The kinetics, substrate and inhibitor specificity of the lactate transporter of Ehrlich–Lettré tumour cells studied with the intracellular pH indicator BCECF

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1. Suspensions of cultured Ehrlich–Lettré tumour cells were loaded with the pH-sensitive fluorescent indicator 2',7'-bis-(carboxyethyl)-5(6)-carboxyfluorescein (BCECF), and changes in intracellular pH upon addition of L-lactate and other monocarboxylates were continuously monitored by fluorimetry using dual-wavelength excitation (450/500 nm) and single-wavelength emission (> 520 nm). 2. The rapid fluorescence changes were analysed by first-order regression analysis, and with suitable calibration procedures this enabled calculation of initial rates of proton uptake associated with monocarboxylate transport. 3. The stoichiometry was shown to be one proton per lactate molecule transported. 4. The kinetics of carrier-mediated transport of a wide range of monocarboxylates were determined at 25 °C. The K_m values for L-lactate, pyruvate and D-lactate were found to be 4.54, 0.72 and 27.5 mM respectively, similar to values found previously for rat erythrocytes. This similarity was shared with a wide range of variously substituted C₂, C₃ and C₄ monocarboxylates, all of which were transported with similar

V_{max} . No stereoselectivity was found in the K_m values for D- and L-2-chloropropionate (0.75 mM) or D- and L-3-hydroxybutyrate (11 mM), but in the latter case the V_{max} of the D-isomer was twice that of the L-isomer. 5. The temperature-dependence of L-lactate transport demonstrated a transition point, with activation energies of 60 and 109 kJ·mol⁻¹ above and below 19 °C respectively. The K_m for L-lactate below the transition temperature was about half that above it. 6. Inhibition of lactate transport into tumour cells by a wide range of compounds known to inhibit the erythrocyte monocarboxylate carrier was analysed. Patterns of inhibition were similar to those seen in the erythrocyte, but the K_i values were 2–4-fold higher in the tumour cells. 7. It is concluded that tumour cells contain an isoform of the monocarboxylate carrier with functional properties almost identical with that found in erythrocytes. This is probably identical with MCT1, which was recently cloned and sequenced from Chinese Hamster Ovary cells [Kim Garcia, Goldstein, Pathak, Anderson and Brown (1994) *Cell* 76, 865–873].

INTRODUCTION

Most tumour cells produce the ATP that they require for their growth and cell division by glycolytic conversion of glucose into lactic acid, rather than by total oxidation of glucose around the citric acid cycle (McKeehan, 1982; Newsholme et al., 1985; Gonzalez-Mateos et al., 1993). Such anaerobic metabolism may represent an advantage for rapidly growing tumours, whose vascular supply might prove inadequate for the provision of sufficient oxygen for ATP synthesis through oxidative phosphorylation. However, the resulting high rates of glycolysis lead to the formation of large quantities of lactic acid, which must leave the cell rapidly. If this were to be prevented, the accumulation of lactic acid within the cell would cause intracellular acidification and consequent inhibition of glycolysis.

Extensive work from this and other laboratories has identified the existence of a family of monocarboxylate transporters which catalyse the net transport of lactic acid and a wide range of other short-chain monocarboxylic acids across cell plasma membranes (see Poole and Halestrap, 1993). The best characterized of these is that found in erythrocytes. In isolated heart cells, studies of the kinetics and substrate and inhibitor specificities of monocarboxylate transport have led us to propose that there are two additional isoforms of the carrier present in these cells, both

distinct from that present in erythrocytes (Poole et al., 1989, 1990; Wang et al., 1993, 1994). Furthermore, the relative insensitivity to inhibition by 4,4'-di-isothiocyanostilbene-2,2'-disulphonate (DIDS) and 4-acetamido-4'-isothiocyanostilbene-2,2'-disulphonate (SITS) of lactate transport into liver cells and sarcolemmal vesicles from skeletal muscle suggests that yet other isoforms may exist in these tissues (Edlung and Halestrap, 1988; Roth and Brooks, 1990; Juel, 1991). Earlier studies on Ehrlich ascites-tumour cells showed that they also possess a proton-linked monocarboxylate carrier (Spencer and Lehninger, 1976; Belt et al., 1979; Johnson et al., 1980). However, the lack of stereoselectivity for L- over D-lactate, and a K_m for pyruvate substantially higher than has been observed in other tissues (Spencer and Lehninger, 1976), suggest that this might be yet another distinct isoform of the carrier. If evidence could be found for a unique isoform in tumour cells, it might provide a locus for effective chemotherapy. Indeed, inhibition of lactic acid efflux from Ehrlich ascites cells with bioflavonoids such as quercetin has been shown to decrease intracellular pH (pH_i) and hence to inhibit glycolysis and cell growth (Belt et al., 1979). Thus we decided to characterize monocarboxylate transport into these cells more fully by performing a detailed study of the kinetics of the carrier for a range of substrates and inhibitors. For this purpose we have developed a continuous fluorimetric assay

Abbreviations used: BCECF, 2',7'-bis-(carboxyethyl)-5(6)-carboxyfluorescein; BCECF/AM, BCECF acetoxymethyl ester; DIDS, 4,4'-di-isothiocyanostilbene-2,2'-disulphonate; DBDS, 4,4'-dibenzamidostilbene-2,2'-disulphonate; DNDS, 4,4'-dinitrostilbene-2,2'-disulphonate; NBDS, 4-nitro-4'-benzamidostilbene-2,2'-disulphonate; TBenzDS, *NNN'*-tetrabenzyl-4,4'-diaminostilbene-2,2'-disulphonate; SITS, 4-acetamido-4'-isothiocyanostilbene-2,2'-disulphonate; UK 5099, α -cyano- β -(1-phenylindol-3-yl)-acrylate; CHC, α -cyano-4-hydroxycinnamate; pH_i, intracellular pH; pH_o, extracellular pH.

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of transport, using the intracellular pH indicator 2',7'-bis-(carboxyethyl)-5(6)-carboxyfluorescein (BCECF).

Since lactate transport involves co-transport of a proton, its uptake into cells will be accompanied by a slight acidification, which can be detected using BCECF. This has already been demonstrated by others in several cell types, although detailed kinetic studies have not been reported (Bonanno, 1990; Best et al., 1992; Cairns et al., 1993; Nedergaard and Goldman, 1993; Sekine et al., 1994). We have recently developed the technique successfully for use in single isolated heart cells and demonstrated that kinetic parameters measured this way are in excellent agreement with those measured by conventional radioactive procedures (Wang et al., 1993, 1994). Indeed, since fluorescence can be measured continuously with a very rapid time resolution, the technique both produces more accurate measurements of transport rates and allows them to be made at higher, more physiological, temperatures. Furthermore, the technique can be used to study the proton-linked transport of any monocarboxylate, since it is not dependent on obtaining the substrate in a radioactively labelled form. In the present studies, these advantages are used to the full to characterize the kinetics of transport of a wide range of substrates and the inhibition produced by a range of inhibitors. Our results represent the most complete study of the substrate specificity of any lactate carrier studies to date, and strongly suggest that the isoform present in tumour cells is the same as that found in erythrocytes.

EXPERIMENTAL

Materials

Chemicals and biochemicals were obtained from sources given previously (Poole and Halestrap, 1988, 1991; Poole et al., 1991), with the following additions. BCECF, acetoxymethyl ester (BCECF/AM) was obtained from Calbiochem, Nottingham, U.K., or Boehringer Mannheim U.K., Lewes, East Sussex, U.K. Minimal Essential Medium (with Earle's salts) and newborn-calf serum (heat-inactivated) were obtained from Gibco BRL (Life Technologies), Paisley, Renfrewshire, Scotland, U.K. Benzylpenicillin sodium was obtained from Britannia Pharmaceutica, Redhill, Surrey, U.K., and streptomycin sulphate was obtained from Evans Medical, Horsham, West Sussex, U.K. Sodium butyrate, trimethylamine/HCl and nigericin were obtained from Sigma Chemical Co., Poole, Dorset, U.K. 4-Nitro-4'-benzamido-stilbene-2,2'-disulphonate (NBDS) was generously given by Dr. R. C. Poole of this Department and was synthesized from 4-nitro-4'-aminostilbene-2,2'-disulphonate by the same methodology as used to synthesize 4,4'-dibenzamidostilbene-2,2'-disulphonate (DBDS) (Wang et al., 1993). Ehrlich-Lettre mouse ascites-tumour cells (Lettre et al., 1972) were kindly donated by Dr. C. L. Bashford, Department of Biochemistry, St George's Hospital Medical School, London SW17 0RE, U.K.

Cell culture

Ehrlich-Lettre ascites cells were initially isolated from white Balb/C mice 7–10 days after intraperitoneal implantation and diluted into 20 ml of PBS containing heparin (2 international units/ml). The cells were sedimented by centrifugation at 600 *g* for 10 min and resuspended in 20 ml of PBS, from which they were taken for culture in 75 cm² flasks containing 50 ml of medium A [Minimal Essential Medium with Earle's salts, containing 10% (v/v) newborn-calf serum, 50 units/ml penicillin, 50 µg/ml streptomycin, 2 mg/ml D-glucose and 0.6 mg/ml L-glutamine] at 37 °C under an atmosphere of air/CO₂ (19:1). The

initial cell density was approx. 2×10^5 cells per flask, and reached 5×10^7 cells per flask after 8 days culture with a change of medium every 2 days. Cells were maintained at this density and harvested by a 1:2 split, half being used for subculture and half sedimented by centrifugation followed by resuspension of the cells from eight culture vessels in 20 ml of PBS. Cells were used from passage nos. 10–90 without any detectable change in their behaviour.

Preparation of cells for fluorimetric measurements

The harvested cells were washed once in PBS and twice in a standard assay buffer (see below). In each case cells were incubated at 6 °C for 20 min before centrifugation, to remove all endogenous lactate. This is important to ensure transport of monocarboxylates is measured under *zero-trans* conditions (Poole and Halestrap, 1993). The standard buffer used was slightly modified from that employed by Lambert et al. (1989) for Ehrlich ascites-tumour cells, and contained 150 mM Na⁺, 5 mM K⁺, 150 mM Cl⁻, 1 mM Ca²⁺, 1 mM sulphate, 1 mM phosphate, 3.3 mM Mops, 3.3 mM Tris and 5 mM Hepes. The buffer was adjusted to pH 7.4 at 25 °C with NaOH. Cells (1×10^7 /ml) were loaded with BCECF by incubation for 30 min at room temperature in buffer containing 10 µM BCECF/AM before washing three times in standard buffer to remove extracellular BCECF. The loaded cells were stored on ice, protected from light, at 1×10^7 cells/ml until use, and showed no detectable deterioration for at least 3 h.

Measurement of monocarboxylate transport

To 3 ml of buffer contained in a 4 ml fluorescence cuvette was added a 0.5 ml portion of the cells loaded with BCECF (final concentration of approx. 1.5×10^6 cells/ml). The cells were kept in suspension by constant stirring using an overhead paddle-stirrer integral to the cuvette holder of the fluorimeter, which was maintained at the required temperature (usually 25 °C) by water circulation. The fluorescence was measured with a modified double-beam spectrophotometer which allowed dual-wavelength excitation, the incident light switching between 450 and 500 nm (2 nm slit widths) at a frequency of 397 Hz by means of an oscillating mirror. The emitted light was passed through an optical cut-off filter (> 520 nm) on to a photomultiplier tube orientated at 90° to the incident light and in close proximity to the cuvette. The ratio of fluorescent light corresponding to the two excitation wavelengths (the '450/500' ratio) was determined electronically and the resulting signal averaged by computer to give 2 data points per second, which were displayed in real time and stored for further data analysis using our own software. The '450/500' ratio provides a measure of pH_i which minimizes errors due to light-scattering of the sample and loss of BCECF from the cell by bleaching or diffusion (Eisner et al., 1989). Once the fluorescence signal from the cell suspension had reached a steady state, usually after 1 min, substrates (neutralized as the sodium salts) were added to the suspension through a light-sealed addition port, and the subsequent fluorescence change was monitored. The initial rate of the fluorescence change was determined by fitting the data to a first-order progress curve by using Fig P and PFit software (Biosoft, Cambridge, U.K.). The first two data points were omitted, since these incorporated errors associated with the mixing time and addition artefacts. Whenever additions of coloured reagents were made to the standard assay buffer, it was confirmed that these had no effect on the '450/500' fluorescence ratio.

Calibration and measurement of pH gradients

To calibrate the BCECF 450/500 fluorescence ratio in terms of pH_i , two techniques were employed. For the most accurate calibration the standard buffer was replaced with one in which 150 mM K^+ was substituted for 150 mM Na^+ and the K^+/H^+ ionophore nigericin ($5 \mu M$) was added to equilibrate pH_i with extracellular pH (pH_o) (Thomas et al., 1979). The cell suspension was then titrated with sequential additions of either HCl or NaOH, the 450/500 fluorescence ratio recorded and the pH of the medium measured with a pH electrode in a parallel incubation. The second method, which was used for routine measurements of pH_i , was the 'null-point' technique (Eisner et al., 1989). This avoids the use of nigericin and the associated risk of contaminating the apparatus with the ionophore. In this technique, defined mixtures of sodium butyrate (a weak permeant acid) and trimethylamine hydrochloride (a weak permeant base), were added to the cell suspension until a mixture was found that gave no change in fluorescence. The pH_i was then calculated by using the formula derived by Eisner et al. (1989). The two techniques consistently gave the same value for pH_i of 7.3 under our normal incubation conditions.

Presentation of results

To calculate the rate of carrier-mediated transport, the initial rate of fluorescence change, determined by first-order curve fitting as described above, was corrected for free diffusion of the non-dissociated monocarboxylic acid by subtracting the rate observed in the presence of 5 mM α -cyano-4-hydroxycinnamate (see Poole and Halestrap, 1993). Conversion of the rate of fluorescence change into a rate of proton uptake in nmol/ μl of intracellular volume was accomplished by using the pH_i /fluorescence calibration curve and the calculated buffering power of the cells as explained in the Results and discussion section. Calculation of kinetic constants was performed by using non-linear least-squares regression analysis to the relevant kinetic equation using Fig P and PFit software (Biosoft). These fits routinely produced standard errors for each parameter of < 20% of the parameter value, but in most cases several experiments were performed on separate cell preparations and the derived parameter values were meaned and expressed as mean $\pm$ S.E.M.

RESULTS AND DISCUSSION

Calibration of BCECF signal for measurement of pH_i and proton uptake

Cells incubated in a high- $[K^+]$ medium in the presence of nigericin to allow rapid K^+/H^+ exchange across the plasma membrane equilibrate their pH_i with the pH of the medium (Thomas et al., 1979). Measurement of the external pH and the BCECF 450/500 fluorescence ratio with sequential additions of either HCl or NaOH allowed calibration of the fluorescence ratio in terms of pH_i as shown in Figure 1. No correction was made for autofluorescence, since this was found to be less than 10% of the BCECF fluorescence signal at both wavelengths, and showed no change under any of the conditions used. The change in fluorescence ratio with pH obeyed a linear relationship over the pH range used in the present experiments, with a slope of 245.5 mV/pH unit. This value was extremely consistent from day to day, as indicated by the very low errors shown in Figure 1. In order to convert the perturbation of pH_i observed upon monocarboxylate addition to cells into nmol of H^+ /monocarboxylate transported, the maximum changes in fluorescence ratio observed after addition of increasing concentrations of

Figure 1 Calibration of BCECF 450/500 fluorescence ratio for measurement of pH_i

Cells were incubated in a high- $[K^+]$ medium (150 mM) in the presence of $5 \mu M$ nigericin to allow pH_i to equilibrate with pH_o as described in the Experimental section. Measurements of changes in the 450/500 fluorescence ratio (mV) and pH were made in parallel incubations, following sequential additions of either HCl or NaOH. The black circles represent mean data from three separate cell preparations, with error bars showing the S.E.M. only where this is greater than the symbol diameter. The white circles represent a separate experiment performed over a more acid range of pH. The data were fitted by linear regression to give a slope of 245.5 mV/pH unit.

Figure 2 Calibration of BCECF 450/500 fluorescence ratio for measurement of monocarboxylate/ H^+ transport

The magnitude (mV) of the increase in 450/500 BCECF fluorescence ratio after addition of the concentrations of butyrate (●) or L-lactate (○) shown was measured at the point of maximal acidification. The smooth curves drawn gave initial slopes (by differentiation) of 14.4 and 13.8 $mV \cdot l \cdot mol^{-1}$.

butyrate were determined. Data are illustrated in Figure 2, where the fluorescence change in mV is plotted against [butyrate]. In three such experiments, the mean initial slope ($\pm$ S.E.M.) of the curve obtained was 14.35 ± 0.41 mV/mM butyrate added. Butyrate equilibrates rapidly across the membrane by free diffusion of the undissociated acid, and this was confirmed by demonstrating that addition of 5 mM α -cyano-4-hydroxycinnamate (CHC) to inhibit the monocarboxylate transporter had no effect on the change in fluorescence signal observed. At equilibrium, butyrate will equilibrate according to the equation:

$$[butyrate]_{in}/[butyrate]_{out} = [H^+]_{out}/[H^+]_{in}$$

Thus, from the resting transmembrane pH gradient (0.1 pH unit acid inside) determined as described above, $[butyrate]_{in}$ can be calculated to be $0.8 \times [butyrate]_{out}$ (the concentration of butyrate added) at the point corresponding to the initial slope of Figure

Figure 3 Time courses of the BCECF fluorescence changes associated with the uptake of increasing concentrations of L-lactate in the absence (a) and presence (b) of 5 mM CHC

After incubation of the cell suspension for approx. 1 min at 25 °C in the fluorimeter, L-lactate was added at the concentrations shown. In (b) 5 mM CHC was present from the start of the incubation. The traces shown are typical of many experiments whose results are summarized in Table 1.

2. Since all the butyrate entering the cell must have been accompanied by a proton, the value (in mM) determined for $[\text{butyrate}]_{\text{in}}$ at any time point must also represent the amount of H^+ that has entered the cell (in nmol of $\text{H}^+/\mu\text{l}$ of intracellular space). Thus, from the initial slope of Figure 2 divided by 0.8, we obtained a value of 17.94 mV for the change in fluorescence ratio associated with the entry of 1 nmol of $\text{H}^+/\mu\text{l}$ of intracellular volume. This correction factor allows us to convert any change (or rate of change) of the fluorescence ratio into a rate of H^+ entry into the cell in units of nmol of $\text{H}^+/\mu\text{l}$ of intracellular volume (per min), the same units as we have usually used in the past when determining transport rates by radioactive techniques (Poole and Halestrap, 1993). By combining the data of Figures 1 and 2, it is also possible to calculate the buffering power of the cells under our conditions, which was 13.7 mM H^+/pH unit.

Lactate/proton stoichiometry of transport

In Figure 2 we also show that the total change in fluorescence that occurs after the addition of low concentrations of L-lactate is the same as that induced by butyrate at the same concentration. Since the entry of butyrate is known to occur by free diffusion of the undissociated acid, and therefore must have a stoichiometry of 1 proton per butyrate molecule, it follows that the uptake of lactate must also occur with the same stoichiometry. Although this has been assumed, the present data formally prove it. At higher lactate concentrations the fluorescence change at equilibrium is slightly less than that caused by butyrate. This probably reflects pH-regulatory mechanisms operating to decrease the acid load induced by the monocarboxylate. Because butyrate enters the cell much more rapidly than lactate at higher concentrations, these compensating mechanisms decrease the equilibrium pH change caused by butyrate less than they do that for lactate.

Figure 4 Analysis of L-lactate transport kinetics

The time courses of the lactate-induced fluorescence changes shown in Figure 3(a) were fitted to a first-order rate equation by using $R_i - (F_i - R_0)e^{-kt}$, where R_0 and R_i are the initial and final fluorescence ratios respectively and k the first-order rate constant. The initial rate, $k(R_i - R_0)$, was then calculated from the derived parameter values (Halestrap and McGivan, 1979). The resulting values were converted into nmol of H^+/min per μl as described in the text and plotted against [L-lactate] in (b). Data obtained in the presence of 5 mM CHC (■) were then fitted by linear regression, and the slope was used as a measure of the diffusion constant for free diffusion of the undissociated lactic acid (k_{diff}). Data in the absence of inhibitor (●) were fitted to the Michaelis-Menten equation modified to incorporate the diffusion rate, i.e. $v = V_{\text{max}}[S]/(K_m + [S]) + k_{\text{diff}}[S]$. From the data shown, values for k_{diff} , K_m and V_{max} ($\pm$ S.E.M.) were $0.124 \pm 0.002 \text{ min}^{-1}$, $4.17 \pm 0.15 \text{ mM}$ and $22.69 \pm 0.41 \text{ nmol/min per } \mu\text{l}$ respectively. Mean values from several experiments are shown in Table 1.

Measurement of transport kinetics and substrate specificity

The data of Figure 3 show the changes in pH_i associated with the addition of increasing concentrations of L-lactate to BCECF-loaded cells in the absence (Figure 3a) and presence (Figure 3b) of 5 mM CHC. At all concentrations of lactate used in the present experiments, CHC gave > 90% inhibition of the rate of lactate transport. To determine the initial rates of transport accurately, and so derive kinetic constants, the time courses of the fluorescence changes were fitted to a first-order rate equation by non-linear least-squares regression, as illustrated in Figure 4(a); the fit of the data is very good. In order to determine the kinetic parameters for the transporter, it was necessary to correct the initial rate of lactate uptake for the component occurring by free diffusion of the undissociated acid (Poole and Halestrap, 1993). This was achieved by subtracting from the observed rate of fluorescence change the rate observed in the presence of 5 mM CHC. The latter shows a linear relationship with [lactate], as expected for a diffusion-mediated process, and this allows calculation of the rate constant for diffusion, k_{diff} (Figure 4b). The rate of transport was then fitted to the Michaelis-Menten equation modified to take into account the diffusion component,

Table 1 Kinetic parameters for the transport of various monocarboxylates into Ehrlich–Lettré tumour cells

For each substrate, experiments were performed as illustrated for L-lactate in Figures 3 and 4. For each separate experiment, kinetic parameters were derived by regression analysis and the resulting values were meaned and are presented as means $\pm$ S.E.M. K_i values for the monocarboxylates acting as inhibitors of lactate transport into rat erythrocytes were taken from Poole et al. (1990), except for those marked with an asterisk. These values are from unpublished work by R. C. Poole and C. Mann, and represent K_m values measured by proton flux as described by Poole and Halestrap (1991).

Substrate	<i>n</i>	K_m (mM)	V_{max} (nmol/min per μ l)	k_{diff} (min^{-1})	Rat erythrocytes K_i (mM)
C₁					
Formate	2	> 100	30.1 $\pm$ 5.69	0.061 $\pm$ 0.001	> 100
C₂					
Acetate	3	3.73 $\pm$ 1.26	17.4 $\pm$ 3.68	1.170 $\pm$ 0.240	3.5
Glyoxylate	3	63.3 $\pm$ 7.43	11.5 $\pm$ 1.78	0.017 $\pm$ 0.003	70
Oxamate	2	49.2 $\pm$ 5.0	11.9 $\pm$ 0.92	0.014 $\pm$ 0.003	17
Glycollate	2	14.6 $\pm$ 1.46	27.6 $\pm$ 1.77	0.051 $\pm$ 0.004	6.6
Chloroacetate	3	0.72 $\pm$ 0.07	15.0 $\pm$ 1.23	0.427 $\pm$ 0.016	—
Dichloroacetate	3	0.60 $\pm$ 0.09	10.7 $\pm$ 2.67	0.460 $\pm$ 0.033	—
C₃					
Pyruvate	4	0.72 $\pm$ 0.05	13.2 $\pm$ 2.71	0.378 $\pm$ 0.267	0.64
L-Lactate	7	4.54 $\pm$ 0.27	20.7 $\pm$ 1.81	0.105 $\pm$ 0.010	2.2
D-Lactate	4	27.5 $\pm$ 2.15	23.3 $\pm$ 6.69	0.018 $\pm$ 0.001	51
D-2-Chloropropionate	6	0.74 $\pm$ 0.17	12.3 $\pm$ 1.97	0.440 $\pm$ 0.033	0.98
L-2-Chloropropionate	6	0.76 $\pm$ 0.10	12.9 $\pm$ 0.93	0.391 $\pm$ 0.020	0.92
Fluoropyruvate	2	8.95 $\pm$ 0.41	19.2 $\pm$ 2.64	0.322 $\pm$ 0.172	—
C₄					
2-Oxobutyrate	2	0.16 $\pm$ 0.08	7.76 $\pm$ 2.41	0.614 $\pm$ 0.159	0.19
3-Oxobutyrate	2	5.48 $\pm$ 0.56	27.7 $\pm$ 2.84	0.093 $\pm$ 0.002	3.5
D/L-2-Hydroxybutyrate	3	2.62 $\pm$ 0.19	15.9 $\pm$ 1.34	0.054 $\pm$ 0.004	—
D/L-3-Hydroxybutyrate	3	12.5 $\pm$ 0.26	15.6 $\pm$ 0.70	0.063 $\pm$ 0.002	8.1
D-3-Hydroxybutyrate	3	10.1 $\pm$ 0.53	21.7 $\pm$ 1.31	0.060 $\pm$ 0.006	6.4*
L-3-Hydroxybutyrate	3	11.4 $\pm$ 0.82	9.08 $\pm$ 1.31	0.047 $\pm$ 0.001	10.3*
4-Hydroxybutyrate	3	7.70 $\pm$ 0.76	24.8 $\pm$ 1.46	0.110 $\pm$ 0.005	7.2
2-Hydroxy-2-methyl propionate	1	> 100	6.11	0.013	> 100

again by least-squares regression analysis, to generate values for the K_m and V_{max} for transport as shown in Figure 4(b). Mean values from several experiments are given in Table 1.

Kinetic data are also presented in Table 1 for a variety of other monocarboxylates with side chains of various chain lengths and chemical substitutions. In most cases the fit of the data was very good and consistent between experiments, but with either very poor substrates (high K_m) such as formate, oxamate and glyoxylate, or very good substrates (low K_m) such as 2-oxobutyrate, the errors on the K_m were significantly greater. For 2-oxobutyrate (K_m 0.18 mM) the magnitude of the change in pH_i at concentrations of the substrate below its K_m was too small to measure initial rates with much precision. Without such a range of transport rates at substrate concentrations both above and below the K_m , the accuracy of the data fit is inevitably poorer. In addition, the inhibition by 5 mM CHC, which, although largely non-competitive, has some competitive component (see Table 4), may not be sufficient at higher substrate concentrations to provide a reliable estimate of the non-carrier-mediated rate of transport. This is reflected in the higher than expected values for k_{diff} for 2-oxobutyrate. However, it should be noted that such errors in k_{diff} will have very little effect on the derived value for the K_m , but may lead to an underestimation of V_{max} . For substrates with a high K_m , the imprecision of the kinetic parameters reflects the difficulty of adding monocarboxylates at concentrations above their K_m without producing unacceptable addition artefacts and osmotic stress to the cells. Despite the high K_m values, the main entry route of these monocarboxylates into the cell

Figure 5 CHC-sensitive transport of oxamate, glyoxylate and formate into tumour cells

The protocol was the same as that for Figure 3, but with additions of formate, glyoxylate and oxamate at 10 or 20 mM as indicated. Where present, 5 mM CHC was added 1 min before the substrate.

appears to be through the carrier, since uptake was largely abolished by 5 mM CHC (Figure 5). It should be noted that the data presented in Figure 5 are the first direct demonstrations that

Table 2 Temperature-dependence of the kinetic parameters for L-lactate transport into Ehrlich-Lettre tumour cells

The kinetic parameters were determined at the temperature shown by using the protocol described in Figures 3 and 4. The errors given are the S.E. of the parameters derived by least-squares regression analysis of the data (at least eight substrate concentrations) to the Michaelis-Menten equation after correction for the CHC-insensitive rate of transport. This was performed by using the value for k_{diff} determined from the rates of transport measured in the presence of 5 mM CHC as described in Figure 4. Each experiment number indicates an experiment performed on a different day.

Expt. no.	Temperature (°C)	K_m (mM)	V_{max} (nmol/min per μ l)	k_{diff} (min^{-1})
1	37	5.34 ± 0.56	41.5 ± 2.31	0.50
2	36	4.57 ± 0.63	51.2 ± 4.35	0.51
3	25	4.78 ± 0.56	27.2 ± 1.63	0.15
4	25	4.17 ± 0.67	22.7 ± 0.40	0.12
1	25	5.42 ± 0.25	24.6 ± 0.60	0.11
3	12	2.75 ± 0.28	4.45 ± 0.20	0.09
4	7	2.63 ± 0.24	1.34 ± 0.05	0.01

substrates such as formate, glyoxylate and oxamate enter the cell primarily on the monocarboxylate carrier. Previous data had merely shown their poor ability to inhibit the transport of lactate and pyruvate (Poole et al., 1990). Accurate estimates of the kinetic parameters for some other monocarboxylates were impossible, because their rate of transport by free diffusion of the undissociated acid was very large compared with that through the transporter. This was particularly the case for unsubstituted monocarboxylates, whose transport, except that of acetate, was barely affected by 5 mM CHC. Even for acetate the rate of diffusion was high, and this is reflected in the value for k_{diff} shown in Table 1. It should be noted that for most of the monocarboxylates tested the V_{max} values were very similar (about 20 nmol/min per μ l intracellular volume at 25 °C). Lower values were found for substrates with low K_m values, such as 2-oxobutyrates, but these may be underestimates, as outlined above.

To facilitate comparison of the kinetics of the tumour cell monocarboxylate transporter with those of rat erythrocytes, the K_i values of the latter for the different substrates acting as competitive inhibitors of lactate transport are also shown in Table 1. K_i values are given, rather than K_m values, since the latter are not available in most cases. However, it has been shown that substrates acting as competitive inhibitors have K_i values that closely match their K_m values (Poole and Halestrap, 1993). It is clear that the substrate specificity of both carriers is very similar. Thus both show significant stereoselectivity for L-over D-lactate, but not for L-over D-3-hydroxybutyrate or for L-over D-2-chloropropionate, although in the case of 3-hydroxybutyrate the V_{max} for the D-isomer (the naturally occurring form) was found to be about twice that of the L-isomer. In rat erythrocytes, values (A. P. Halestrap, R. C. Poole and C. Mann, unpublished work) reported in Table 1 and those of others (Regen and Tarpley, 1978; Anderson et al., 1978) have also suggested the D-isomer of 3-hydroxybutyrate to be the better substrate, but in this case it is a lower K_m that is responsible. Both the tumour cell and erythrocyte carriers accept substitutions on C-2 or C-3, with substitution on C-2 with a chloro or oxo group greatly enhancing the affinity of the carrier for the substrate. The transporter also accepts double substitution of C-2 with chloride, as in dichloroacetate, or with the hydroxyl group, as in fluoropyruvate, which exists primarily in the diol form (Meany and Tienhaara, 1973), but not when a methyl group is present, as in

Figure 6 Transport of branched-chain keto acids and phenylpyruvate into tumour cells

L-Lactate (LAC), phenylpyruvate (PHE), α -ketoisocaproate (KIC) or α -ketoisovalerate (KIV) was added at 5 mM (i–iii, vii, viii) or 1 mM (iv–vi) where indicated by the arrow marked MC. After a new steady-state fluorescence ratio was obtained, a second addition of 5 mM L-lactate was made as indicated. In trace vii 5 mM CHC was also present.

2-hydroxy-2-methylpropionate. For both carriers oxamate, glyoxylate and formate are very poor substrates. In most instances, the K_m values measured for the tumour cell carrier are similar to or a little higher than those measured for the erythrocyte carrier. Some differences may reflect the different species from which the cells are derived, erythrocytes from the rat and the tumour cells from the mouse. There may also be differences in the phospholipid composition between the two cell types, which can alter carrier kinetics (Grunze et al., 1980). Another factor may be that the measurements with rat erythrocytes were performed at 6 °C, whereas those with the tumour cells were made at 25 °C. Deuticke (1982) has reported that the K_m for lactate efflux from human erythrocytes is decreased as the temperature is lowered, and we have confirmed that is the case for the tumour cells, as shown in Table 2.

There are two substrates whose kinetics of transport we have attempted to measure without success; these are α -ketoisocaproate (2-oxo-4-methylpentanoate) and α -ketoisovalerate (2-oxo-3-methylbutyrate), which are the transamination products of leucine and valine respectively. When these substrates were added to the BCECF-loaded cells there was a fast initial decrease in pH_i , which slowed before the expected equilibrium had been reached. This is illustrated in Figure 6. Addition of L-lactate after the initial response to these substrates gave a very much slower decrease in pH_i than was observed if sequential additions of L-lactate were made in their absence. These data support our previous suggestion that monocarboxylates with hydrophobic side chains may inhibit the transporter strongly by binding to an internal binding site with high affinity (Halestrap et al., 1974; Halestrap, 1976; Poole and Halestrap, 1993). It is envisaged that the substrate is transported by the carrier to the inner surface, where it may either be released rapidly, as is the case for most substrates, or form a tight-binding complex which prevents return of the empty carrier to the external face of the plasma membrane. A similar mode of action is thought to operate for CHC, the most widely used inhibitor of monocarboxylate transport (Halestrap and Denton, 1974; Halestrap, 1976; Leeks and Halestrap, 1978; Poole and Halestrap, 1993). Surprisingly, phenylpyruvate did not demonstrate the same rapid uptake followed by inhibition, and appeared to show very little carrier-

Figure 7 Concentration-dependence of the inhibition of lactate transport into tumour cells by DBDS (■), phloretin (●) and mersalyl (▲)

Cells were preincubated with the required concentration of inhibitor for approx. 2 min before addition of 5 mM L-lactate. Initial rates of change of fluorescence ratio were measured as described in Figures 3 and 4 and are expressed as a percentage of the rate in the absence of inhibitor. Data shown represent means $\pm$ S.E.M. (error bars) from three separate experiments. For each experiment, data were fitted by non-linear least-squares regression to the equation $v = 100 / (1 + ([I]/K_{0.5})^n)$ for DBDS and phloretin and $v = 100 / (1 + ([I]/K_{0.5})^n)$ for mersalyl as explained in the text. Mean values $\pm$ S.E.M. for $K_{0.5}$ derived from several experiments are reported in Table 3.

mediated transport. However, previous studies on the human erythrocyte transporter showed that, when added simultaneously with the substrate (pyruvate), phenylpyruvate and α -ketoisocaproate had competitive K_i values of 0.6 and 1.3 mM respectively (Halestrap, 1976). This suggests that in human erythrocytes both these monocarboxylates bind to the external substrate binding site of the carrier with higher affinity than pyruvate (K_m 1.9 mM) or L-lactate (K_m 9.1 mM). The difference in behaviour between the two noticed here could be explained if phenylpyruvate, unlike the branched-chain keto acids, is only transported very slowly by the carrier and does not readily form a tightly bound complex with the internal substrate-binding site. Phenylpyruvate and the branched-chain keto acids accumulate in Phenylketonuria and Maple Syrup Urine Disease respectively, both of which are diseases associated with disturbances of the central nervous system (CNS). It is possible that inhibition of the transport of lactic acid out of cells in the CNS may play a part in these effects, although the inhibition of pyruvate transport across the mitochondrial inner membrane is a more likely locus of action (Halestrap et al., 1974).

Inhibitors of L-lactate transport

The inhibition of the lactate transport into tumour cells by a variety of established inhibitors of the erythrocyte monocarboxylate transporter was investigated, and data are reported in Figure 7 and Tables 3 and 4. In most cases a single concentration of L-lactate was used (5 mM) and the concentration of inhibitor was varied. From such studies the data could be fitted well by least-squares regression analysis to the equation $v = V / (1 + [I]/K_{0.5})$, where v is the rate of change of pH_i in the presence of inhibitor concentration $[I]$, V is the rate in the absence of inhibitor and $K_{0.5}$ the concentration of inhibitor giving half-maximal inhibition under the conditions used. This is exemplified by the data for DBDS and phloretin in Figure 7. However, for the mercurial thiol reagents mersalyl and *p*-chloromercuribenzenesulphonate this equation was not appropriate, since these reagents are not simple reversible inhibitors. Rather, $K_{0.5}$ was calculated from the equation

$v = V / (1 + ([I]/K_{0.5})^n)$, which gave a good fit to the data. This was also found to be the case in erythrocytes by Deuticke et al. (1978, 1982), although Spencer and Lehninger (1976) reported simple competitive inhibition by mersalyl in their studies on tumour cells. It is probable that, at the lower concentrations of such mercurial reagents, very reactive thiol groups are attacked before the lactate carrier, and thus little inhibition is observed until these more reactive groups have been saturated.

Values for the $K_{0.5}$ of a variety of inhibitors are shown in Table 3, where data for the rat erythrocyte are also presented. The overall pattern of inhibitor potency is the same for both cell types, but the absolute values are significantly higher for the tumour cells than for the erythrocytes. The lower temperature (6 °C) used in the erythrocyte studies might lead to a 2–3-fold lower K_i than for the tumour cells, in analogous fashion to the effect of temperature on the K_m value for L-lactate (Table 2). However, two additional factors may also lead to substantial increases in the $K_{0.5}$ values observed in the present experiments. Firstly they were performed at 5 mM L-lactate, which is just above the K_m , rather than 0.5 mM (about one-fifth of the K_m value) used for the erythrocyte studies. This was necessary to obtain accurate rates of change of pH_i at higher inhibitor concentrations. Stilbene disulphonates are impermeant and probably inhibit transport by binding to the external substrate-binding site (Poole and Halestrap, 1991); this produces primarily competitive inhibition, as shown in Table 4. Thus the $K_{0.5}$ would be expected to be about 2-fold higher at 5 mM L-lactate than at 0.5 mM L-lactate. Secondly, the present experiments were performed in a saline medium in which the intracellular pH was similar to the extracellular pH, whereas in the erythrocyte experiments a citrate buffer was used to create a substantial pH gradient across the plasma membrane (0.6–0.8 pH unit alkaline inside). It was not possible to generate a similar pH gradient across the plasma membrane of tumour cells under such conditions (results not shown), and so direct comparison of data obtained in the presence and absence of a pH gradient was not possible. However, we have previously performed such experiments with human erythrocytes (Halestrap, 1976; Leeks and Halestrap, 1978), where it was shown that the K_i for CHC increased from about 50 μ M in citrate medium (pH gradient 0.8 unit alkaline inside) to 400–500 μ M in saline medium (pH gradient 0.2 unit acid inside). A similar observation has been made for the effects of a transmembrane pH gradient on the inhibition of mitochondrial pyruvate transport by CHC and is believed to be a result of the inhibitor binding tightly to the inner surface of the carrier (Halestrap, 1978a,b). This is favoured by an alkaline transmembrane gradient, which drives the carrier into the inward-facing conformation.

More detailed analyses of the inhibition by DBDS, CHC and phloretin, which represent three different classes of inhibitor, were performed by studying the inhibition of transport observed when concentrations of both L-lactate and inhibitor and inhibitor were varied. Results are reported in Table 4. In all cases data were fitted to the equation for mixed inhibition and also for the simpler models of competitive and non-competitive inhibition. Where the simpler model gave as good a fit as for mixed inhibition (i.e. F value giving $P < 0.05$), this is noted and the single K_i value is quoted. Phloretin inhibition could be fitted well to a non-competitive model (i.e. K_{i1} and K_{i2} were similar), with a mean K_i of $6.9 \pm 0.5 \mu$ M ($n = 3$) compared with a value of 1.4 μ M in rat erythrocytes (Wang et al., 1993). The inhibition by CHC could also be fitted well to the non-competitive model with a mean K_i of $211 \pm 17.8 \mu$ M ($n = 3$), although a small competitive component cannot be dismissed, since the mean values of K_{i1} and K_{i2} were statistically different. In the present experiments

Table 3 Inhibition of lactate transport into Ehrlich–Lettré tumour cells by a variety of reagents

$K_{0.5}$ values for the inhibitors were obtained as described in the legend to Figure 7; at least six concentrations of inhibitor were used, distributed either side of the $K_{0.5}$. Data for each individual experimental were fitted by using non-linear least-squares regression analysis to the equations shown in Figure 7 legend. The co-operative model was only used for mersalyl and *p*-chloromercuribenzenesulphonate (PCMBs). Where more than one experiment was performed, $K_{0.5}$ values are given as the means $\pm$ S.E.M., with *n* values indicated in parentheses. Data for the rat erythrocyte are taken from Poole and Halestrap (1993), except where indicated by an asterisk. These data are unpublished work by A. P. Halestrap, R. C. Poole and C. Mann.

Inhibitor	Ehrlich–Lettré cells $K_{0.5}$ at 5 mM L-lactate (μ M)	Rat erythrocytes $K_{0.5}$ at 0.5 mM L-lactate (μ M)
α -Cyanocinnamate analogues		
α -Cyanocinnamate	1700 (1)	400
α -Cyano-4-hydroxycinnamate (CHC)	165.6 ± 20.9 (3)	27
α -Fluorocinnamate	724.4 ± 126 (2)	660
Phenylcinnamate	60.7 ± 10.8 (2)	44*
UK5099	8.1 ± 0.5 (3)	20
Stilbene disulphonates		
DIDS	434.1 ± 29.4 (5)	50
SITS	1180 ± 210 (3)	130
DBDS	215.1 ± 21.4 (5)	35
DNDS	> 5000 (3)	1200
TBenzDS	6.7 ± 1.3 (2)	1.5
NBDS	396.5 (1)	89*
Other inhibitors		
Phloretin	5.1 ± 0.2 (4)	1.4
Niflumic acid	6.1 ± 0.9 (3)	—
3-Isobutyl-1-methylxanthine	287.5 ± 19.8 (3)	200
Mersalyl	49.9 ± 2.1 (5)	—
PCMBs	112.3 ± 22.7 (3)	—
5-Nitro-2-(3-phenylpropylamino)benzoate	9.3 ± 0.7 (3)	—

Table 4 Kinetic parameters for inhibition of L-lactate transport into Ehrlich–Lettré tumour cells by CHC, DBDS and phloretin

Initial rates of lactate transport were measured for at least five substrate and five inhibitor concentrations, and the data were fitted to the equation for mixed inhibition by least-squares regression analysis, and the competitive and uncompetitive K_i values (K_{i1} and K_{i2} respectively) were derived. For each inhibitor three separate experiments were performed, and the derived parameter values are presented as the means $\pm$ S.E.M. The data were also fitted to the simpler competitive and non-competitive models, and where statistical analysis (*F* value, $P < 0.05$) showed the simpler model to be as good a fit, this is indicated under the type of inhibition. Where mixed inhibition gave a better fit, this is shown together with the dominant component of the inhibition. In both cases the K_i value ($\pm$ S.E.M.) for the simpler fit is also shown.

Inhibitor	Mixed inhibition		Type of inhibition	K_i for simplest model (μ M)
	K_{i1} (μ M)	K_{i2} (μ M)		
CHC	140.2 ± 16.5	294.5 ± 17.0	Non-competitive	211 ± 17.8
DBDS	118.5 ± 10.8	1340 ± 681	Mixed/competitive	92.4 ± 10.6
Phloretin	5.9 ± 0.6	8.4 ± 1.6	Non-competitive	6.9 ± 0.7

cells were preincubated with CHC for 1 min before transport was initiated, which would give sufficient time for the inhibitor to reach the high-affinity internal substrate-binding site, as discussed above. Non-competitive inhibition is the predicted form of inhibition under such conditions (Halestrap, 1978a; Deves and Krupka, 1989; 1990). In contrast, previous kinetic studies on the nature of CHC inhibition of the human erythrocyte lactate transporter reported competitive inhibition, but in those experiments CHC and lactate were added simultaneously (Halestrap, 1976). Inhibition by DBDS was best fitted to the equation for mixed inhibition, although with $K_{i1} \ll K_{i2}$ and large errors on K_{i2}

the fit to the competitive model was almost as good. This implies that the major element of inhibition is competitive, and so a competitive K_i ($92.4 \pm 10.6 \mu$ M; $n = 3$) is also given in Table 4. A similar pattern of inhibition was also found in rat erythrocytes (Poole and Halestrap, 1991), although, once again, the competitive K_i value measured here is significantly higher than the value of 22μ M for rat erythrocytes (Poole and Halestrap, 1991).

Activation energy of the transporter

The continuous assay of transport by using BCECF fluorescence makes it possible to measure transport rates accurately over a range of temperatures. This allows the rate of transport at physiological temperature and the activation energy for transport to be determined. The former value has been impossible to obtain directly by conventional radioactive techniques, which lack the necessary time resolution (Poole and Halestrap, 1993). The data presented in Figure 8 show the temperature-dependence of transport determined in two separate experiments and are presented as an Arrhenius plot ($1/T$ against $\ln v$). In these experiments rates of transport were measured at a single concentration of pyruvate (5 mM) which ensured that the carrier was operating near to V_{max} , even when temperature-dependent changes in K_m are taken into account, while avoiding large errors due to diffusion of the undissociated acid. The Arrhenius plot is clearly biphasic, with a transition point at about 19°C , which is often observed in studies on the temperature-dependence of transporters (Halestrap, 1976; McGivan et al., 1977; Baur and Heldt, 1977). The transition point probably indicates a phase change in the phospholipids at this temperature. It is significant that K_m values measured below this temperature were lower than those measured above it (Table 2), suggesting that this parameter

Figure 8 Temperature-dependence of the monocarboxylate transporter of tumour cells

Initial rates of transport of 5 mM pyruvate were measured at the temperatures indicated by using BCECF fluorescence. Corrections were made for changes in the response of BCECF at different temperatures by measuring the magnitude of the response to 5 mM butyrate at each temperature. The carrier-specific rates were calculated by subtraction of the rate in the presence of 10 mM CHC. Data from two separate experiments ($\circ$ and $\bullet$) are presented as an Arrhenius plot. This was analysed by performing two linear regressions, one for temperatures of 19 °C and above, which gave an activation energy of 59.9 kJ·mol⁻¹, and one for temperatures of 19 °C and below, which gave an activation energy of 109.2 kJ·mol⁻¹.

is influenced more by the state of the membrane phospholipids rather than by a direct effect of temperature on the transporter. From the data of Figure 8, the activation energy at temperatures below the transition point was calculated to be 109.2 kJ·mol⁻¹ and that above the transition temperature 59.9 kJ·mol⁻¹. From the latter value it can be calculated that the rate of L-lactate transport at 37 °C will be 2.55 times that at 25 °C, giving a $V_{\max}$ value of about 65 nmol/min per μ l intracellular volume.

Conclusions

The data presented in this paper confirm that BCECF measurement of changes in pH_i provides an excellent means of performing detailed kinetic analysis of the monocarboxylate carrier of tumour cells. The K_m and K_i values derived for a variety of substrates and inhibitors demonstrate that the carrier in these cells is very similar to that found in erythrocytes, although in many cases the absolute parameter values are 2–4-fold higher in the tumour cells. This might reflect either a species difference (the tumour cells are derived from mouse and the erythrocytes from rat) or a difference in the phospholipid composition. The transition temperature of the biphasic Arrhenius plot was found to be about 10 °C in human erythrocytes (Halestrap, 1976), compared with the value of 19 °C found in the present experiments. If this reflects a phase change in the membrane phospholipids, as has been suggested (Halestrap, 1976), then the composition and properties of the tumour-cell and erythrocyte membranes may well differ sufficiently to account for some of the differences in the kinetics of the monocarboxylate carrier observed in the two cell types. We have recently purified the lactate carrier from rabbit erythrocytes sufficiently to obtain a 16-residue N-terminal amino acid sequence (Poole and Halestrap, 1994). This sequence is identical in all but one residue, where there is a conservative substitution of Val for Ile, with that predicted from the cDNA that has been cloned and sequenced for a monocarboxylate carrier found in Chinese Hamster Ovary cells and named MCT1 (Kim Garcia et al., 1994). Thus it is likely

that tumour cells also contain MCT1, although definitive proof will require the carrier cDNA to be cloned and sequenced.

Our data indicate that the maximal rate of carrier-mediated L-lactate transport into the tumour cells at 37 °C is about 65 nmol/min per μ l intracellular volume. The reported rates of lactic acid output by Ehrlich ascites cells range from 2 to 6 nmol/min per mg of protein (Belt et al., 1979; Johnson et al., 1980; Gonzalez-Mateos et al., 1993; Halder et al., 1993). Under the conditions used in the present studies the intracellular volume was found to be 1.98 ± 0.16 μ l/mg of protein (mean $\pm$ S.E.M. of 12 separate experiments) when determined with [¹⁴C]sucrose and ³H₂O. Thus the carrier would appear to have considerable spare capacity to allow for rapid efflux of the lactic acid produced by glycolysis. Nevertheless Belt et al. (1979) demonstrated that, when quercetin was added to Ehrlich ascites cells at a concentration sufficient to inhibit the lactate transporter by more than 90 %, pH_i fell significantly and glycolysis was inhibited. We have confirmed that all transport inhibitors produce a decrease in pH_i when added at concentrations that inhibit transport by more than about 80 % (results not shown). Thus, if a sufficiently specific and potent inhibitor of the tumour monocarboxylate transporter could be found, it might be of interest as a chemotherapeutic agent. The cloning of MCT1 opens up the possibility of identifying other isoforms of the lactate carrier and their tissue distribution with greater certainty. Clearly it will be important to establish whether the isoform present in tumour cells is essential for the function of other cells dependent on rapid lactic acid influx or efflux. Only if tumour isoform-specific inhibitors can be identified are they likely to be of potential use, although inhibition of the lactate transporter of erythrocytes might be tolerated, since bovine and sheep erythrocytes lack the carrier, yet function normally (Poole and Halestrap, 1993).

We thank the Royal Society for financial support, Dr Robert Poole for providing us with several stilbenedisulphonate inhibitors and for helpful discussions, and Dr. Lindsay Bashford for provision of tumour cells.

REFERENCES

- Andersen, B. L., Tarpley, H. L. and Regen, D. M. (1978) *Biochim. Biophys. Acta* **508**, 525–538
- Baur, H. and Heldt, H. W. (1977) *Eur. J. Biochem.* **74**, 397–403
- Belt, J. A., Thomas, J. A., Buchsbaum, R. N. and Racker, E. (1979) *Biochemistry* **19**, 3506–3511
- Best, L., Trebilcock, R. and Tomlinson, S. (1992) *Mol. Cell. Endocrinol.* **86**, 49–56
- Bonanno, J. A. (1990) *Curr. Eye Res.* **9**, 707–712
- Cairns, S. P., Westerblad, H. and Allen, D. G. (1993) *J. Physiol. (London)* **464**, 561–574
- Deuticke, B. (1982) *J. Membr. Biol.* **70**, 89–103
- Deuticke, B., Rickert, I. and Beyer, E. (1978) *Biochim. Biophys. Acta* **507**, 137–155
- Deuticke, B., Beyer, E. and Forst, B. (1982) *Biochim. Biophys. Acta* **684**, 96–110
- Deves, R. and Krupka, R. M. (1989) *Methods Enzymol.* **171**, 113–132
- Deves, R. and Krupka, R. M. (1990) *Biochim. Biophys. Acta* **1030**, 24–31
- Edlung, G. L. and Halestrap, A. P. (1988) *Biochem. J.* **249**, 117–126
- Eisner, D. A., Kenning, S. C., O'Neil, S. C., Pocock, G., Richard, C. D. and Valdeolmillos, A. (1989) *Pflügers Arch.* **413**, 553–558
- Gonzalez-Mateos, F., Gomez, M. E., Garcias-Alguero, L., Sanchez, V. and Aragon, J. J. (1993) *J. Biol. Chem.* **268**, 7809–7817
- Grunze, M., Forst, B. and Deuticke, B. (1980) *Biochim. Biophys. Acta* **600**, 860–869
- Halder, J., Ray, M. and Ray, S. (1993) *Int. J. Cancer* **54**, 443–449
- Halestrap, A. P. (1976) *Biochem. J.* **156**, 193–207
- Halestrap, A. P. (1978a) *Biochem. J.* **172**, 377–387
- Halestrap, A. P. (1978b) *Biochem. J.* **172**, 389–398
- Halestrap, A. P. and Denton, R. M. (1974) *Biochem. J.* **138**, 313–316
- Halestrap, A. P. and McGivan, J. D. (1979) In: *Techniques in Metabolic Research* (Kornberg, H. L., Metcalfe, J. C., Northcote, D. H., Pogson, C. I. and Tipton, K. F., eds.), vol. B206, pp. 1–23, Elsevier/North-Holland, Amsterdam
- Halestrap, A. P., Brand, M. D. and Denton, R. M. (1974) *Biochim. Biophys. Acta* **367**, 102–108
- Johnson, J. H., Belt, J. A., Dubinsky, W. P., Zimniak, A. and Racker, E. (1980) *Biochemistry* **19**, 3836–3840

- Juel, C. (1991) *Biochim. Biophys. Acta* **1065**, 15–20
- Kim Garcia, C., Goldstein, J. L., Pathak, R. K., Anderson, R. G. W. and Brown, M. S. (1994) *Cell* **76**, 865–873
- Lambert, I. H., Hoffmann, E. K. and Jorgensen, F. (1989) *J. Membr. Biol.* **111**, 113–132
- Leeks, D. R. and Halestrap, A. P. (1978) *Biochem. Soc. Trans.* **6**, 1363–1366
- Lettre, R., Paweletz, N., Werner, D. and Granzow, C. (1972) *Naturwissenschaften* **59**, 59–63
- McGivan, J. D., Bradford, N. M. and Mendes-Mourao, J. (1977) *FEBS Lett.* **80**, 380–384
- McKeehan, W. L. (1982) *Cell Biol. Int. Rep.* **6**, 635–650
- Meany, J. E. and Tienhaara, J. E. (1973) *Biochemistry* **12**, 2067–2070
- Nedergaard, M. and Goldman, S. A. (1993) *Am. J. Physiol.* **265**, R282–R289
- Newsholme, E. A., Crabtree, B. and Ardawi, M. S.-M. (1985) *Biosci. Rep.* **5**, 393–400
- Poole, R. C. and Halestrap, A. P. (1988) *Biochem. J.* **254**, 385–390
- Poole, R. C. and Halestrap, A. P. (1991) *Biochem. J.* **275**, 307–312
- Poole, R. C. and Halestrap, A. P. (1993) *Am. J. Physiol.* **264**, C761–C782
- Poole, R. C. and Halestrap, A. P. (1994) *Biochem. J.* **303**, 755–759
- Poole, R. C., Cranmer, S. L., Halestrap, A. P. and Levi, A. J. (1990) *Biochem. J.* **269**, 827–829
- Poole, R. C., Cranmer, S. L., Holdup, D. W. and Halestrap, A. P. (1991) *Biochim. Biophys. Acta* **1070**, 69–76
- Poole, R. C., Halestrap, A. P., Price, S. J. and Levi, A. J. (1989) *Biochem. J.* **264**, 409–418
- Regen, D. M. and Tarpley, H. L. (1978) *Biochim. Biophys. Acta* **508**, 539–550
- Roth, D. A. and Brooks, G. A. (1990) *Arch. Biochem. Biophys.* **279**, 377–385
- Sekine, N., Cirulli, V., Regazzi, R., Brown, L. J., Gine, E., Tamarit-Rodriguez, J., Girotti, M., Marie, S., Macdonald, M. J., Wollheim, C. B. and Rutter, G. A. (1994) *J. Biol. Chem.* **269**, 4895–4902
- Spencer, T. L. and Lehninger, A. L. (1976) *Biochem. J.* **154**, 405–414
- Thomas, J. A., Buchsbaum, R. N., Zimniak, A. and Racker, E. (1979) *Biochemistry* **18**, 2210–2218
- Wang, X., Levi, A. J. and Halestrap, A. P. (1994) *Am. J. Physiol.*, in the press
- Wang, X. M., Poole, R. C., Halestrap, A. P. and Levi, A. J. (1993) *Biochem. J.* **290**, 249–258

Received 11 May 1994/20 July 1994; accepted 26 July 1994