate transporter is eluted from a Mono-Q anion-exchange column at 0.1–0.15 M-NaCl, and so it was of interest to determine whether any proteins labelled by DIDS were fractionated in a similar manner. For these experiments we fractionated rat erythrocyte membranes prepared from cells pretreated with 100 $\mu$M-DIDS for 1 h at 37 °C. The $\alpha$-cyano-4-hydroxycinnamate-sensitive pyruvate transport activity reconstituted from unfractionated solubilized protein was $2.7 \pm 0.23$ nmol/mg (mean $\pm$ S.E.M., $n = 3$ experiments) at 10 s for DIDS-treated membranes, as compared with $7.0 \pm 0.94$ nmol/mg ($n = 3$) for control membranes (prepared from cells treated with 5 $\mu$M-DIDS). This confirmed that DIDS had caused irreversible inhibition of the monocarboxylate transporter, the magnitude being as predicted from the earlier study with intact cells (Poole & Halestrap, 1991).

The results of a typical fractionation experiment with DIDS-labelled rat erythrocyte membranes, showing reconstituted transport activity and immunoblots of DIDS-labelled proteins in the fractions, are shown in Fig. 4. The elution profile for protein, as judged by the $A_{280}$, was almost identical to that shown in Fig. 3, and so is not shown. As described above, it was much more difficult to detect the DIDS-labelled 35–45 kDa protein in unfractionated rat erythrocyte membranes than the similar protein from rabbit and guinea pig. However, Fig. 4 shows that, upon fractionation of DIDS-treated rat erythrocyte membranes, the 35–45 kDa DIDS-labelled polypeptide could be detected more readily, and that most of this protein was eluted at 0.1–0.15 M-NaCl, as was the reconstituted transport activity in the same experiment. As expected, the total pyruvate transport activity eluting in this peak of protein was considerably less than for the control experiment (Fig. 3*b*), in which the same quantity of membrane protein was fractionated. The correlation between fractionation of the 35–45 kDa DIDS-labelled polypeptide and transport activity provides further evidence that this protein may be involved in lactate transport. Although the level of the 35–45 kDa labelled polypeptide in the fractions within this peak of protein did not correlate exactly with the residual transport activity, this might be explained by the introduction of two negative charges upon reaction of the protein with a single DIDS molecule. This could be responsible for a slight shift in elution towards higher salt concentrations relative to the unmodified, active carrier. A proportion of the DIDS-labelled 35–45 kDa protein was eluted at > 0.25 M-NaCl, along with the DIDS-labelled band 3 protein, and this did not correlate with transport activity. This may represent a denatured form of the protein which could also be present in the unmodified fractions, but would not be detected by a functional assay. Alternatively, this labelled protein that was eluted at > 0.25 M-NaCl may be a distinct 35–45 kDa polypeptide which is unrelated to the lactate transporter.

There were two other DIDS-labelled proteins which eluted in a very similar manner to the 35–45 kDa polypeptide; these were of approx. 80 kDa and 125 kDa. The broad band centred on 125 kDa was the most heavily labelled protein eluted in parallel with lactate transport activity. Interestingly, it was visible only in silver-stained SDS/PAGE gels of the active fractions of DIDS-labelled rat erythrocytes, and not in those from control erythrocytes (Fig. 5). This observation implies that the 125 kDa band may have arisen by cross-linking of two or more polypeptides mediated by DIDS, which possesses two reactive isothiocyanate groups. The band 3 protein is not a component of the 125 kDa band, since this band showed no reactivity with anti-(band 3)

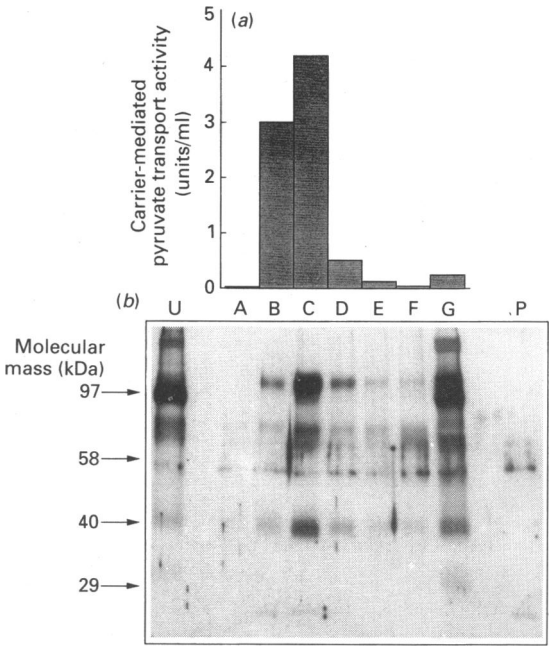


**Fig. 4. Mono-Q fractionation of membrane proteins from erythrocytes treated with 100 $\mu$M-DIDS**

Mono-Q chromatography of rat erythrocyte membranes, prepared from cells treated with 100 $\mu$M-DIDS for 1 h at 37 °C, and reconstitution of monocarboxylate transporter activity were performed as described in the Experimental section. The elution profile was essentially the same as that shown in Fig. 3. Samples A–G were pooled from the same column fractions as shown in Fig. 3, and either reconstituted into liposomes prior to determination of transport activity (*a*), or 5 $\mu$l samples were probed with anti-DIDS antibodies on an immunoblot (*b*). Panel (*b*) also shows immunoreactivity with unfractionated solubilized protein (U; 2 $\mu$g) and with 5 $\mu$l of the fraction containing the peak of the transport activity for the experiment shown in Fig. 3, performed with control erythrocyte membranes (P).

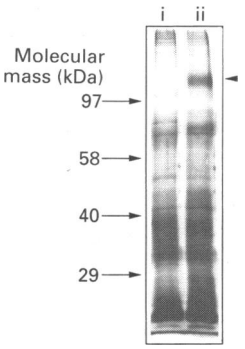


**Fig. 5. Proteins present in the fractions containing the peak of lactate transport activity from control and 100 $\mu$M-DIDS-treated rat erythrocytes**

Fractions containing the peak of lactate transport activity from a separation of control erythrocyte membranes (lane i) or membranes prepared from cells treated with 100 $\mu$M-DIDS (lane ii) were subjected to SDS/PAGE and proteins were stained with silver. For each sample, 5 $\mu$l (approx. 2 $\mu$g) of the fraction was loaded. The migration of a protein of approx. 130 kDa is indicated with an arrowhead.

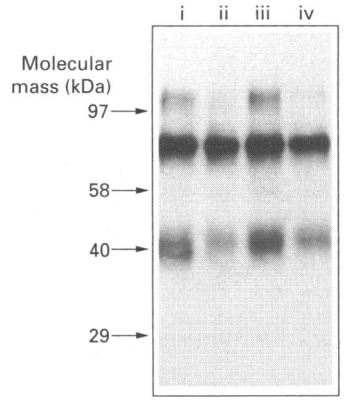


**Fig. 6. Labelling of rat erythrocyte membrane proteins by 100 μM-DIDS, and its protection by inhibitors, in a fraction eluted from Q-Sepharose by 200 mM-NaCl**

Erythrocytes were labelled by 100 μM-DIDS in the absence or presence of inhibitors (indicated below), and membranes free of peripheral proteins were prepared as described in the Experimental section. Membrane proteins were solubilized as in the reconstitution experiments, and 0.8 ml of solubilized protein (approx. 1 mg) was mixed with 0.5 ml of Q-Sepharose, pre-equilibrated with reconstitution buffer, for 30 min at 7 °C on a rotating stirrer. The Q-Sepharose was washed three times with reconstitution buffer containing 0.5% (w/v) MEGA-10 and then eluted with the same buffer containing 0.2 M-NaCl. The eluates were concentrated by centrifugal filtration using a Centricon 10 (Amicon), and 2 μg of each sample was subjected to immunoblotting with an anti-DIDS antibody. The lanes show labelling in the absence of protecting agent (lane i), and in the presence of DBDS (lane ii), DNDS (lane iii) and α-cyano-4-hydroxycinnamate (lane iv).

antibodies on immunoblots (results not shown). Since the 125 kDa band cofractionated with the 35–45 kDa polypeptide, it is possible that the lactate transporter is part of the cross-linked conjugate (see below).

### Protection against DIDS labelling of rat erythrocyte membrane proteins by inhibitors of monocarboxylate transport

In order to establish more firmly that the rat DIDS-labelled 35–45 kDa protein coeluting with transport activity during ion-exchange fractionation is indeed the carrier protein, we carried out labelling with 100 μM-DIDS in the presence or absence of DBDS, DNDS and α-cyano-4-hydroxycinnamate. Since it was difficult to detect labelling of this protein in unfractionated membranes, we prepared a partially purified fraction. It proved impractical to perform gradient elutions for a large number of samples, so we performed batch preparations of a fraction containing the labelled 35–45 kDa polypeptide using Q-Sepharose, and used the protein fraction eluted by 0.2 M-NaCl for immunoblotting with anti-DIDS antibodies. It was confirmed that the polypeptide composition of this fraction was similar to the active fractions eluted from the Mono-Q column. Fig. 6 shows that DBDS and α-cyano-4-hydroxycinnamate, but not DNDS, caused considerable protection against labelling of the 35–45 kDa polypeptide by DIDS. The data for the protection of the labelled 35–45, 80 and 125 kDa polypeptides in a number of experiments are quantified in Table 2. Labelling of the 80 kDa polypeptide was inhibited to some extent by each of DBDS, DNDS and α-cyano-4-hydroxycinnamate. However, each caused a similar degree of protection, in contrast to the expected observations for the lactate transporter. It was more difficult to assess labelling of the 125 kDa band derived from cross-linking (see above) since, for reasons which are unclear, this was not detected as readily as the 35–45 kDa and 80 kDa polypeptides

**Table 2. Summary of protection by inhibitors of lactate transport against DIDS labelling of rat erythrocyte membrane proteins**

The experimental protocol was as described in the legend to Fig. 6. Labelling was quantified by densitometric scanning of the ECL-developed blots as described in Table 1. Labelling of the protein bands indicated is expressed as a percentage of the labelling of the same band in control incubations (100 μM-DIDS alone). Results are given as mean values ± S.E.M. for the numbers of experiments indicated in parentheses.

| | Labelling (% of control) | | |
|---|---|---|---|
| Inhibitor | 35–45 kDa band | 80 kDa band | 125 kDa band |
| None | 100 | 100 | 100 |
| DBDS (1 mM) | 26 ± 3.6 (4) | 65 ± 11 (4) | 35 ± 2.5 (4) |
| DNDS (1 mM) | 94 ± 12 (3) | 89 ± 4.0 (3) | 121 ± 10 (3) |
| α-Cyano-4-hydroxycinnamate (5 mM) | 45 ± 5.7 (4) | 76 ± 11 (4) | 57 ± 14 (4) |

using this batch elution procedure. However, despite the lower level of this band, both DBDS and α-cyano-4-hydroxycinnamate appeared to afford some protection against its labelling by DIDS, whereas DNDS caused no decrease in its labelling. This protection against labelling of the 125 kDa band was similar quantitatively to that obtained for the 35–45 kDa polypeptide. Again, this might suggest that this band is a cross-linked product containing the 35–45 kDa polypeptide.

### General conclusions

Perhaps the most convincing evidence for the molecular identity of a carrier protein is the demonstration of a transport function mediated by a purified protein when incorporated into a suitable artificial membrane system. We have developed a method by which solubilized rat and rabbit erythrocyte membrane proteins can be incorporated into liposomes, to result in the functional reconstitution of specific lactate transporter activity (Poole & Halestrap, 1988). This assay of activity can be used to follow the transporter through purification. However, attempts to purify the protein by such means as affinity chromatography on α-cyanocinnamate–Sepharose, DBDS–Sepharose and immobilized lectins have met with failure (see Poole, 1989; Halestrap & Poole, 1989; R. C. Poole, unpublished work). Therefore in the present work we describe an alternative approach, using anti-DIDS antibodies to detect membrane proteins labelled by DIDS. This reagent has been shown to behave as an affinity label for the erythrocyte lactate transporter (Poole & Halestrap, 1991). The protection against labelling by DIDS afforded by specific substrates and inhibitors of the transporter has been used as a criterion to assess the involvement of particular proteins in lactate transport. Correlation of the fractionation of DIDS-labelled polypeptides with reconstituted transport activity during a partial purification has been used as a separate and complementary strategy.

The data presented here provide strong evidence for the involvement of polypeptides of approx. 35–50 kDa in monocarboxylate transport in rat, rabbit and guinea pig erythrocytes. Each of these proteins in the different species is a broad band on SDS/PAGE, suggesting heterogeneous glycosylation, and its labelling by 100 μM-DIDS is protected against by the unrelated compound α-cyano-4-hydroxycinnamate, a specific inhibitor of lactate transport. Furthermore, the protection afforded by DBDS and DNDS against labelling of the protein in rabbit and rat

erythrocytes is as expected for the lactate transporter. There are, however, two discrepancies in these results. Firstly, the polypeptides do not have exactly the same electrophoretic mobility; the protein in rat erythrocytes is approx. 35–45 kDa, whereas those from rabbit and guinea pig are approx. 40–50 kDa. This small difference may be explained readily by different extents of glycosylation of the protein in different species. Indeed, the data shown in Fig. 2 reveal that the band 3 protein also migrates differently in different species (compare rabbit and guinea pig). Secondly, despite the similar level of lactate transport activity in rat and rabbit erythrocytes (Deuticke *et al.*, 1978), there is considerably less labelling of the rat protein than that from rabbit. There are a number of possible explanations for this observation. Firstly, the rat erythrocyte transporter may have a higher turnover number than that from rabbit, either through an inherently greater catalytic efficiency or perhaps because of its lipid environment in the membrane. It is known that the latter parameter has effects on lactate transport (Grunze *et al.*, 1980). Secondly, part of the difference may reflect a slightly greater sensitivity of rabbit erythrocytes to inhibition of lactate transport by DIDS than those from rat (R. C. Poole, unpublished work). Thirdly, it is possible that, upon reaction with DIDS, the rat erythrocyte monocarboxylate transporter can become cross-linked to another protein, or even with other monocarboxylate carriers in the membrane, so that labelling of the 35–45 kDa band underestimates the true level of the protein. There is some evidence to support this possibility. A DIDS-labelled polypeptide of approx. 125 kDa was eluted in the same fractions as lactate transport activity from the Mono-Q anion exchange column loaded with DIDS-labelled rat erythrocyte membranes. The protein appears to be a cross-linked product, since it is absent in control membranes. There is considerably more of this DIDS-labelled protein than of the 35–45 kDa polypeptide, and some evidence that its labelling by DIDS is protected against by α-cyano-4-hydroxycinnamate and DBDS, but not DNDS. The cross-linked product is not detected readily in unfractionated DIDS-labelled rat erythrocyte membranes because it migrates very close to the band 3 protein on 10% (w/v) polyacylamide gels. However, it is detectable by immunoblotting of 6% (w/v) polyacrylamide gels, which show it to be present in rat erythrocytes, but not those of rabbit or guinea pig (results not shown).

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## REFERENCES

Bennet, V. (1983) Methods Enzymol. **96**, 313–324
Blake, M. S., Johnston, K. H., Russell-Jones, G. J. & Gotschlich, E. C. (1984) Anal. Biochem. **136**, 175–179
Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
Cabantchik, Z. I. & Rothstein, A. (1972) J. Membr. Biol. **10**, 311–330
Deuticke, B. (1979) Biophy. Membr. Transp. **2**, 159–189
Deuticke, B. (1982) J. Membr. Biol. **70**, 89–103
Deuticke, B. (1989) Methods Enzymol. **173**, 300–329
Deuticke, B., Rickert, I. & Beyer, E. (1978) Biochim. Biophys. Acta **507**, 137–155
Donovan, J. A. & Jennings, M. L. (1985) Biochemistry **24**, 561–564
Donovan, J. A. & Jennings, M. L. (1986) Biochemistry **25**, 1538–1545
Garcia, A. M. & Lodish, H. F. (1989) J. Biol. Chem. **264**, 19607–19613
Grunze, M., Forst, B. & Deuticke, B. (1980) Biochim. Biophys. Acta **600**, 860–869
Halestrap, A. P. (1976) Biochem. J. **156**, 193–207
Halestrap, A. P. & Poole, R. C. (1989) in Anion Transport Protein of the Red Blood Cell Membrane (Hamasaki, N. & Jennings, M. L., eds.), pp. 73–86, Elsevier, Amsterdam
Halestrap, A. P., Poole, R. C. & Cranmer, S. L. (1990) Biochem. Soc. Trans. **18**, 1132–1135
Hochstrasser, D., Patchornik, A. & Merril, C. (1988) Anal. Biochem. **173**, 412–423
Hudson, L. & Hay, F. C. (1976) Practical Immunology, Blackwell Scientific Publications, Oxford
Jennings, M. L. & Adams-Lackey, M. (1982) J. Biol Chem. **257**, 12866–12871
Jennings, M. L. & Passow, H. (1979) Biochim. Biophys. Acta **554**, 498–519
Jentsch, T. J., Garcia, A. M. & Lodish, H. F. (1989) Biochem. J. **261**, 155–166
Laemmli, U. K. (1970) Nature (London) **227**, 680–683
Nielsen, B. L. & Brown, L. R. (1984) Anal. Biochem. **141**, 311–315
Poole, R. C. (1989) Ph.D. Thesis, University of Bristol
Poole, R. C. & Halestrap, A. P. (1988) Biochem. J. **254**, 385–390
Poole, R. C. & Halestrap, A. P. (1990) Biochem. Soc. Trans. **18**, 1245–1246
Poole, R. C. & Halestrap, A. P. (1991) Biochem. J. **275**, 307–312
Poole, R. C., Halestrap, A. P., Price, S. J. & Levi, A. J. (1989) Biochem. J. **264**, 409–418
Poole, R. C., Cranmer, S. L., Halestrap, A. P. & Levi, A. J. (1990) Biochem. J. **269**, 827–829
Poole, R. C., Cranmer, S. L., Holdup, D. W. & Halestrap, A. P. (1991) Biochim. Biophys. Acta **1070**, 69–76
Spencer, T. L. & Lehninger, A. L. (1976) Biochem. J. **154**, 405–414
Towbin, H., Staehelin, T. & Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354

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