Reversible and irreversible inhibition, by stilbenedisulphonates, of lactate transport into rat erythrocytes

Identification of some new high-affinity inhibitors

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1. Inhibition of L-lactate transport into rat erythrocytes by stilbenedisulphonates was studied under conditions which allowed the contribution of reversible and irreversible inhibition to be assessed. 2. At low temperatures (7 °C), 4,4'-isothiocyanostilbene-2,2'-disulphonate (DIDS) and other stilbenedisulphonates were found to inhibit lactate transport instantaneously, in a manner which was fully reversible. The most potent reversible inhibitors were 4,4'-dibenzamidostilbene-2,2'-disulphonate (DBDS), DIDS and 4-acetamido-4'-isothiocyanostilbene-2,2'-disulphonate (SITS), for which apparent K_i values at 0.5 mM-L-lactate were approx. 36, 53 and 130 μM respectively. 3. DIDS and DBDS were competitive inhibitors with respect to L-lactate, with K_i values of approx. 40 μM and 22 μM respectively. 4. After incubation for 1 h at 37 °C with DIDS or its dihydro derivative (H_2DIDS), which contain the amino-reactive isothiocyanate group, most of the inhibition observed was irreversible. Under these conditions the IC_{50} value (concn. causing 50 % inhibition) for irreversible inhibition by both compounds was approx. 100 μM . SITS was much less potent as an irreversible inhibitor of L-lactate transport, approx. 20 % inhibition being obtained at 1000 μM . 5. The reversible inhibitor DBDS (1 mM) afforded protection against irreversible inhibition by DIDS and H_2DIDS (100 μM); protection was 60 and 65 % respectively after a 60 min incubation. This indicates that specific binding of the irreversible inhibitors is required before covalent modification can take place. 6. These compounds may be useful high-affinity probes for lactate transport in other tissues and might act as affinity labels for the transport protein(s).

INTRODUCTION

Stilbenedisulphonates are among the most potent inhibitors of inorganic anion exchange in erythrocytes (Cabantchik & Rothstein, 1972; Cabantchik *et al.*, 1978). The most effective of these compounds are 4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid (DIDS) and its dihydro derivative (H_2DIDS). These compounds initially inhibit anion transport in a reversible manner, with K_i values of approx. 0.03 μM and 0.04–0.05 μM respectively (Shami *et al.*, 1978; Janas *et al.*, 1989). Subsequent to this binding step, one of the isothiocyanate groups on the inhibitor reacts with the ϵ -amino group of a lysine residue on the transporter, rendering the inhibition irreversible (see Passow, 1986). This has allowed [^3H]DIDS and [^3H] H_2DIDS to be used as affinity labels for the erythrocyte anion transporter, and thus has enabled the identification of band 3 (nomenclature according to Fairbanks *et al.*, 1971) as the protein responsible (Cabantchik & Rothstein, 1974; Lepke *et al.*, 1976; Ship *et al.*, 1977). Other stilbenedisulphonate derivatives, which do not possess the reactive isothiocyanate group, inhibit anion transport in a manner which is fully reversible (see Cabantchik *et al.*, 1978).

Lactate and a variety of other short-chain monocarboxylates are transported across the plasma membrane of mammalian erythrocytes by three parallel pathways: (1) a specific H^+ /monocarboxylate co-transporter (which accounts for > 90 % of transport at physiological concentrations of L-lactate), (2) band 3 and (3) diffusion of the free acid (Halestrap, 1976; Deuticke *et al.*, 1978, 1982). Deuticke *et al.* (1982) found that DIDS, at concentrations just sufficient for maximal inhibition of inorganic anion transport via band 3, had almost no effect on lactate transport. However, Jennings & Adams-Lackey (1982) reported that incubation with a higher (100 μM) concentration of H_2DIDS , for

1 h at 37 °C, resulted in approx. 80 % inhibition of the specific monocarboxylate carrier in rabbit erythrocytes. These workers showed the inhibition to be irreversible, and used [^3H] H_2DIDS to label a broad band of 43–60 kDa on SDS/PAGE in parallel with inhibition of transport (Jennings & Adams-Lackey, 1982; Donovan & Jennings, 1985, 1986). We have also found the amino-reactive stilbenedisulphonate DIDS (500 μM) to inhibit the monocarboxylate transporters of hepatocytes and heart cells by approx. 50 and 70 % respectively (Edlund & Halestrap, 1988; Poole *et al.*, 1989). It is unclear from previous work whether DIDS and dihydro-DIDS interact specifically with monocarboxylate transporters or whether inhibition is a consequence of a more general reactivity of isothiocyanates with susceptible amino groups. The erythrocyte monocarboxylate carrier is known to be inhibited by a variety of amino reagents (Halestrap, 1976; Donovan & Jennings, 1986).

Here we present a more detailed investigation of the effects of stilbenedisulphonates on the specific monocarboxylate carrier of rat erythrocytes. Our data show that some of these compounds are potent reversible inhibitors of this transporter. The structure–activity relationships for a range of these compounds are discussed. We also examine the relationship between reversible and irreversible inhibition of L-lactate transport by the reactive isothiocyanate derivatives.

EXPERIMENTAL

Materials

Unless stated otherwise, all chemicals and biochemicals were obtained from the sources given previously (Poole & Halestrap, 1988; Poole *et al.*, 1990). 4,4'-Diaminostilbene-2,2'-disulphonic

Abbreviations used: DIDS, 4,4'-di-isothiocyanostilbene-2,2'-disulphonate; H_2DIDS , 4,4'-di-isothiocyanodihydrostilbene-2,2'-disulphonate; SITS, 4-acetamido-4'-isothiocyanostilbene-2,2'-disulphonate; DADS, 4,4'-diaminostilbene-2,2'-disulphonate; DazDS, 4,4'-diazidostilbene-2,2'-disulphonate; DNDS, 4,4'-dinitrostilbene-2,2'-disulphonate; DBDS, 4,4'-dibenzamidostilbene-2,2'-disulphonate.

acid (DADS) was obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K. Benzoyl chloride and BSA (essentially fatty-acid-free) were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Disodium 4-acetamidostilbene-2,2'-disulphonate (SITS) and disodium 4,4'-diazidostilbene-2,2'-disulphonate (DAzDS) were from Fluka, Glossop, Derbyshire, U.K. 4,4'-Dinitro-2,2'-stilbenedisulphonic acid (DNDS) was supplied by Phase Separations Ltd., Queensbury, Clywd, U.K. 4,4'-Diisothiocyano-2,2'-dihydrostilbenedisulphonate (H_2 DIDS) was obtained from HSC Research Development Corporation, Toronto, Canada. 4,4'-Dibenzamido-2,2'-stilbenedisulphonate (DBDS) was synthesized by benzylation of DADS with benzoyl chloride by the method of Kotaki *et al.* (1971). DADS and benzoyl chloride were used at a molar ratio of 1:2.2. The crude preparation was characterized by t.l.c. on silica-gel F₂₅₄ (Merck) in pyridine/acetic acid/water (10:1:40, by vol.) and was found to contain some contamination with the monobenzoylelated compound; no DADS, however, could be detected. The DBDS was purified by chromatography on silica gel (Kotaki *et al.*, 1971). Stock solutions of stilbenedisulphonates in water, neutralized with NaOH if necessary, were made fresh each day and were protected from light. DBDS was added from concentrated stock solutions in dimethyl sulphoxide/methanol (4:1, v/v); an equivalent volume of this solvent was included in the control incubations.

Methods

Preparation of erythrocyte suspensions for measurement of transport. Washed suspensions of rat erythrocytes were prepared as described previously (Poole *et al.*, 1990), except that the citrate buffer was modified to include 15 mM-Hepes (78 mM-sodium citrate/15 mM-Hepes/1 mM-EGTA, neutralized to pH 7.4 with NaOH). Unless stated otherwise, all incubations and assays of initial rates of transport were carried out in this buffer at 10% haematocrit. The washed cell suspension was incubated for 1 h at 37 °C with 5 μ M-DIDS, a concentration sufficient to inhibit inorganic-anion exchange in erythrocytes irreversibly (Halestrap, 1976). The cells were then sedimented by centrifugation (2000 g, 5 min) and resuspended in citrate buffer at 10% haematocrit. This cell suspension was used directly for measurement of transport or incubated with inhibitors at 37 °C. After incubation with inhibitors, the cells were either cooled rapidly to 7 °C for measurement of transport, or were first washed free of excess and reversibly bound inhibitor. This was achieved by adding an aliquot of cell suspension to at least 10 vol. of ice-cold citrate buffer supplemented with 0.5% (w/v) BSA. It is known that albumin binds stilbenedisulphonates (Cabantchik & Rothstein, 1972). The cells were sedimented by centrifugation and washed again with 40 vol. of citrate/BSA. Finally the cell pellet was washed once in citrate buffer (containing no BSA), resuspended in the same buffer at 10% haematocrit and maintained at 7 °C for measurement of transport.

Measurement of initial rates of L-lactate transport. Initial rates of zero-trans uptake of L-[¹⁴C]lactate into rat erythrocytes were measured over a 15 s time period at 7 °C by a centrifuge-stop technique as described previously (Poole *et al.*, 1990). If reversible inhibition by stilbenedisulphonates was to be studied, the inhibitor was introduced to the cell suspension with the L-[¹⁴C]lactate used to initiate the assay of transport. All measurements were performed in duplicate for each cell preparation, and rates of transport are given as percentages of the control carrier-mediated rate. This was obtained by subtraction of the component of transport (diffusion) that occurred in the presence of 2 mM- α -cyano-4-hydroxycinnamate, which gives maximal inhibition of the monocarboxylate transporter [see Poole *et al.* (1990) and Fig. 3 (below) of the present work].

In some experiments lactate transport was monitored by measuring the proton flux that accompanies transport of lactate on the monocarboxylate carrier (Dubinsky & Racker, 1978; Leeks & Halestrap, 1979). This was achieved using a standard combination pH electrode to measure extracellular pH in a suspension of erythrocytes, prepared as described above, but suspended in a lightly buffered citrate medium (84 mM-sodium citrate/1 mM-EGTA, adjusted to pH 7.4 with NaH_2PO_4). Data acquisition was performed with a microcomputer.

Unless stated otherwise, all results are typical of at least two experiments on different cell preparations. Where kinetic data were fitted to appropriate equations, this was performed by using non-linear least-squares regression analysis. Any additions or modifications to the methods are given in the Figure legends.

RESULTS AND DISCUSSION

Irreversible inhibition of L-lactate transport in rat erythrocytes by DIDS, H_2 DIDS and SITS

In Fig. 1 we show the inhibition of transport of 0.5 mM-L-lactate into rat erythrocytes after incubation for 1 h at 37 °C with different concentrations of DIDS, H_2 DIDS and SITS. These compounds all contain at least one isothiocyanate group, which enables them to react with susceptible amino groups. The data of Fig. 1 show that, under these conditions, washing with buffer containing BSA reversed little of the inhibition caused by DIDS and H_2 DIDS. Thus most of the inhibition reflects covalent modification of the transporter. Irreversible inhibition of 50% by DIDS and H_2 DIDS under these conditions required approx. 100 μ M-inhibitor; the data for H_2 DIDS were in good agreement with those of Jennings & Adams-Lackey (1982) for rabbit erythrocytes. SITS was significantly less potent than DIDS or H_2 DIDS, and most of the inhibition observed under these conditions could be reversed by washing the erythrocytes (Fig. 1c). This difference between total and irreversible inhibition indicates that, at least for SITS, some inhibition of transport can occur by a reversible interaction with the carrier. It is noteworthy that the extent of irreversible inhibition of band 3-mediated inorganic-anion exchange by SITS is also much less than that observed for DIDS and H_2 DIDS, even after prolonged incubation at 37 °C (Cabantchik & Rothstein, 1972).

In Fig. 2 we show a time course for the onset of irreversible inhibition of the rat erythrocyte monocarboxylate transporter by 500 μ M-DIDS. Under these conditions 50% inhibition required approx. 10 min to develop, but the extent of inhibition at 60 min was less than predicted on the basis of strict first-order kinetics. A much better fit was obtained if an additional parameter was included, representing a component of transport which was not inhibited, even after prolonged incubation. A component of inorganic-anion transport which is apparently insensitive to irreversible inhibition by isothiocyanates has previously been reported (Rakitzis *et al.*, 1978). Such observations may also be explained by removal of reactive DIDS from solution. Hydrolysis of isothiocyanates to unreactive amino derivatives and reaction with amino groups of phospholipids on the surface of the erythrocyte membrane are known to occur (see Cabantchik & Rothstein, 1972; Ho & Guidotti, 1975).

Reversible inhibition of L-lactate transport by stilbenedisulphonates

At 7 °C, addition of 100 μ M-DIDS to rat erythrocytes resulted in instantaneous inhibition of proton fluxes accompanying L-lactate transport (Fig. 3a, traces ii and iv). The level of inhibition observed was not increased if cells were preincubated with 100 μ M-DIDS for 2 min at 7 °C (trace iii). The data of Fig. 2

Fig. 1. Total and irreversible inhibition of L-lactate transport after incubation of rat erythrocytes with DIDS, H_2DIDS , and SITS for 1 h at 37 °C

Suspensions of rat erythrocytes at 10 % haematocrit were incubated for 1 h at 37 °C with the concentrations of (a) DIDS, (b) H_2DIDS or (c) SITS as indicated. At the end of this incubation the cells were divided into two aliquots. One of these was cooled rapidly to 7 °C and transport activity assayed immediately (●), whereas the other was washed free of excess and reversibly bound inhibitor before assay (▲). The former treatment allowed total inhibition to be assayed, whereas the latter measured only that component of inhibition which was irreversible.

indicate that, during such short incubations at 7 °C, there should be little or no irreversible inhibition of L-lactate transport by DIDS. This was found to be the case, since if cells were incubated with 100 or 500 μM -DIDS for 30 s at 7 °C and then washed with buffer containing BSA, then initial rates of transport of 0.5 mM-L-lactate were $101 \pm 3\%$ and $104 \pm 2\%$ (means $\pm$ S.E.M. for four experiments) of untreated cells washed in an identical manner. The extent of the reversible inhibition by 100 μM -DIDS shown in Fig. 3(a) was similar to total inhibition after prolonged incubation

Fig. 2. Time course for the onset of irreversible inhibition of L-lactate transport by 500 μM -DIDS at 37 °C

Rat erythrocytes at 10 % haematocrit and 37 °C were mixed with 500 μM (final concn.)-DIDS. Samples were withdrawn at the times indicated, and reversibly bound inhibitor was removed as described under 'Methods'. The L-lactate transport activity of each sample was then determined.

at 37 °C (see Fig. 1a). Rapid reversal of inhibition could be achieved by addition of BSA as shown in Fig. 3(b). Whereas BSA itself caused some inhibition of L-lactate transport, within 5 s it reversed the inhibitory effect of 100 μM -DIDS, to the rate observed with BSA alone (compare traces ii and iii).

Fig. 4 shows the kinetics of the reversible inhibition of L-lactate transport by DIDS and by some other stilbene-disulphonate derivatives that do not contain the reactive isothiocyanate group. The most effective of these compounds was DBDS, the derivative with the most hydrophobic substitution, DNDS and DAZDS being poor inhibitors. Data were analysed using non-linear regression to the equation:

$$V = V_0 / [1 + ([I]/K_i)]$$

where V is the observed rate of transport, V_0 is the rate of transport in the absence of inhibitor and K_i is the apparent dissociation constant of the transporter-inhibitor complex. It should be emphasized that apparent K_i values were calculated for the particular concentration of L-lactate employed (0.5 mM). The structures of a range of stilbenedisulphonates, and their apparent K_i values for reversible inhibition of L-lactate transport, determined as described above, are given in Table 1. The data in the Table provide some information on those factors which influence the inhibitory potency of these compounds. Clearly the difference in K_i values for DIDS and H_2DIDS (53 μM as against 398 μM) indicates the importance of the central double bond in the stilbene molecule. Different substitutions at the 4 and 4' position resulted in apparent K_i values which spanned a 30-fold range. It is clear that either relatively non-polar (-NCS) or bulky aromatic substitutions (-benzamido) yield potent inhibitors, whereas the more polar nitro and amino substitutions are at least 20-fold less effective. Perhaps the most interesting comparison is between DIDS and DAZDS; -NCS and -NNN groups are similar in their size and shape, suggesting that the less polar nature of the isothiocyanate group is the most important factor leading to its higher affinity for the transporter.

The data presented here are in contrast with what is known about reversible inhibition of band 3-mediated inorganic-anion exchange by stilbenedisulphonates (see Cabantchik *et al.*, 1978). For example DBDS was found to be a very much more potent inhibitor of lactate transport ($K_i = 36 \mu\text{M}$) than were DNDS and DAZDS ($K_i > 1000 \mu\text{M}$); IC_{50} (concn. causing 50 % inhibition) values for inhibition of band 3 are approx. 1.3, 2 and 6 μM respectively (Cabantchik *et al.*, 1978). In addition, DBDS is a more potent inhibitor of lactate transport than is DIDS, the most

Fig. 3. Rapid reversible inhibition of L-lactate transport by DIDS

Transport of 2.0 mM-L-lactate was monitored continuously by measuring the proton fluxes accompanying its uptake as described under 'Methods'. Incubations were in a 4 ml water-jacketed container at 7 °C. A downward deflection corresponds to extracellular alkalinization (from the starting pH of 7.4), and the traces were calibrated with standard solutions of HCl. Addition of 250 nmol of HCl (shown) caused acidification by 0.02 pH unit; 2.0 mM-L-lactate was added to all incubations at the point indicated. In (a) the conditions were: (i) control; (ii) control with 100 μ M-DIDS added at the point indicated; (iii) erythrocytes preincubated with 100 μ M-DIDS for 2 min at 7 °C; (iv) 100 μ M-DIDS added with L-lactate; and (v) 2 min preincubation with 2 mM- α -cyano-4-hydroxycinnamate. In (b) the traces are for the following incubations: (i) control; (ii) cells preincubated with 0.5% BSA for 2 min before addition of L-lactate; and (iii) 100 μ M-DIDS added with L-lactate, followed by 0.5% BSA at the point indicated. BSA was added from a stock solution of 20% (w/v) that had been dialysed overnight against 84 mM-sodium citrate/1 mM-EGTA, pH 7.4.

Fig. 4. Comparison of reversible inhibition of L-lactate transport by different stilbenedisulphonates

Initial rates of L-lactate transport were measured as described under 'Methods'. DIDS (●), DBDS (▲), DNDS (■) and DAzDS (○) were added, at the concentration indicated, with L-[14 C]lactate used to initiate transport.

potent known inhibitor of band 3 (Janas *et al.*, 1989). Our data indicate that DBDS and DIDS are two of the most potent known reversible inhibitors of the erythrocyte monocarboxylate carrier, with K_i values similar to, or lower than, the most effective of the α -cyanocinnamate derivatives (Halestrap, 1976; Poole *et al.*, 1990).

To determine the nature of reversible interaction of stilbenedisulphonates with the rat erythrocyte lactate transporter, the effects of substrate concentration on the kinetics of inhibition were investigated. In Fig. 5 we show the kinetics of reversible inhibition of L-lactate transport by DIDS and DBDS at three different concentrations of the substrate. The data are presented

in the form of comparative Dixon plots and demonstrate that the apparent K_i values for inhibition of L-lactate transport increase as the concentration of L-lactate is increased. At 0.5 mM-, 2.5 mM- and 6 mM-L-lactate, apparent K_i values for inhibition by DIDS were 50 ± 2.1 , 87 ± 2.9 and 124 ± 1.7 μ M respectively (means $\pm$ S.E.M. for four experiments). These data provide a clear indication that inhibition is largely competitive. When the data from four separate experiments were fitted to the equation for competitive inhibition, which in three out of four experiments gave as good a statistical fit as for mixed inhibition, the mean derived K_i value ($\pm$ S.E.M.) was 39.5 ± 5.7 μ M and the K_m value was 2.5 ± 0.51 mM. The K_m value is very similar to that reported previously (Edlund & Halestrap, 1988; Poole *et al.*, 1990). For DBDS, competitive K_i values were determined as 22.5 ± 2.7 μ M and 22.4 ± 1.9 μ M in two experiments (means $\pm$ S.E.M.).

Protection, by DBDS, against irreversible inhibition of lactate transport caused by DIDS and H₂DIDS

We have shown that DIDS and H₂DIDS can inhibit lactate transport reversibly and that, upon prolonged incubation, irreversible inhibition develops. However, these observations do not necessarily imply that covalent modification occurs at the same site as the reversible interaction. If this were the case, then reversible inhibitors should be able to protect against irreversible inhibition by DIDS and H₂DIDS. In Table 2 we show that DBDS (1 mM) afforded considerable protection (approx. 60%) against irreversible inhibition of L-lactate transport after incubation of rat erythrocytes with 100 μ M-DIDS or -H₂DIDS for 60 min at 37 °C. The protection was slightly greater (70–75%) at 30 min.

General conclusions

In the present paper we have shown that a variety of stilbenedisulphonates are reversible inhibitors of the specific monocarboxylate transporter in rat erythrocytes. It is perhaps

Table 1. Kinetic parameters for reversible inhibition of L-lactate transport in rat erythrocytes by stilbenedisulphonate derivatives

The data from experiments performed as described for Fig. 4 were fitted by non-linear least-squares regression analysis to the equation $V = V_0/[1 + ([I]/K_i)]$ as described in the text. The derived apparent K_i values ($\pm$ S.E.M.) are given for individual experiments at an L-lactate concentration of 0.5 mM. The 4 and 4' substitutions of these stilbenedisulphonates are also indicated. The general structure of these inhibitors, along with the structure of H₂DIDS, in which the central double bond of the stilbene is reduced, are shown below:

Stilbenedisulphonates			H ₂ DIDS		
Inhibitor	R	R'	Apparent K_i at 0.5 mM-L-lactate (μ M)		
			Expt. 1	Expt. 2	Mean
DIDS	-NCS	-NCS	49 $\pm$ 4	57 $\pm$ 2	53
H ₂ DIDS	-NCS	-NCS	348 $\pm$ 87	448 $\pm$ 61	398
SITS	-NHCOCH ₃	-NCS	111 $\pm$ 12	153 $\pm$ 27	132
DBDS	-NHCO-C ₆ H ₅	-NHCO-C ₆ H ₅	38 $\pm$ 1.5	34 $\pm$ 1.4	36
DNDS	-NO ₂	-NO ₂	1080 $\pm$ 63	1161 $\pm$ 154	1120
DAZDS	-NNN	-NNN	1079 $\pm$ 145	1314 $\pm$ 147	1196
DADS	-NH ₂	-NH ₂	> 1500	> 1500	> 1500

Fig. 5. Kinetics of inhibition of L-lactate transport by DIDS and DBDS

Results are shown for reversible inhibition of L-lactate transport by (a) DIDS and (b) DBDS. The concentrations of L-lactate were 0.25 (●), 2.5 (▲) and 6.0 (■) mM. The data are presented as comparative Dixon plots, i.e. $1/[\text{rate (\% of control)}]$ versus inhibitor concentration; the intercepts on the x-axis represent apparent K_i values for the particular concentrations of L-lactate employed. The data for individual concentrations of L-lactate were fitted separately to the equation $V = V_0/[1 + ([I]/K_i)]$ as described in the text. The lines drawn are those derived by non-linear least-squares regression analysis to this equation; derived parameter values are given in the text.

surprising that these compounds compete with the transported substrate for binding. One explanation might be that the sulphonate group behaves as an analogue of the carboxy group of the substrate, but this seems unlikely since ethane- and propane-sulphonates were found to be neither substrates nor inhibitors of the erythrocyte lactate transporter (R. C. Poole, unpublished work).

The present data indicate clearly that caution should be exercised in the use of stilbenedisulphonates to discriminate

between different pathways of monocarboxylate transport. Some workers have used 0.5–1.0 mM-DIDS or -SITS to inhibit transport of inorganic anions in various tissues when studying processes involving monocarboxylate transport (Rice & Steck, 1977; Siebens & Boron, 1989). Clearly, these concentrations of inhibitors are likely to inhibit monocarboxylate transport.

DBDS may prove to be a useful non-permeant (see Cabantchik *et al.*, 1978) inhibitor of lactate transport in other tissues, most of which appear to have a specific transporter similar to that in

Table 2. Protection of lactate transport against irreversible inhibition by DIDS and H₂DIDS, using DBDS

Erythrocytes were incubated for 30 or 60 min with DIDS or H₂DIDS (100 µM) in the presence or absence of 1 mM-DBDS as indicated below. At the end of these incubations, the cells were washed free of reversibly bound inhibitor as described under 'Methods'. Initial rates of uptake of 0.5 mM-L-lactate were measured as described under 'Methods'. Data are presented as percentages ± s.e.m. (for four experiments) of the control α-cyano-4-hydroxycinnamate-sensitive rates of transport. Since the inhibition caused by DBDS was only reversed to 93 ± 1.0 % of control rates under these conditions (mean ± s.e.m. for four experiments), the percentage protection against irreversible inhibition by DIDS and H₂DIDS was calculated in the following manner:

$$\text{Percentage protection} = \frac{C - (93 \cdot B/A)}{93 - (93 \cdot B/A)} \times 100$$

Irreversible modifier	Period of incubation (min)	Rate (% of control)			Protection (%)
		No additions (A)	Modifier alone (B)	Modifier + DBDS (C)	
DIDS	30	100	56 ± 0.4	83 ± 1.6	76
	60	100	38 ± 2.3	70 ± 2.0	60
H ₂ DIDS	30	100	45.5 ± 2.1	78 ± 2.9	70
	60	100	30 ± 3.1	70 ± 1.5	65

erythrocytes (see Edlund & Halestrap, 1988; Halestrap & Poole, 1989). It is a more stable compound than the isothiocyanate derivatives, which also suffer the disadvantage of reacting non-specifically with amino groups. The K_i value for DBDS is similar to those of the most effective α-cyanocinnamate derivatives in erythrocytes (Halestrap, 1976; Poole *et al.*, 1990), but these inhibitors can enter cells and interfere with metabolism of lactate and pyruvate at the level of mitochondrial pyruvate transport (Halestrap & Denton, 1975; Thomas & Halestrap, 1981). Other inhibitors of monocarboxylate transport, such as *p*-chloro-mercuribenzenesulphonate and quercetin, have many non-specific effects [see Suolinna *et al.* (1975), Salter *et al.* (1978) and Deuticke (1986)]. Thus the membrane-impermeant DBDS may prove useful in studies to determine the importance and possible regulatory role of plasma-membrane lactate/pyruvate transport in a variety of tissues.

Those stilbenedisulphonates which possess an isothiocyanate group can subsequently react with the transporter, causing irreversible inhibition. Reversible binding of the inhibitor to the exofacial substrate binding site (or a site interacting with it), appears to be a prerequisite for such covalent modification. Thus these compounds can be regarded as affinity labels for the carrier. Jennings and co-workers (Jennings & Adams-Lackey, 1982; Donovan & Jennings, 1985) found a rabbit erythrocyte membrane protein of 43–60 kDa to be labelled by [³H]H₂DIDS in parallel with inhibition of L-lactate transport. Clearly, such circumstantial evidence for an involvement of this protein in the transport process would be strengthened if DBDS or substrates of the transporter could afford protection against labelling of the carrier. DIDS and H₂DIDS, and the potent reversible inhibitor DBDS, may also prove to be useful probes for the specific monocarboxylate transporter in other cells.

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REFERENCES

- Cabantchik, Z. I. & Rothstein, A. (1972) *J. Membr. Biol.* **10**, 311–330
 Cabantchik, Z. I. & Rothstein, A. (1974) *J. Membr. Biol.* **15**, 207–226
 Cabantchik, Z. I., Knauf, P. A. & Rothstein, A. (1978) *Biochim. Biophys. Acta* **515**, 239–302

- Deuticke, B. (1986) *Membr. Biochem.* **6**, 309–326
 Deuticke, B., Rickert, I. & Beyer, E. (1978) *Biochim. Biophys. Acta* **507**, 137–155
 Deuticke, B., Beyer, E. & Forst, B. (1982) *Biochim. Biophys. Acta* **684**, 96–110
 Donovan, J. A. & Jennings, M. L. (1985) *Biochemistry* **24**, 561–564
 Donovan, J. A. & Jennings, M. L. (1986) *Biochemistry* **25**, 1538–1545
 Dubinsky, W. P. & Racker, E. (1978) *J. Membr. Biol.* **44**, 25–36
 Edlund, G. L. & Halestrap, A. P. (1988) *Biochem. J.* **249**, 117–126
 Fairbanks, G., Steck, T. L. & Wallach, D. F. H. (1971) *Biochemistry* **10**, 2606–2617
 Halestrap, A. P. (1976) *Biochem. J.* **156**, 193–207
 Halestrap, A. P. & Denton, R. M. (1975) *Biochem. J.* **148**, 97–106
 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 Science Publishers, Amsterdam
 Ho, M. K. & Guidotti, G. (1975) *J. Biol. Chem.* **250**, 675–683
 Janas, T., Bjerrum, P. J., Brahm, J. & Wieth, J. O. (1989) *Am. J. Physiol.* **257**, C601–C606
 Jennings, M. L. & Adams-Lackey, M. (1982) *J. Biol. Chem.* **257**, 12866–12871
 Kotaki, A., Naoi, M. & Yagi, K. (1971) *Biochim. Biophys. Acta* **229**, 547–556
 Leeks, D. R. & Halestrap, A. P. (1979) in *Function and Molecular Aspects of Biomembrane Transport* (Quagliariello, E., Papa, S., Palmieri, F., Slater, E. C. & Siliprandi, N., eds.), pp. 427–430, Elsevier/North-Holland, Amsterdam
 Lepke, S., Fasold, H., Pring, M. & Passow, H. (1976) *J. Membr. Biol.* **29**, 147–177
 Passow, H. (1986) *Rev. Physiol. Biochem. Pharmacol.* **103**, 61–204
 Poole, R. C. & Halestrap, A. P. (1988) *Biochem. J.* **254**, 384–390
 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
 Rakitzis, E. T., Gilligan, P. J. & Hoffman, J. F. (1978) *J. Membr. Biol.* **41**, 101–115
 Rice, W. R. & Steck, T. L. (1977) *Biochim. Biophys. Acta* **468**, 305–317
 Salter, D. W., Custead-Jones, S. & Cook, J. S. (1978) *J. Membr. Biol.* **40**, 67–76
 Shami, Y., Rothstein, A. & Knauf, P. A. (1978) *Biochim. Biophys. Acta* **508**, 357–363
 Ship, S., Shami, Y., Breuer, W. & Rothstein, A. (1977) *J. Membr. Biol.* **33**, 311–323
 Siebens, A. W. & Boron, W. F. (1989) *Am. J. Physiol.* **256**, F354–F365
 Suolinna, E.-M., Buchsbaum, R. N. & Racker, E. (1975) *Cancer Res.* **35**, 1865–1872
 Thomas, A. P., Halestrap, A. P. (1981) *Biochem. J.* **198**, 551–564