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Inhibition of L-lactate transport and band 3-mediated anion transport in erythrocytes by the novel stilbenedisulphonate *N,N,N',N'*-tetrabenzyl-4,4'-diaminostilbene-2,2'-disulphonate (TBenzDS)

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(1) The synthesis of the novel stilbenedisulphonate *N,N,N',N'*-tetrabenzyl-4,4'-diaminostilbene-2,2'-disulphonate (TBenzDS) is described, and its interaction with the lactate transporter and band 3 protein of erythrocytes investigated. At 10% haematocrit the IC_{50} (concn. required for 50% inhibition) for inhibition of transport of 0.5 mM L-lactate into rat erythrocytes at 7 °C was approx. 1.6 μM, as low as any other inhibitor of the transporter. In human erythrocytes at 10% haematocrit the IC_{50} value was increased from approx. 3 μM to 9 μM upon raising the temperature from 7 °C to 25 °C. (2) TBenzDS inhibited transport of L-lactate into rat erythrocytes in a manner that was competitive with the substrate, as is the case for some other stilbene disulphonate derivatives (Poole, R.C. and Halestrap, A.P. (1991) Biochem. J. 275, 307–312). (3) Increasing the haematocrit from 5 to 20% caused a 3-fold increase in the IC_{50} value for inhibition of L-lactate transport in rat erythrocytes. (4) TBenzDS was found to bind to erythrocyte membranes, with a partition coefficient (P_m) of 6000–7000 under all conditions tested. (5) TBenzDS also inhibited band 3-mediated sulphate transport in rat erythrocytes; 50% inhibition required approx. 2.5 μM TBenzDS for cells at 10% haematocrit. (6) TBenzDS is fluorescent, and an enhancement of this fluorescence occurs upon addition of BSA or erythrocyte membranes. The fluorescence enhancement caused by erythrocyte membranes is due to binding of the inhibitor to the band 3 protein at the same site as the stilbenedisulphonate 4,4'-diisothiocyanodihydrostilbene-2,2'-disulphonate (H_2DIDS).

Introduction

Transport of L-lactate across the plasma membrane is of major physiological importance for the metabolism of many mammalian cells [1] and in most tissues is catalysed by proton-linked monocarboxylate carriers. L-Lactate transport has been studied most extensively in erythrocytes, whose carrier accepts a range of substituted and unsubstituted aliphatic monocarboxylates, is stereoselective for L- over D-lactate, and is inhibited by aromatic carboxylic acids, most notably derivatives of α-cyanocinnamate [2–4]. Other tissues such as liver and skeletal muscle have transporters with similar properties [1,5,6]. However, the different stereoselectivity and affinity for some substrates in heart cells and tumour cells suggest the presence of distinct transporters in these tissues [4,7,8].

Specific inhibitors of proton-linked monocarboxylate transport may be of considerable use in investigating the metabolic role of these carriers, and as kinetic and structural probes of the transporter itself. However, most of the inhibitors known are not specific for lactate transport, and there are very few which bind to the carrier with high affinity ($K_i < 50$ μM). Amongst the most potent and more selective of these inhibitors are the derivatives of α-cyanocinnamate, and the stilbenedisulphonates 4,4'-diisothiocyanostilbene-2,2'-di-

Abbreviations: DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulphonate; H_2DIDS, 4,4'-diisothiocyanodihydrostilbene-2,2'-disulphonate; DADS, 4,4'-diaminostilbene-2,2'-disulphonate; DBDS, 4,4'-dibenzamidostilbene-2,2'-disulphonate; TBenzDS, *N,N,N',N'*-tetrabenzyl-4,4'-diaminostilbene-2,2'-disulphonate.

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sulphonate (DIDS) and 4,4'-dibenzamidostilbene 2,2'-disulphonate (DBDS) [9,10]. Poole and Halestrap [10] have shown recently that DBDS and DIDS bind reversibly to an exofacial site on the transporter, in competition with substrate. Subsequently DIDS, which possesses isothiocyanate groups, reacts with an amino group within this site to produce irreversible inhibition of transport [10]. In the present paper we describe a novel stilbenedisulphonate derivative which is as potent as any known inhibitor of lactate transport in erythrocytes. The interactions of this compound with the lactate transporter and band 3 protein are investigated. The inhibitor is also shown to be concentrated in red cell membranes, with a partition coefficient of approx. 6000–7000.

Materials and Methods

Materials

Unless stated otherwise, all chemicals, biochemicals and radiochemicals were obtained from the sources given previously [10]. Benzyl bromide was obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K., and all organic solvents were of "AnalaR" grade from BDH Chemicals, Poole, Dorset, U.K. Sodium [^{35}S]-sulphate was obtained from Amersham International plc, Amersham, Bucks, U.K.

Methods

Synthesis of N,N,N',N'-tetrabenzyl-4,4'-diaminostilbene-2,2'-disulphonate (TBenzDS)

TBenzDS was synthesised by tetrasubstitution of 4,4'-diaminostilbene-2,2'-disulphonate (DADS) with benzyl bromide, as follows. 4,4'-Diaminostilbene-2,2'-disulphonate (DADS, 0.002 mole) was dissolved in 30 ml of DMSO/H_2O (1:2, v/v) containing 0.024 mole $NaHCO_3$. Benzyl bromide (0.012 mole) was added and the mixture stirred overnight at room temperature. At this stage a brown slurry had precipitated, and this was washed with H_2O and ice-cold diethyl ether (2 × 20 ml of each). The solid was then dissolved in hot methanol (25 ml) and the product precipitated with ice-cold diethyl ether (100 ml). The TBenzDS was collected on a Buchner funnel, washed with diethyl ether, and then dried under vacuum over P_2O_5, yielding approx. 0.8 g of light green solid. TBenzDS remaining in the supernatant of the original reaction mixture could be extracted with 2 vol. chloroform/methanol (2:1, v/v) and, following removal of the solvent, dissolved in hot methanol and precipitated with diethyl ether, as above. TLC analysis of TBenzDS on silica-gel F_{254} (Merck), using chloroform/ methanol/ acetic acid/ water (25:15:2:2, by vol.) as solvent, showed a single fluorescent and iodine staining spot, with an R_f value of approx. 0.8.

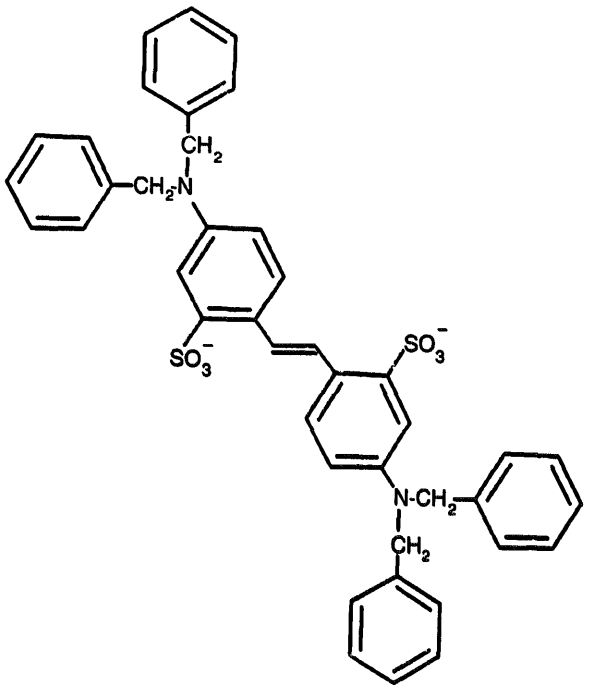

Fig. 1. Structure of *N,N,N',N'*-tetrabenzyl-4,4'-diaminostilbene-2,2'-disulphonate (TBenzDS).

The structure of TBenzDS, confirmed by NMR spectra, is shown in Fig. 1. The compound had an absorption maximum at 375 nm with ε_{375} (mM) of 27. Stock solutions of TBenzDS were stable when stored at −20 °C in DMSO/methanol (4:1, v/v).

Preparation of erythrocyte suspensions and measurement of transport of L-lactate

Suspensions of rat and human erythrocytes were pretreated with 5 μM DIDS for 1 h at 37 °C to inhibit irreversibly band 3-mediated anion transport, as described previously [10]. Initial rates of zero-*trans* uptake of [U-^{14}C]-L-lactate were measured by a centrifuge-stop technique [4,10] under the conditions indicated in the text and figure legends. Unless stated otherwise, all incubations were carried out at an haematocrit of 10%. When TBenzDS was used, the cell suspensions were preincubated with the inhibitor for 5 min prior to assay of initial rates of transport. All results were corrected for α-cyano-4-hydroxycinnamate-insensitive transport (free diffusion), which represents < 5% of total transport (see Ref. 10).

Measurement of band 3-mediated sulphate transport in rat erythrocytes

Transport of 4 mM SO_4^{2-} into rat erythrocytes, in exchange for intracellular chloride, was measured at 30 °C in a buffer lacking any other permeant anions (84 mM sodium citrate, 1 mM EGTA, adjusted to pH 6.5 with citric acid). To initiate transport 1 ml of cell

suspension, preincubated for 5 min at 30 °C in the absence or presence of inhibitor, was mixed with 12.5 μl of buffer containing $^{35}SO_4^{2-}$ (final concentration 4 mM; 0.15 μCi/ml). To terminate transport, 20 μM DIDS was added, and the cells sedimented by centrifugation in a microcentrifuge (10000 × *g*, 1 min). The cell pellet was washed once with 1 ml ice-cold citrate buffer (pH 6.5) containing 20 μM DIDS, and then lysed with 0.8 ml H_2O. Protein was precipitated with 2% perchloric acid prior to determination of radioactivity by liquid scintillation counting, using appropriate quench corrections. Uptake of $^{35}SO_4$ was essentially linear with respect to time up to 15 min; a 7 min time point was used for the measurement of initial rates of transport. Results were corrected for any SO_4^{2-} uptake remaining in the presence of 20 μM DIDS; this was always less than 2% of control uptake.

Preparation of erythrocyte membranes ('ghosts')

Erythrocyte membranes were prepared either from untreated cells or from those pretreated with 5 μM DIDS for1 h at 37 °C (citrate buffer at 10% haematocrit). The membranes were prepared by hypotonic lysis as described by Poole and Halestrap [11], except that the initial lysis step was performed in the presence of the proteinase inhibitor phenylmethylsulphonyl fluoride (0.4 mM). Membrane protein concentrations were determined by the method of Bradford [12].

Measurement of the partition of TBenzDS into erythrocyte membranes

The association of TBenzDS with erythrocyte membranes was determined using membranes prepared from DIDS-treated red cells (see above), to prevent any binding to the band 3 protein. TBenzDS (20 μM) was added to suspensions of erythrocyte membrane protein, in 1.2 ml of citrate buffer (prefiltered through a nitrocellulose filter, 0.4 μm pore size). After incubation for 5 min at the appropriate temperature, the membranes were sedimented (at the same temperature) for 5 min at 10000 × *g* in a microcentrifuge. The supernatants (1 ml of each) were taken and their absorbance at 375 nm measured. The free concentration of TBenzDS, measured in this manner, could be used to determine the partition coefficient, P_m ($[TBenzDS]_m/[TBenzDS]_w$) by fitting the data for a range of membrane protein concentrations to the following equation:

$$[TBenzDS]_w = [TBenzDS]_{tot}/(1 + P_m(V_m/V_w))$$

where the subscripts w and m refer to the aqueous and membrane phases, respectively, V the relative volume of the appropriate phase, and $[TBenzDS]_{tot}$ the total concentration of TBenzDS in the incubation. Values of V_m/V_w were calculated from the value of 0.001 for erythrocytes at 10% haematocrit [13], which represents a membrane protein concentration of 0.5 mg/ml [14].

Treatment of kinetic data

Kinetic data were analysed by non-linear least-squares regression analysis. The equation for linear inhibition $V = V_0/(1 + I/K_i)$, to give apparent K_i values, or an equation describing cooperative inhibition $V = V_0/(1 + (I/IC_{50})^n)$ were used, where V was the observed rate of transport, V_0 the rate of transport in the absence of inhibitor, I the concentration of inhibitor, IC_{50} the concentration of inhibitor causing 50% inhibition of transport, and n the Hill coefficient. In some experiments where [L-lactate] and [TBenzDS] were varied independently, data were fitted to an equation which describes a competitive interaction between the inhibitor and transportable substrate:

$$V = V_{max} \cdot S/(K_m(1 + (I/K_{0.5})^n) + S)$$

where S is the concentration of substrate, and $K_{0.5}$ the concentration of TBenzDS required for 50% inhibition of lactate transport, extrapolated to zero [lactate].

Results

Inhibition of erythrocyte L-lactate transport by TBenzDS

Inhibition of the transport of 0.5 mM L-lactate by TBenzDS in rat and human erythrocytes is shown in

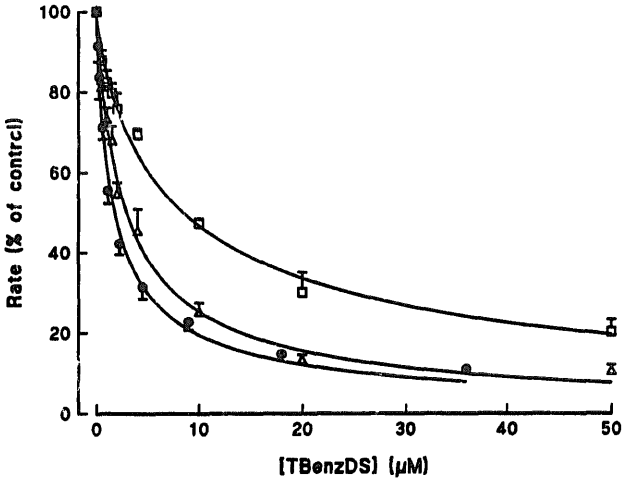

Fig. 2. Inhibition of L-lactate transport in rat and human erythrocytes by TBenzDS. Rat (●) and human (△, □) erythrocytes at 7 °C (●, △) and 25 °C (□) were preincubated with TBenzDS for 5 min. Initial rates of transport of 0.5 mM L-lactate were then measured as described in Methods. For rat erythrocytes at 7 °C, transport was terminated by centrifugation after 15 s, whereas for human erythrocytes at 7 °C and 25 °C, transport was terminated at 15 min and 90 s, respectively. These conditions were chosen so that uptake of substrate was linear with respect to time over the period chosen (see Refs. 4 and 10). Data are shown as mean values ± S.E. for the number of experiments indicated in the text. The lines drawn through the data points were obtained by fitting the data to the equation $V = V_0/(1 + (I/IC_{50})^n)$ as described in the Experimental section.

Fig. 2. The data show that this compound is a potent inhibitor of L-lactate transport; indeed, the concentrations required for 50% inhibition were much lower than any other stilbenedisulphonate or α-cyanocinnamate derivative examined previously [4,10]. The data could be fitted to the equation for linear inhibition (see Methods), but at high concentrations of TBenzDS, inhibition was not as great as expected when using this model. The equation for cooperative inhibition resulted in a better fit to this data; for rat erythrocytes at 7 °C, IC_{50} and n were 1.65 ± 0.25 μM and 0.79 ± 0.03, respectively (mean values ± S.E. for four experiments). It is unclear whether these data are a true reflection of negative cooperativity, or are related in some complex manner to the association of TBenzDS with the erythrocyte membrane (see below). However, all kinetic data relating to inhibition of lactate transport by TBenzDS will be treated in terms of IC_{50} values (concentration required for 50% inhibition of transport), rather than dissociation constants (K_i values) for the transporter-inhibitor complex. The data of Fig. 2 show that at 7 °C, the IC_{50} value for TBenzDS inhibition of L-lactate transport in human erythrocytes was 3.07 ± 0.4 μM (n = 3), approx. 2-fold higher than in rat erythrocytes under the same conditions. Inaddition, the IC_{50} value was increased approx. 3-fold to 9.1 ± 1.2 μM (n = 5) upon raising the temperature to 25 °C.

The data in Table I show the effects of both L-lactate concentration and haematocrit upon the IC_{50} values for inhibition of lactate transport in rat erythrocytes by TBenzDS. In these cells it is known that the stilbenedisulphonates DIDS and DBDS bind at an exofacial site in competition with substrate [10]. The table shows that the IC_{50} value for TBenzDS became higher upon increasing [L-lactate]. When the pooled data for three experiments were fitted to an equation describing a competitive interaction between substrate and inhibitor (see Methods), the following parameters were obtained (±S.E.): K_m, 3.27 ± 0.82 mM; $K_{0.5}$, 1.01 ± 0.30 μM; n, 0.89 ± 0.11. In Fig. 3 we demonstrate the competitive nature of the inhibition graphically, using these parameter values to generate the curves for each [L-lactate], using the equation:

$$V = V_0/(1+(I/(K_{0.5}(1+S/K_m)))^n)$$

i.e. the term $K_{0.5}\cdot(1+S/K_m)$ representing the IC_{50} at each lactate concentration. The inhibition curves for each [L-lactate] are a good fit to the data; for non-competitive inhibition, the data for each [L-lactate] would be superimposed on a single curve.

Table I also shows a considerable effect of haematocrit upon the IC_{50} value for inhibition of lactate transport by TBenzDS; this value was increased by 3.2-fold upon a 4-fold increase in haematocrit.

TABLE I

Effect of L-lactate concentration and haematocrit upon inhibition of L-lactate transport by TBenzDS

Rat erythrocytes were preincubated with seven concentrations of TBenzDS (0.15 to 9 μM) for 5 min prior to assay of initial rates of α-cyano-4-hydroxycinnamate-sensitive L-lactate transport at 7 °C. Results are shown as mean IC_{50} values ± S.E. for the number of experiments indicated in parentheses.

Haematocrit (%)	[L-Lactate] (mM)	IC_{50} (μM)
10	0.25	1.30 ± 0.24 (3)
10	2.5	2.11 ± 0.36 (3)
10	6.0	3.05 ± 0.33 (3)
5	0.5	0.84 ± 0.11 (4)
10	0.5	1.46 ± 0.12 (4)
20	0.5	2.06 ± 0.25 (4)

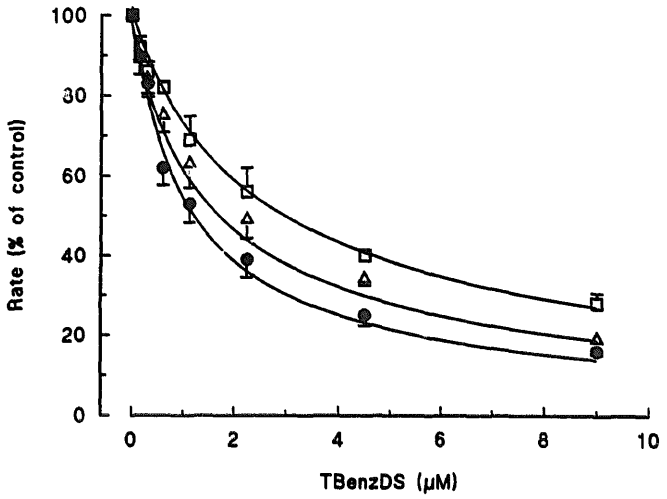

Fig. 3. Competitive inhibition of L-lactate transport in rat erythrocytes by TBenzDS. Initial rates of transport of 0.25 mM (●), 2.5 mM (△), and 6.0 mM (□) L-lactate were measured in the presence of the concentrations of TBenzDS as indicated. Data are presented as plots of rates of transport (as a percentage of control rate ± S.E. for three experiments) for each [L-lactate] versus [TBenzDS]. The lines were drawn as follows: The pooled data from three experiments (total of 72 duplicate data points) were fitted to an equation describing competitive inhibition:

$$V = V_{max}\cdot S/(K_m(1+(I/K_{0.5})^n)+S)$$

as described in Methods. The fit derived parameter values (see text) were then inserted into the equation

$$V = V_0/(1+(I/(K_{0.5}(1+S/K_m)))^n)$$

and used to generate the inhibition curves for the three concentrations of substrate given by the fit to the original equation.

Association of TBenzDS with the erythrocyte membrane

The effect of haematocrit upon IC_{50} values for inhibition of L-lactate transport by TBenzDS suggests that the inhibitor may associate with the erythrocyte membrane. This might be expected since it is more hydrophobic than other stilbenedisulphonates. The

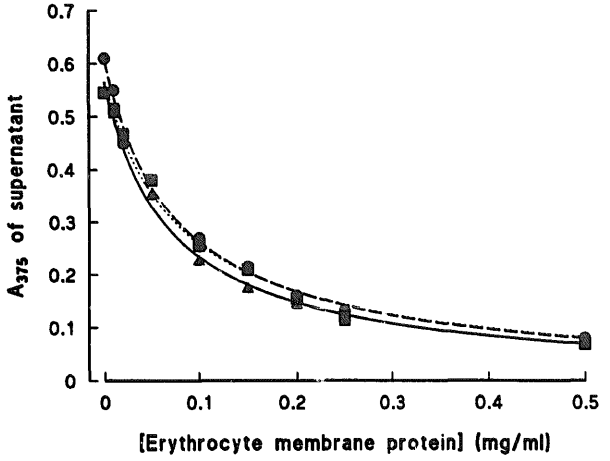

Fig. 4. Partition of TBenzDS into erythrocyte membranes. Measurement of partition of TBenzDS into erythrocyte membranes, prepared from red cells treated with 5 μM DIDS, was performed as described in Methods, using 20 μM TBenzDS and the concentrations of membrane protein as indicated. The data are given as the concentration of TBenzDS remaining in the supernatant (measured as A_{375}) after sedimentation of the erythrocyte membranes. Results are shown for rat erythrocyte membranes at 7 °C (●) and human erythrocyte membranes at 7 °C (▲) and 25 °C (■). The lines drawn are those obtained by fitting the data to an equation describing partition of the compound between aqueous and membrane phases as described in Methods. Derived values for P_m are given in the text.

data of Fig. 4 show that TBenzDS partitions very strongly into erythrocyte ghost membranes (prepared from cells pretreated with 5 μM DIDS to irreversibly inhibit band 3, as in the lactate transport experiments). Inspection of the data at 0.5 mg/ml membrane protein shows that approx. 17 μM of the total 20 μM TBenzDS was bound to the membranes. This compares with the concentration of the band 3 protein of 2 μM at this concentration of membranes (see below), but specific binding of TBenzDS to this protein should be minimal because the membranes were prepared from cells pretreated with 5 μM DIDS. Indeed, when control erythrocyte membranes were used in these experiments, the binding curves could not be distinguished from those shown in Fig. 4 for DIDS-treated membranes (not shown). For rat erythrocyte membranes at 7 °C, the derived P_m value was 6700 ± 370 (value ± S.E. for the data in Fig. 3). As expected, no non-specific binding of the less hydrophobic DBDS to erythrocyte membranes, prepared from DIDS-treated cells (see above), could be detected (Poole, R.C., unpublished observations). For human erythrocyte membranes, partition coefficients (± S.E.) of 7048 ± 455 and 5913 ± 454 were obtained at 7 °C and 25 °C, respectively. Thus TBenzDS binds to human erythrocyte membranes as tightly as to those from rat, with only a small effect of temperature on P_m. This implies that the effect of temperature upon inhibition of lactate transport by TBenzDS, described above, is not due to a change in partition of the inhibitor into the membrane.

Inhibition of band 3-mediated sulphate transport by TBenzDS

Fig. 5 shows that transport of 4 mM SO_4^{2-} into rat erythrocytes was inhibited by TBenzDS, in a manner dependent on the haematocrit. Inspection of the figure reveals that 50% inhibition required approx. 1.5, 2.5 and 5 μM TBenzDS at 5, 10 and 20% haematocrit, respectively. DIDS binds to band 3 with extremely high affinity (K_i approx. 30 nM, see Janas et al. [15]), and so titrates out SO_4^{2-} transport in a manner which is linear with respect to concentration of the inhibitor (see Fig. 5). This determines that the upper limit of the band 3 concentration is 2 μM (approx. 10^6 sites/cell) in rat erythrocytes at 10% haematocrit, assuming a 1 : 1 binding stoichiometry. Comparison of the data for DIDS with that for TBenzDS at 10% haematocrit demonstrates that DIDS is the more potent inhibitor of sulphate transport. However, whilst low concentrations of TBenzDS caused less inhibition than the reversible inhibitor DBDS (K_i approx. 2 μM in human erythrocytes [16,17]) in erythrocytes at 10% haematocrit, complete inhibition by TBenzDS was achieved at lower concentrations than with DBDS (see Fig. 5). These unusual inhibition curves for TBenzDS are probably related to the competition between binding of the inhibitor to phospholipids and the band 3 protein. This makes it very difficult to estimate the true K_i for inhibition of band 3-mediated sulphate transport by TBenzDS, especially since it is unclear whether the band 3 protein senses the aqueous or membrane concentration of the inhibitor.

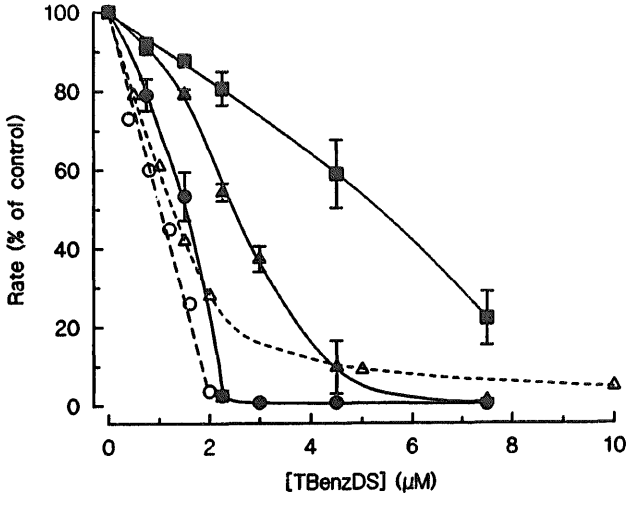

Fig. 5. Inhibition of band 3-mediated sulphate transport in rat erythrocytes by TBenzDS. Rat erythrocytes at 5% (●), 10% (▲) and 20% (■) haematocrit were incubated for 5 min at 30 °C with TBenzDS before assay of initial rates of sulphate transport as described in Methods. Data are shown as mean values ± S.E. for three separate experiments using different erythrocyte suspensions. Inhibition of sulphate transport, with cells at 10% haematocrit, by DIDS (○) and DBDS (△), in single experiments, are shown for comparison (broken lines). Smooth curves are drawn through the data points.

TBenzDS as a fluorescent probe

DBDS has been used as a fluorescent probe, since an enhancement of fluorescence is observed upon increasing the hydrophobicity of its solvent environment, or upon binding to specific sites on albumin and band 3 [16,18]. This fluorescence technique has provided valuable information on the stilbenedisulphonate binding site on the band 3 protein, and the relationship between this and the substrate binding site [16,17]. TBenzDS is also fluorescent, and excitation and emission spectra of the compound are shown in Fig. 6. In an aqueous medium, excitation and emission maxima were approx. 385 nm and 445 nm, respectively. Fluorescence spectra were also recorded in solvents with lower dielectric constants (ε) than water ($\varepsilon = 80$): methanol ($\varepsilon = 33$), ethanol ($\varepsilon = 24$), and ethyl acetate ($\varepsilon = 6$). In these solvents there was a blue shift in both the excitation and emission spectra of TBenzDS. The excitation spectrum was broadened considerably with these solvents, and the emission peak split into two maxima at approx. 420 and 440 nm. Whilst there was a small fluorescence enhancement with methanol and ethanol, this was not observed with the more hydrophobic ethyl acetate. Thus, the extent of fluorescence enhancement is not a reliable indicator of the hydrophobicity of the solvent environment. However, in a viscous medium (50% sucrose), a significant enhancement of TBenzDS fluorescence, without any shift in the spectra, was observed. This is similar to the observation for DBDS [18], and is taken to reflect immobilisation of the fluorophore.

The effects of BSA, which is known to bind stilbene disulphonates, and erythrocyte membranes on the fluorescence of TBenzDS are also shown in Fig. 6. Both BSA and control rat erythrocyte membranes caused an enhancement in the fluorescence of TBenzDS. They also induced a slight blue shift in excitation and emission spectra as with organic solvents. If membranes

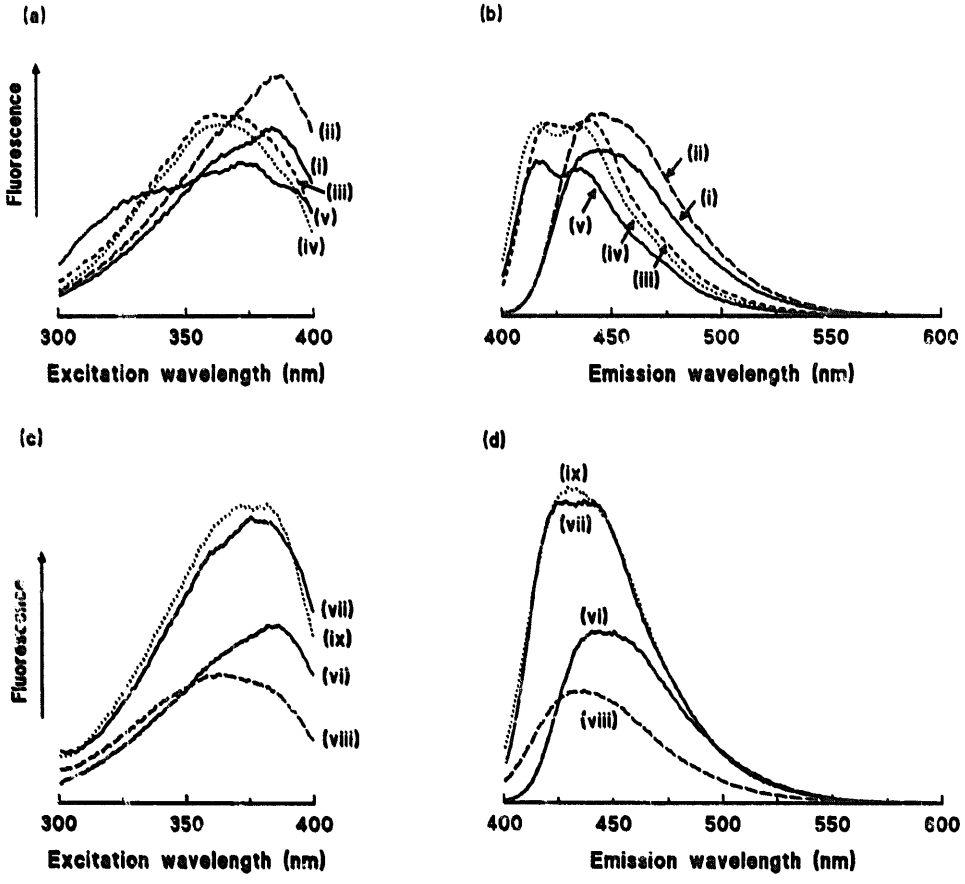

Fig. 6. Fluorescence properties of TBenzDS and its binding to band 3. Fluorescence spectra were recorded using a Perkin-Elmer LS-5B fluorimeter, connected to a microcomputer. All measurements were performed in a volume of 3 ml at 25 °C, with TBenzDS at a concentration of 2 μM, and both excitation and emission slit widths set at 2.5 nm. Excitation spectra (*a* and *c*) were recorded with the emission monochromator set at 440 nm, whereas for emission spectra (*b* and *d*), the excitation wavelength was set at 375 nm. The effects of varying the dielectric constant of the solvent, and changes in viscosity, on the fluorescence spectra of TBenzDS are shown in panels (a) and (b). The conditions were as follows; (i), H_2O; (ii), 50% (w/v) sucrose in H_2O; (iii), methanol; (iv), ethanol; (v), ethyl acetate. In panels (c) and (d) the effects of erythrocyte membrane preparations and BSA on the excitation and emission spectra of TBenzDS are shown. These spectra were recorded in citrate buffer, as used for cell incubations (78 mM sodium citrate/15 mM Hepes/1 mM EGTA, pH 7.4). Spectra are shown for (vi), buffer alone; (vii), 0.2 mg/ml erythrocyte membrane protein; (viii), 0.2 mg/ml erythrocyte membranes from cells treated with 5 μM H_2DIDS for 1 h at 37 °C; and (ix), 0.3 mg/ml BSA.

prepared from erythrocytes pretreated with 5 μM 4,4'-diisothiocyanodihydrostilbene-2,2'-disulphonate (H_2DIDS) for 1 h at 37 °C (to permanently occupy the stilbene disulphonate binding site on the band 3 protein) were used, there was a blue shift in both excitation and emission spectra, as with the control membranes. This implies that the inhibitor is in a hydrophobic environment when bound to membrane lipids (see above). However, there was a slight decrease, rather than an increase, in fluorescence intensity with the H_2DIDS-treated membranes. These data indicate that the enhancement of TBenzDS fluorescence observed with control membranes is a reflection of specific binding to the stilbene disulphonate site on the band 3 protein. Similar results to those observed with H_2DIDS-treated membranes were obtained using membranes prepared from erythrocytes pretreated with 5 μM DIDS, but addition of DIDS to solutions of TBenzDS was found to cause some quenching of fluorescence (data not shown).

The enhancement of fluorescence upon binding to the band 3 protein and BSA may be due to immobilisation of the probe. In both cases, the slight blue shifts observed in the spectra imply that TBenzDS is in a more hydrophobic environment when bound to these proteins than when free in aqueous solution. It is of note that TBenzDS is more bulky and hydrophobic than the other stilbenedisulphonates known to bind to band 3 [21,22].

We could find no evidence to indicate that binding of TBenzDS to the erythrocyte lactate transporter could be measured using the fluorescence technique. This was confirmed using membranes from guinea pig erythrocytes that had been pretreated with 5 μM DIDS. Erythrocytes from this species have the highest capacity for monocarboxylate transport [23], but no decrease in TBenzDS fluorescence was observed upon addition of substrates for the transporter was observed at either 7 °C or 25 °C (not shown). These data imply that either inhibitor binding to this protein does not result in an increase in fluorescence of TBenzDS, or more probably, that the lactate transporter is not sufficiently abundant to be detected by this technique.

Discussion

The data presented here indicate that TBenzDS is as potent as any known inhibitor of L-lactate transport in erythrocytes. The IC_{50} value of approx. 1.7 μM for rat erythrocytes (0.5 mM L-lactate; 10% haematocrit) compares with a value of 36 μM for DBDS, the next most potent stilbene disulphonate [10] and approx. 30 μM for α-cyano-4-hydroxycinnamate [1,4]. Only quercetin and phloretin have IC_{50} values that are comparable with that of TBenzDS in rat erythrocytes (approx. 2 μM and 1.5 μM, respectively; Poole, R.C., unpublished data), but these compounds inhibit many membrane permeability pathways [24–28]. As with DBDS and DIDS, inhibition of lactate transport by TBenzDS appears to be competitive with substrate, an observation which would be difficult to predict from their structures [10].

The most notable feature of TBenzDS is its hydrophobicity, which results in partition into the erythrocyte membrane, yielding a very high local concentration in this environment. The two sulphonate groups on the molecule probably prevent rapid permeation of the membrane, and perhaps it is only partially embedded in the membrane. There are two explanations for the inhibitory potency of TBenzDS. One is that the inhibitor binds more tightly to the transporter than other stilbenedisulphonates because of its greater bulk and hydrophobicity. The other is that the inhibitor partitions into the membrane, resulting in a high local concentration, prior to binding to the carrier, perhaps by 'sliding' into its binding site by lateral movement from the bulk phospholipid. It is impossible to prove from the data presented here whether inhibition of L-lactate transport is a consequence of interaction of aqueous or membrane-associated TBenzDS with the transporter, since the effects of haematocrit will cause parallel changes in both concentrations. The effect of temperature on the IC_{50} value for TBenzDS in human erythrocytes is not secondary to changes in P_m. However, lipid phase changes/separations occur in this temperature range [29,30], and so if TBenzDS is preferentially associated with a particular lipid species, this could result in a higher concentration of inhibitor in the immediate environment of the carrier protein as the temperature is lowered. Alternatively, a change in dissociation constant of the transporter-inhibitor complex may be responsible, if binding is associated with a decrease in entropy.

TBenzDS may prove to be useful as a fluorescent probe, complementary to DBDS, to study the stilbenedisulphonate binding site on the band 3 protein. Upon purification of sufficient quantities of lactate transporter, it may also be possible to detect binding of the compound to this protein using the fluorescence technique. If this were the case, then the inhibitor may provide valuable information on the stilbenedisulphonate binding site on this protein and its association/interaction with the sites which bind substrates and other inhibitors of transport.

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