Biochem. J. (1988) 249, 117–126 (Printed in Great Britain)

The kinetics of transport of lactate and pyruvate into rat hepatocytes

Evidence for the presence of a specific carrier similar to that in erythrocytes

Gillian L. EDLUND and Andrew P. HALESTRAP
Department of Biochemistry, University of Bristol, Bristol BS8 1TD, U.K.

Time courses of L-lactate and pyruvate uptake into isolated rat hepatocytes were measured in a citrate-based medium to generate a pH gradient (alkaline inside), by using the silicone-oil-filtration technique at 0 °C to minimize metabolism. At low concentrations of lactate and pyruvate (0.5 mM), transport was inhibited by over 95% by 5 mM-α-cyano-4-hydroxycinnamate, whereas at higher concentrations (> 10 mM) a significant proportion of transport could not be inhibited. The rate of this non-inhibitable transport was linearly related to the substrate concentration, was less with pyruvate than with L-lactate, and appeared to be due to diffusion of undissociated acid. Uptake of D-lactate was not inhibited by α-cyano-4-hydroxycinnamate and occurred only by diffusion. Kinetic parameters for the carrier-mediated transport process were obtained after correction of the initial rates of uptake of lactate and pyruvate in the absence of 5 mM-α-cyano-4-hydroxycinnamate by that in the presence of inhibitor. Under the conditions used, the K_m values for L-lactate and pyruvate were 2.4 and 0.6 mM respectively and the K_i for α-cyano-4-hydroxycinnamate as a competitive inhibitor was 0.11 mM. K_m values for the transport of L-lactate and pyruvate into rat erythrocytes under similar conditions were 3.0 and 0.96 mM. The $V_{max.}$ of lactate and pyruvate transport into hepatocytes at 0 °C was 3 nmol/min per mg of protein. Carrier-mediated transport of 0.5 mM-L-lactate was inhibited by 0.2 mM-*p*-chloromercuribenzenesulphonate (> 90%), 0.5 mM-quercetin (80%), 0.6 mM-isobutylcarbonyl-lactyl anhydride (70%) and 0.5 mM-4,4′-di-isothiocyanostilbene-2,2′-disulphonate (50%). A similar pattern of inhibition of lactate transport is seen in erythrocytes. It is suggested that the same or a similar carrier protein exists in both tissues. The results also show that L-lactate transport into rat hepatocytes is very rapid at physiological temperatures and is unlikely to restrict the rate of its metabolism. Differences between our results and those of Fafournoux, Demigne & Remesy [(1985) J. Biol. Chem. **260**, 292–299] are discussed.

INTRODUCTION

The existence of a specific transport mechanism for L-lactate and pyruvate was first demonstrated in human erythrocytes by the use of α-cyanocinnamate derivatives to inhibit their uptake into the cell (Halestrap & Denton, 1974; Halestrap, 1976). The transporter has since been shown to catalyse the net transport of lactic acid (Leeks & Halestrap, 1977; Deuticke *et al.*, 1978; Dubinsky & Racker, 1978) and to be sensitive to both thiol and amino reagents (see Deuticke, 1982). Evidence has also been presented for the presence of a specific transport mechanism in Ehrlich ascites cells (Spencer & Lehninger, 1976), skeletal muscle (Koch *et al.*, 1981; De Hemptinne *et al.*, 1983; Mason & Thomas, 1985; Fishbein, 1986), cardiac muscle (Watts & Randle, 1967; Mowbray & Ottaway, 1973*a*,*b*; Mann & Yudilevich, 1981; Mann *et al.*, 1985), smooth muscle (Kutchai *et al.*, 1978), placenta (Kastendieck & Moll, 1977; Moll *et al.*, 1980; Leichtweiss & Schroder, 1981; Illsley *et al.*, 1986), kidney tubules (Mengual *et al.*, 1983; Mengual & Sudaka, 1983; Jorgensen & Sheikh, 1984), intestine (Lamers, 1975), blood/brain barrier (Oldendorf, 1972, 1973; Partridge & Oldendorf, 1977; Cremer *et al.*, 1979, 1982) and liver (Leeks & Halestrap, 1979; Schwab *et al.*, 1979; Monson *et al.*, 1982; Fafournoux *et al.*, 1985). However, to characterize the kinetic properties of these transporters with any accuracy, it is necessary to determine initial rates of lactate uptake into the cell in the absence of appreciable metabolism (Halestrap & McGivan, 1979). This is most easily accomplished with isolated cell preparations by using inhibitor-stop or centrifuge-stop techniques (Halestrap & McGivan, 1979), although for lactate transport both extracellular and intracellular pH electrodes have been used successfully (Spencer & Lehninger, 1976; Leeks & Halestrap, 1977; Dubinsky & Racker, 1978; Mason & Thomas, 1985; Mason, 1986).

The liver is a major site of lactate utilization for both glucose and fatty acid synthesis. As such, it might be expected to have a transport mechanism for lactate with high activity. Indeed, it is generally assumed that lactate and pyruvate rapidly equilibrate across the plasma membrane of the liver cell and that the [lactate]/[pyruvate] ratio in the perfusion/incubation medium can be used as an indicator of the intracellular [NADH]/[NAD^+] ratio (Tischler *et al.*, 1977; Groen *et al.*, 1983). Direct measurement of the [lactate]/[pyruvate] ratio in the cytosol and the incubation medium of liver cells has shown this assumption to be justified (Tischler *et al.*, 1977). Preliminary studies from our laboratory confirmed

Abbreviations used: *p*CMBS, *p*-chloromercuribenzenesulphonate; iBCLA, isobutylcarbonyl-lactyl anhydride; DIDS, 4,4′-di-isothiocyanostilbene-2,2′-disulphonate; SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulphonate; DMO, 5,5′-dimethyloxazolidine-2,4-dione.

that transport of pyruvate into isolated rat liver cells was very rapid and could be inhibited by α-cyano-4-hydroxycinnamate and *p*-chloromercuribenzenesulphonate (*p*CMBS) (Leeks & Halestrap, 1979). However, even at 25 °C it was found that transport was too fast and metabolism of the ^{14}C-labelled substrate too extensive to allow accurate assessment of initial rates of transport and thus determination of kinetic parameters. Two other laboratories have studied the uptake of L-lactate into hepatocytes. Monson *et al.* (1982) used a silicone-oil-filtration technique to measure uptake of L- and D-[^{14}C]lactate into hepatocytes from starved rats after a 15 s incubation. They also showed inhibition of uptake by *p*CMBS and α-cyano-3-hydroxycinnamate and provided evidence for a stereoselective saturable transport mechanism and a non-saturable non-selective process. However, they were unable to provide accurate kinetic analysis, because transport appeared to have equilibrated the lactate within about 5 s, again suggesting a very rapid transport process. Fafournoux *et al.* (1985) performed similar experiments in the presence of inhibitors of gluconeogenesis and with hepatocytes from fed rats. They claimed to measure the uptake of lactate at times as short as 10 s at 37 °C and, using this 10 s uptake value, they determined various kinetic parameters. They concluded that the K_m for L-lactate was 1.8 mM and the $V_{max.}$ 3.6 nmol of lactate/min per mg wet wt. of cells. Inspection of the time courses of Fafournoux *et al.* (1985) and comparison with the data of Leeks & Halestrap (1979) and Monson *et al.* (1982) suggest that their methodology is inadequate to measure true initial rates of transport. Thus their kinetic parameters are likely to be suspect. Indeed, the $V_{max.}$ values that they calculate for lactate transport could give a maximal rate of gluconeogenesis of no greater than about 9 nmol of glucose/min per mg of protein. Such rates can be determined in the presence of fatty acids, and yet there is no evidence for lactate entry into the cell restricting gluconeogenesis (Groen *et al.*, 1983, 1986). Thus we decided to attempt to measure the kinetics of lactate and pyruvate transport into hepatocytes at low temperatures and to compare the kinetic values obtained with those for rat erythrocytes.

EXPERIMENTAL

Materials

Unless stated otherwise, the sources of chemicals and biochemicals were the same as those given elsewhere (Halestrap, 1976; Thomas & Halestrap, 1981). Silicone oil MS550 and dinonyl phthalate were obtained from BDH Chemicals, Poole, Dorset, U.K. All radiochemicals were from Amersham International, Amersham, Bucks., U.K.

Preparation of liver cells

Liver parenchymal cells were prepared from male Wistar rats of 250–300 g body wt., starved for 24 h but with free access to water, by the procedure of Berry & Friend (1969) as modified by Williamson *et al.* (1969). The viability of the cells was tested by their ability to synthesize glucose from 10 mM-L-lactate and 1 mM-pyruvate as described by Thomas & Halestrap (1981). Cell proteins were measured by a modified biuret method (Gornall *et al.*, 1949), with corrections made for albumin in the incubation medium.

Measurement of initial rates of transport

For all transport experiments, cells were resuspended in medium containing 84 mM-sodium citrate/2.5 mM-Ca^{2+} and adjusted to pH 7.4 with 1 M-NaH_2PO_4. Albumin was not added, since it is known to bind α-cyano-4-hydroxycinnamate (Thomas & Halestrap, 1981). Initial rates of transport were measured by a silicone-layer-filtration technique similar to that described by McGivan *et al.* (1977). Tubes for the Beckman model 152 centrifuge were prepared as follows: 0.1 ml of oil containing silicone oil MS550 (sp. gr. 1.07) and dinonyl phthalate (sp. gr. 0.98) (7:3, v/v) was layered on top of 0.05 ml of 10 % (v/v) $HClO_4$ containing 25 % (w/v) glycerol. Liver cells were resuspended in the sodium citrate buffer (approx. 16 mg of protein/ml) and equilibrated at 0 °C with constant agitation. Transport was initiated by addition of [U-^{14}C]lactate or [2-^{14}C]pyruvate substrate (0.2 μCi/ml) and [^{3}H]inulin (2 μCi/ml; 50 μg/ml) as an extracellular marker. At appropriate time intervals, a 0.2 ml sample of the cell suspension was removed and layered on top of the oil. Transport was terminated by immediate centrifugation for 15 s, the cells sedimenting through the oil layer into $HClO_4$. The supernatant fraction was acidified, and then the tubes were frozen in liquid N_2 and cut at the silicone oil/$HClO_4$ interface with a sharp blade, as described by McGivan *et al.* (1977). A sample (50 μl) of the supernatant and the tip of the tube containing the pellet and $HClO_4$ were diluted to 0.5 ml with water in separate scintillation vials. After vigorous shaking to disperse the pellet, 10 ml of Unisolve E was added to each vial, and ^{3}H and ^{14}C were measured by dual-label liquid-scintillation counting. The uptake of substrate was calculated as described by Halestrap & McGivan (1979). In some experiments uptake was studied by a centrifuge-stop technique not involving the use of oil (Halestrap & McGivan, 1979). This gave essentially the same results as the silicone-oil-filtration technique, but proved less accurate, especially at higher substrate concentrations, where the intracellular ^{14}C was only a small percentage of the total ^{14}C in the pellet.

Measurement of intracellular volume and pH gradient

Values for the intracellular volume and pH gradient were measured under the same conditions as used for the transport studies. For volume measurements, the liver-cell suspension was preincubated with ^3H_2O (1 μCi/ml) and 0.5 mM-[U-^{14}C]sucrose (0.2 μCi/ml) as an extracellular marker for 1 min at 0 °C, and then 1 ml samples were centrifuged for 15 s as described previously (Quinlan *et al.*, 1983). The values of the intracellular volume measured at 0 °C in the present experiments (expressed as means ± S.E.M. for n observations) were 1.24 ± 0.02 ($n = 4$) and 1.04 ± 0.03 ($n = 32$) μl/mg of protein in Krebs–Henseleit (1932) and citrate media respectively (see also Table 1). When parallel experiments were performed in Krebs–Henseleit medium at 37 °C, values for the intracellular volume were 1.70 ± 0.04 μl/mg of protein (mean ± S.E.M., $n = 5$), similar to values reported previously from our laboratory (Quinlan *et al.*, 1983). It was found that, if the silicone-oil-filtration technique was used to measure the intracellular volume, the values were about 0.6 μl/mg of protein less than those measured in the absence of oil (Table 1). The same difference was found when measurements were performed at 37 °C, the measured volume falling to 1.15 μl/mg of protein. Thus

Table 1. Comparison of measurements of L-lactate uptake, intracellular volumes and the pH gradient in citrate and Krebs–Henseleit/Hepes medium by using the centrifuge-stop and the silicone-oil-filtration technique

Hepatocytes were suspended in either Krebs–Henseleit medium, pH 7.4, containing 25 mM-Hepes in place of bicarbonate, or in sodium citrate medium, pH 7.4 at 0 °C. Uptake of 1 mM-L-lactate at 15 s, intracellular volumes and the accumulation of the pH marker DMO were measured radioactively after the addition of 1 mM-L-[U-^{14}C]lactate (0.1 μCi/ml), 1 mM-[U-^{14}C]sucrose (0.1 μCi/ml), 0.5 mM-[2-^{14}C]DMO (0.1 μCi/ml), $^{3}H_2O$ (1 μCi/ml) or [^{3}H]inulin (50 μg/ml; 1 μCi/ml) as required, by using either the centrifuge-stop technique or silicone-oil filtration as indicated. Each value represents the mean ± S.E.M. of the number of experiments in parentheses carried out on separate hepatocyte preparations. Discussion of the apparent differences in intracellular volume determined by using the centrifuge-stop and silicone-oil-filtration techniques is presented in the text.

Medium	Technique	L-Lactate uptake in 15 s (nmol/mg of protein)	Intracellular volume (μl/mg of protein)	Calculated ΔpH, alkaline inside
Krebs–Henseleit + Hepes	Centrifuge stop	0.23 ± 0.01 (4)	1.24 ± 0.02 (6)	0.16 ± 0.02 (4)
Citrate	Centrifuge stop	0.64 ± 0.02 (4)	1.04 ± 0.03 (6)	0.52 ± 0.03 (4)
Citrate	Silicone-oil filtration	0.53 ± 0.03 (7)	0.48 ± 0.05 (12)	0.79 ± 0.03 (7)

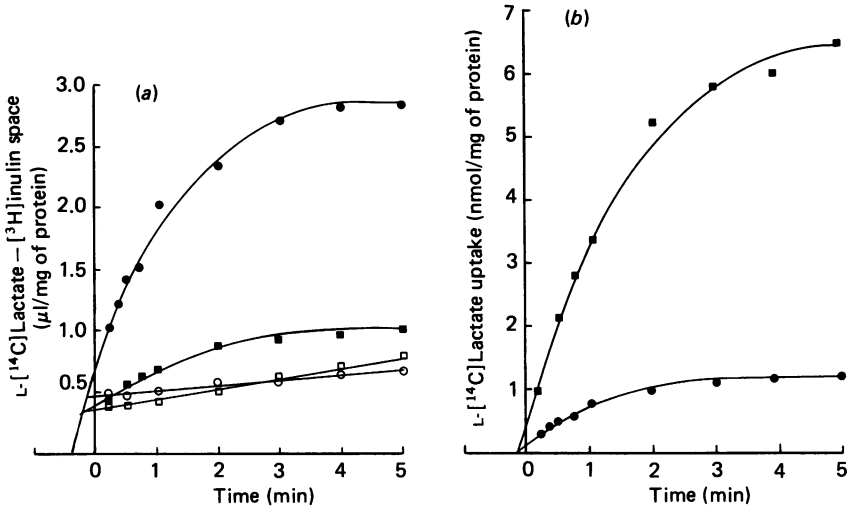

Fig. 1. Time courses of the uptake of L-lactate by isolated liver cells in the presence and the absence of α-cyano-4-hydroxycinnamate

The time courses of the uptake of 0.5 mM- (●, ○) and 10 mM- (■, □)L-[^{14}C]lactate by rat hepatocytes were determined at 0 °C in the absence (●, ■) and presence (○, □) of 5 mM-α-cyano-4-hydroxycinnamate by using the silicone-oil-filtration technique as described in the Experimental section. Typical results are presented from one of four similar experiments, each value representing the mean of two observations on the same hepatocyte preparation. In (*b*) data have been corrected for the apparent uptake of lactate at zero time in the presence of α-cyano-4-hydroxycinnamate as described in the text, where details are also given of regression analysis of the curves and of the pH gradient across the membrane under each condition. Note that in (*a*) lactate uptake is expressed in terms of the [^{14}C]lactate − [^{3}H]inulin 'space', i.e. the intracellular [^{14}C]lactate expressed as the volume of medium containing the same amount of [^{14}C]lactate (see Halestrap & McGivan, 1979). In (*b*) this is corrected to give the lactate uptake in nmol/mg of cell protein.

it appears that some intracellular water is lost during passage through the oil layer. Indeed, when attempts were made to measure the intramitochondrial volume in intact cells by using [^{14}C]mannitol and $^{3}H_2O$ (Quinlan *et al.*, 1983), the silicone-oil technique gave negative values, also about 0.6 μl/mg of protein less than those obtained by normal centrifugation (results not shown). However, if the pH gradient was calculated by using the value for the intracellular volume measured by the silicone-oil-filtration technique, the values were higher than when centrifugation alone was used. This is illustrated in Table 1.

For measurement of the pH gradient, cells were incubated with [^{3}H]inulin (2 μCi/ml; 50 μg/ml) and the pH marker DMO (0.5 mM, 0.2 μCi/ml) before sedimentation by centrifugation either with or without silicone oil as described above. The pH gradient was calculated as described by Addanki *et al.* (1968). Loss of DMO or lactate during passage through the oil seems unlikely. Table 1 gives values of the pH gradient and lactate uptake measured both with and without silicone-oil filtration. The uptake of lactate in 15 s was similar measured by both techniques, as was the equilibrium uptake of DMO. Cohen *et al.* (1987) have reported that

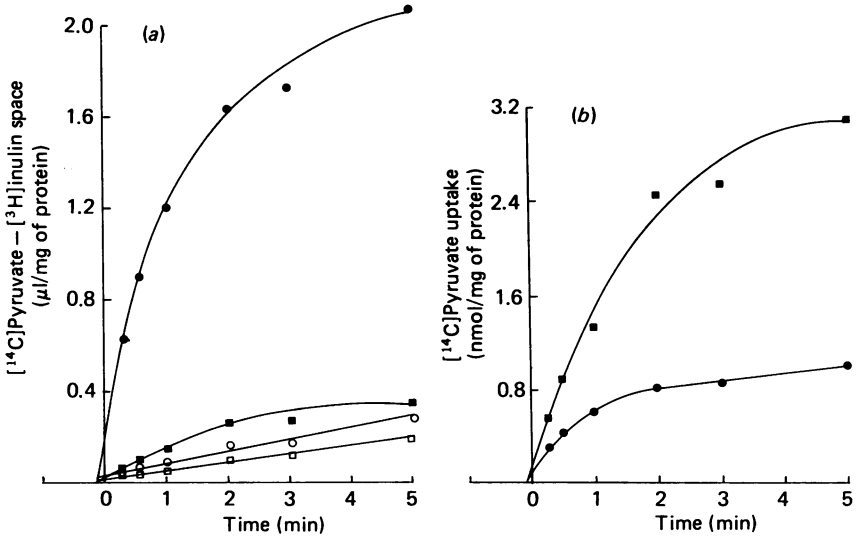

Fig. 2. Time courses of the uptake of pyruvate by isolated liver cells in the presence and the absence of α-cyano-4-hydroxycinnamate

The experimental protocol was the same as for Fig. 1, but [^{14}C]pyruvate instead of lactate was used. Symbols are used in the same way as in Fig. 1.

silicone-oil filtration yields unreliable results when used to measure the matrix volume of isolated mitochondria.

RESULTS AND DISCUSSION

Time courses of pyruvate and lactate entry into hepatocytes at 0 °C

In order to minimize the effects of lactate metabolism, all experiments were performed at 0 °C. Hepatocytes isolated from 24 h-starved rats were employed to prevent generation of intracellular lactate from glycogen stores, which might allow lactate/lactate exchange to occur rather than net uptake of lactic acid. In addition, it was demonstrated that preincubation of cells with 4 mM-quinolinate had no effect on the uptake of lactate. Quinolinate is a well-known inhibitor of phosphoenolpyruvate carboxykinase (McDaniel *et al.*, 1972; Elliot *et al.*, 1977) and inhibits gluconeogenesis by over 50% at this concentration. The time courses of [^{14}C]-lactate and -pyruvate uptake into hepatocytes incubated in a citrate-based medium and measured by the silicone-oil-filtration technique are shown in Figs. 1 and 2. The citrate medium was used to generate an alkaline intracellular pH (Table 1), which caused an accumulation of pyruvate or lactate at equilibrium (see Halestrap, 1976). This allowed much greater accuracy in the measurement of uptake, although time courses could be observed in saline-based media at 0 °C, and data for uptake of L-lactate after 15 s are given for both media in Table 1 to allow comparison. Data are given in Figs. 1 and 2 for two concentrations of substrate (0.5 and 10 mM) in the presence and absence of 5 mM-α-cyano-4-hydroxycinnamate; this compound substantially inhibited uptake of both pyruvate and lactate. No additional inhibition was observed at higher inhibitor concentrations.

The time courses of uptake were fitted by least-squares regression analysis to the first-order rate equation:

$$L_t = L_{tot}(1-e^{-k(t+x)})$$

where L_t is the lactate uptake at time t, L_{tot} is the total uptake of lactate at equilibrium, k is the rate constant and x is the 'dead-time' of centrifugation (the time taken to stop the uptake), normally found to be about 5 s for liver cells (McGivan *et al.*, 1977). Inspection of Fig. 1(*a*) shows that there is a problem in interpreting time courses of lactate uptake. Two parameters are important for the measurement of initial rates: the dead-time and the true zero value for the (L-[^{14}C]lactate – [^{3}H]inulin) space. If the latter value was assumed to be zero, then by regression analysis of four experiments the mean (±S.E.M.) dead time was 17.25±1.23 s. However, after preincubation with 5 mM-α-cyano-4-hydroxycinnamate for 30 min at 0 °C, a measured value for the initial space could be obtained with some accuracy and was found to be 0.32±0.05 ($n = 4$) and 0.30±0.04 ($n = 4$) μl/mg of protein when 0.5 mM- and 10 mM-L-lactate respectively were used. When this value of the initial space was used, a dead-time of centrifugation of about 5 s can be calculated, which is consistent with measurements by McGivan *et al.* (1977) using the silicone-oil-filtration technique. This initial space measured in the presence of 5 mM-α-cyano-4-hydroxycinnamate was apparently independent of lactate concentration, and was much less with pyruvate as substrate (Fig. 2*a*). Its identity is unknown, but may represent binding of some minor impurity in the [^{14}C]lactate to the hepatocytes. In this context it is of interest that Welch *et al.* (1984) have observed covalent labelling of liver plasma membranes with [^{14}C]lactate from the same commercial source as used here, which those authors ascribe to binding of lactate itself. No matter what it represents, the subtraction of this zero-time value from the space measured in the absence of the inhibitor should produce a corrected value for carrier-mediated uptake of lactate. Time courses corrected in this way are shown for both lactate and pyruvate in Figs. 1(*b*) and 2(*b*) respectively, where uptake of substrate is expressed in nmol/mg of protein rather than in terms of the ^{14}C – ^{3}H space.

From Fig. 1(*a*), lactate uptake at equilibrium (L_{tot},

expressed in terms of the [^{14}C]lactate−[^{3}H]inulin space) was estimated by extrapolation to be 2.86 and 1.09 μl/mg of protein for 0.5 mM- and 10 mM-L-lactate respectively in the absence of the inhibitor. The corresponding values for ΔpH measured with [^{14}C]DMO were 0.552 and 0.355, suggesting that as lactate enters the cell so does a proton, leading to a dissipation of ΔpH. If lactate does enter with a proton, it would be predicted that its distribution across the membrane at equilibrium would be determined by the pH gradient. By using the measured value for the intracellular volume of 1.11 μl/mg of protein under these conditions, the expected uptake of lactate at equilibrium would be 1.96 and 25.1 nmol/mg of protein at 0.5 mM- and 10 mM-lactate respectively. The values calculated by least-squares regression analysis of the data of Fig. 1(*b*) (L_{tot}) were 1.29 and 7.16 nmol/mg of protein respectively. Although intracellular accumulation of DMO into other organelles such as mitochondria might lead to an overestimate of the cytoplasmic pH, the discrepancy between calculated and observed uptakes at equilibrium in the presence of 10 mM-lactate is too great to be explained in this manner. An anionic pathway for lactate transport may be present, as is thought to be the case in striated muscle (Mason, 1986). At high [lactate], this would drive lactate out of the cell under the influence of the membrane potential.

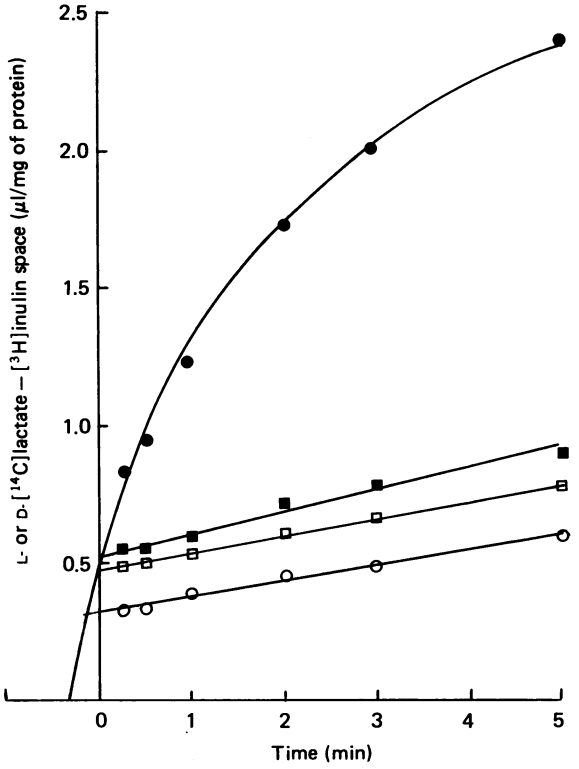

Fig. 3. Comparison of the time courses of the uptake of L- and D-lactate in the presence and the absence of α-cyano-4-hydroxycinnamate

The time courses of 0.5 mM-L-lactate (●, ○) and D-lactate (■, □) uptake by rat hepatocytes in the absence (●, ■) and the presence (○, □) of 5 mM-α-cyano-4-hydroxycinnamate were measured as described in the legend to Fig. 1. The results shown are typical of three separate experiments, each value representing the mean of two observations on the same hepatocyte preparation.

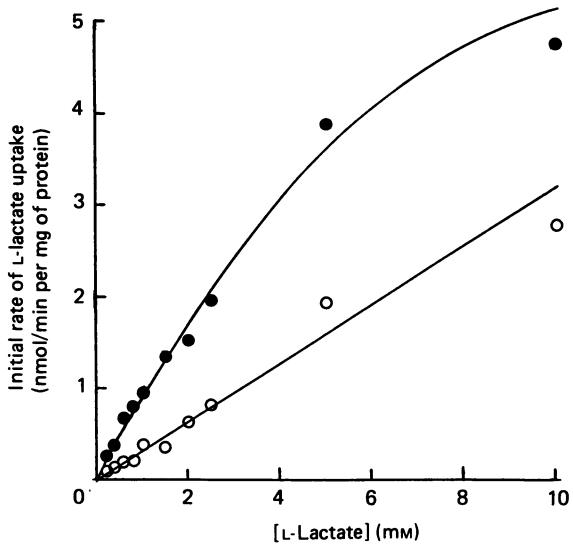

Fig. 4. Concentration-dependence of L-lactate transport into isolated rat liver cells in the presence (○) and the absence (●) of 5 mM-α-cyano-4-hydroxycinnamate

The initial rates of L-lactate transport at increasing concentrations of L-lactate were calculated from the uptake at 15 s, corrected for the α-cyano-4-hydroxycinnamate-insensitive uptake at zero time and assuming a dead-time of 5 s, as explained in the text. Typical results are shown from one of five similar experiments, each value representing the mean of two observations on the same hepatocyte preparation.

The time courses for the uptake of pyruvate presented in Figs. 2(*a*) and 2(*b*) are similar to those for lactate, and again measurements of ΔpH showed that it was decreased from 0.527 at 0.5 mM-pyruvate to 0.422 at 10 mM-pyruvate. The rate of uptake in the presence of 5 mM-α-cyano-4-hydroxycinnamate was less than that observed with lactate, especially at the higher substrate concentrations. These results are consistent with uptake occurring in the presence of α-cyano-4-hydroxycinnamate by diffusion of the free acid, as suggested by Monson *et al.* (1982) and Fafournoux *et al.* (1985). Diffusion of pyruvate would be expected to be slower than that of lactate, because the pK values are 2.49 and 3.86 respectively, leading to a 25-fold greater concentration of lactic acid than of pyruvic acid at the same pH and substrate concentration. Similar observations have been made for mitochondrial pyruvate and lactate transport (Halestrap, 1975, 1978). From Fig. 2(*b*), uptake of pyruvate at equilibrium (P_{tot}) was 0.99 and 3.43 nmol/mg of protein for 0.5 mM- and 10 mM-pyruvate respectively.

Comparison of the uptake of D- and L-lactate

Using erythrocytes from a number of mammalian species, Deuticke *et al.* (1978) demonstrated that those species which catalyse a high rate of exchange at low (5 mM) lactate had a high degree of stereospecificity for the L-lactate over D-lactate. In Fig. 3, the time courses of uptake of 0.5 mM-L- and -D-lactate are compared; clearly L-lactate was transported in preference to D-lactate. In the presence of 5 mM-α-cyano-4-hydroxycinnamate, uptake of L-lactate was almost completely inhibited, but there was very little effect on D-lactate uptake. This

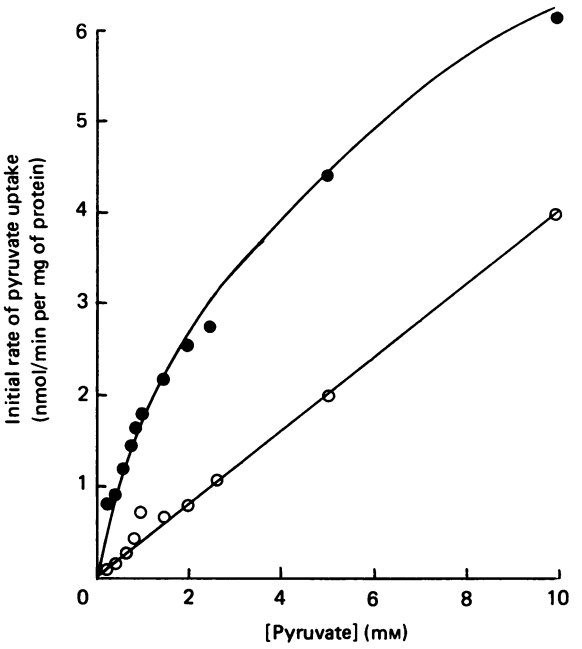

Fig. 5. Concentration-dependence of pyruvate transport into isolated rat liver cells in the presence (○) and the absence (●) of 5 mM-α-cyano-4-hydroxycinnamate

Details are the same as given for Fig. 4, except that pyruvate instead of lactate was used.

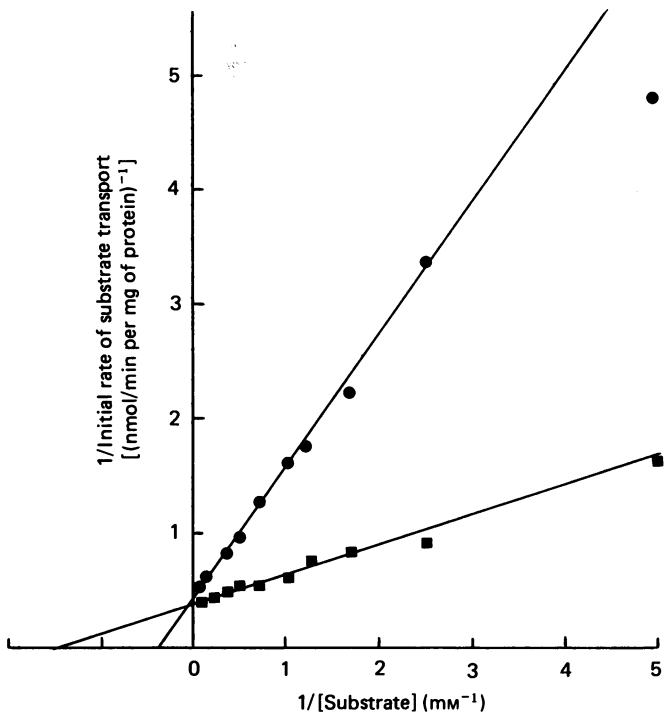

Fig. 6. Kinetics of carrier-dependent L-lactate and pyruvate transport into isolated rat hepatocytes

The initial rates of carrier-mediated transport of L-lactate (●) and pyruvate (■) were calculated from data such as those in Figs. 4 and 5 by subtraction of the uptake of substrate at 15 s in the presence of 5 mM-α-cyano-4-hydroxycinnamate from that in the absence of inhibitor. Data are shown as a Lineweaver–Burk plot, but values for the K_m and $V_{max.}$ were determined by least-squares regression analysis to the Michaelis–Menten equation. Typical results from one of five experiments for lactate and four experiments for pyruvate are shown, each value representing the mean of two observations on the same hepatocyte preparation. Mean values of the calculated $V_{max.}$ and K_m for the two substrates are given in the text.

suggests that in isolated liver cells transport of D-lactate is primarily by free diffusion, whereas carrier-mediated uptake is stereospecific for the L-isomer. This is in agreement with the conclusions of Monson *et al.* (1982), but conflicts with the conclusions of Schwab *et al.* (1979) and Fafournoux *et al.* (1985). Previous studies from our laboratory (Leeks & Halestrap, 1979) also concluded that D-lactate transport into liver cells was carrier-mediated, but we now believe that, at the higher temperatures used in these studies and those of Fafournoux *et al.* (1985), it is not possible to measure true initial rates of transport.

Kinetics of L-lactate and pyruvate transport

In order to obtain values for the kinetic parameters of lactate and pyruvate transport, initial rates of transport were measured at substrate concentrations up to 10 mM. Time courses of the uptake of pyruvate and lactate at both 0.5 mM and 10 mM (Figs. 1 and 2) appear to be linear for the first 15 s. Thus initial rates were calculated from uptake at 15 s, which when corrected for the dead-time gave a total uptake time of 20 s. In all cases uptake was corrected for ^{14}C binding at zero time in the presence of 5 mM-α-cyano-4-hydroxycinnamate as described above. For lactate (Fig. 4) in the absence of 5 mM-α-cyano-4-hydroxycinnamate, rates of uptake were not saturable, and at concentrations of 10 mM and above uptake was linear with increasing concentration of substrate. In some experiments (results not shown) rates of transport were measured at 30 mM-lactate, and it was shown that, at this concentration, uptake measured in the absence of α-cyano-4-hydroxycinnamate was indistinguishable from that obtained in the presence of the inhibitor. This suggests that lactate crosses the plasma membrane primarily via a saturable transport system at low concentrations, whereas, at concentrations of 10 mM and above, diffusion of the undissociated acid is the major route. These observations are in agreement with the conclusions of Monson *et al.* (1982) and Fafournoux *et al.* (1985). Fig. 5 illustrates the effect of increasing pyruvate concentration on the rate of pyruvate uptake under similar conditions. As expected from Fig. 2, the diffusion component of uptake was less prominent than for L-lactate, but was again consistent with a carrier-mediated process and a non-saturable diffusion process for transport.

In order to obtain kinetic parameters for carrier-mediated transport, the diffusion component, determined by measuring the uptake in the presence of 5 mM-α-cyano-4-hydroxycinnamate, was subtracted from the total uptake. The resultant curves obeyed Michaelis–Menten kinetics, and data representative of a number of experiments are shown as Lineweaver–Burk plots in Fig. 6. Data were fitted by a non-linear regression to the Michaelis–Menten equation. The apparent K_m values (expressed as means ± S.E.M. for n experiments) for L-lactate and pyruvate were 2.42 ± 0.35 mM ($n = 5$) and 0.63 ± 0.08 mM ($n = 4$) respectively, with corresponding

Table 2. Kinetic parameters for L-lactate and pyruvate transport into rat hepatocytes and erythrocytes under various conditions

Data are the means ± S.E.M. for the numbers of experiments shown in parentheses. Values for L-lactate transport into rat hepatocytes at 37 °C are taken from Fafournoux *et al.* (1985). Values for the $V_{max.}$ are expressed as nmol of substrate uptake/μl of intracellular space, to allow some comparison between different cell types.

	Present studies		Fafournoux *et al.* (1985)
Cell type...	Erythrocytes	Hepatocytes	Hepatocytes
Buffer...	Citrate	Citrate	Krebs–Ringer
Technique...	Proton flux	Oil filtration	Oil filtration
Temperature (°C)...	7	0	37
L-Lactate			
K_m (mM)	2.99 ± 0.42 (4)	2.42 ± 0.35 (5)	1.9
$V_{max.}$ (nmol/min per μl)	5.02 ± 0.98 (4)	3.32 ± 0.43 (5)	8.0
Pyruvate			
K_m (mM)	0.96 ± 0.17 (3)	0.63 ± 0.08 (4)	–
$V_{max.}$ (nmol/min per μl)	4.26 ± 0.67 (3)	3.17 ± 0.57 (4)	–

Table 3. Kinetic parameters for the inhibition of lactate transport into hepatocytes by α-cyano-4-hydroxycinnamate

Data are presented for three separate experiments, each with L-lactate at 0.2, 0.5, 1.0, 2.0 and 2.5 or 5 mM and α-cyano-4-hydroxycinnamate at 0, 50, 100, 500 and 1000 μM. Initial rates of transport were measured in duplicate for each condition as described in Fig. 4 (50 data points in all) and fitted to the equations for competitive and non-competitive inhibition by least-squares regression analysis as described in the text. Where present, α-cyano-4-hydroxycinnamate was added 30 min before addition of L-lactate and was shown to have no effect on the pH gradient.

	Parameter values and 95% confidence limits for:							
	Competitive inhibition				Non-competitive inhibition			
Expt. no.	K_m (mM)	$V_{max.}$ (nmol/min per mg)	K_i (μM)	Standard error	K_m (mM)	$V_{max.}$ (nmol/min per mg)	K_i (μM)	Standard error
1	2.60 ± 0.72 (1.14–4.05)	4.98 ± 0.81 (3.35–6.61)	167 ± 29 (109–226)	0.185	3.50 ± 0.97 (1.55–5.44)	5.97 ± 1.07 (3.80–8.14)	294 ± 38 (218–376)	0.189
2	1.54 ± 0.29 (0.96–2.12)	3.57 ± 0.32 (2.92–4.21)	64 ± 9 (46–81)	0.122	2.24 ± 0.43 (1.37–3.10)	4.28 ± 0.47 (3.34–5.22)	143 ± 15 113–173	0.134
3	2.88 ± 0.26 (2.35–3.41)	3.33 ± 0.16 (3.01–3.65)	96 ± 8 (81–112)	0.061	4.10 ± 0.27 (3.49–4.57	4.03 ± 0.27 (3.49–4.57)	204 ± 18 (167–241)	0.081
Mean ± S.E.M.	2.34 ± 0.41	3.96 ± 0.51	109 ± 30	–	3.28 ± 0.55	4.76 ± 0.61	214 ± 44	–

values for $V_{max.}$ of 3.13 ± 0.41 and 2.99 ± 0.54 nmol/min per mg of cell protein at 0 °C. In Table 2, the values obtained for transport in hepatocytes are compared with those obtained for rat erythrocytes at 7 °C from time courses obtained by using a proton-flux technique as described by Leeks & Halestrap (1977). Values determined with isolated liver cells at 37 °C by Fafournoux *et al.* (1985) are also included for comparison. The $V_{max.}$ values for transport of pyruvate and lactate into the hepatocyte are similar, as would be expected if they share the same carrier. The same is true for the erythrocyte, and it is noteworthy that the K_m values of the erythrocyte and the hepatocyte carriers are very similar, suggesting that they may be the same protein. Both K_m values are lower than we and others have previously found in human erythrocytes (Halestrap, 1976; Rice & Steck, 1976; Leeks & Halestrap, 1977; Dubinsky & Racker, 1978).

Nature of the inhibition of α-cyano-4-hydroxycinnamate

The effects of α-cyano-4-hydroxycinnamate at 0.05, 0.1, 0.5 and 1 mM on kinetics of lactate transport were studied and used to calculate a K_i value for the inhibitor. Data were fitted by least-squares regression analysis to the equations for both non-competitive and competitive inhibition, and the results of three separate experiments are summarized in Table 3. The data could be fitted satisfactorily to both equations, although the fit was consistently better for the competitive model. In addition, the values of K_m and $V_{max.}$ derived by using the competitive model are more consistent with those obtained in the previous kinetic experiments. The data could not be fitted to the equation for mixed inhibition. With the competitive model, the calculated K_i for α-cyano-4-hydroxycinnamate (mean ± S.E.M. of three separate experiments) was 109 ± 30 μM. Competitive

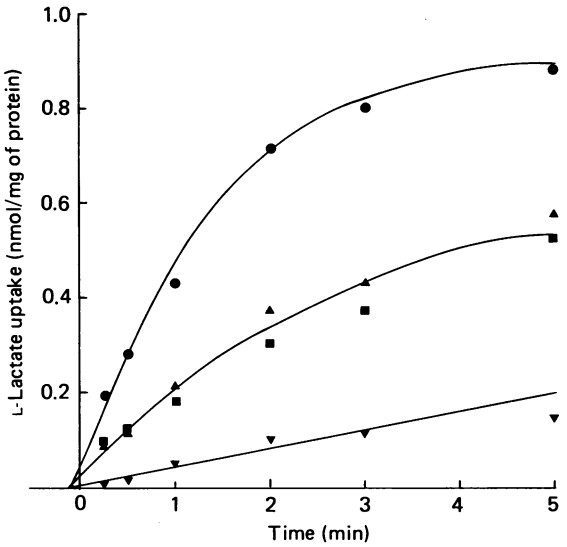

Fig. 7. Comparison of the effects of SITS, DIDS and α-cyano-4-hydroxycinnamate on the uptake of L-lactate into hepatocytes

Hepatocytes were preincubated for 30 min at 0 °C in citrate medium in the absence (●) or the presence of 0.5 mM-SITS (■), 0.5 mM-DIDS (▲) and 5 mM-α-cyano-4-hydroxycinnamate (▼) before initiating transport with 0.5 mM-L-[^{14}C]lactate as described in the Experimental section. Results are shown from one of two experiments, each value representing the mean of two observations on the same hepatocyte preparation. Uptake has been corrected for the α-cyano-4-hydroxycinnamate-insensitive component at zero time as described for Figs. 1(*b*) and 2(*b*). Parallel measurement of the transmembrane pH gradient (inside−outside) with [^{14}C]DMO gave values for control, SITS-, DIDS- and α-cyano-4-hydroxycinnamate-incubated cells of 0.61, 0.65, 0.54 and 0.54 respectively.

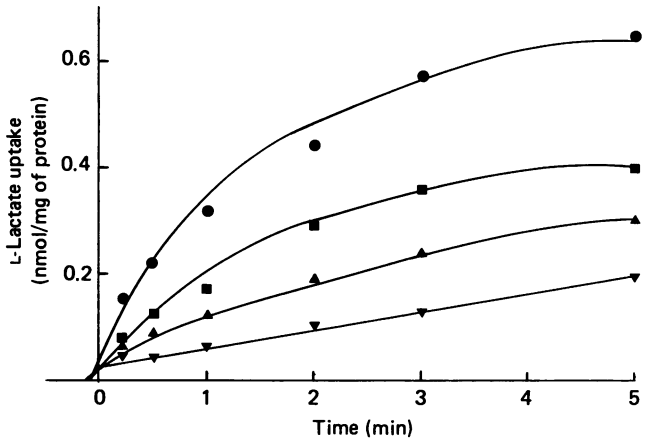

Fig. 8. Effect of *p*CMBS, quercetin and iBCLA on the uptake of L-lactate into hepatocytes

Hepatocytes were preincubated for 30 min at 0 °C in citrate medium in the absence (●) or the presence of 0.2 mM-*p*CMBS (▼), 0.5 mM-quercetin (▲) or 0.6 mM-iBCLA (■) before initiating transport with 0.5 mM-L-[^{14}C]lactate as described in the Experimental section. Results are shown from one of two experiments, each value representing the mean of two observations on the same hepatocyte preparation. Uptake has been corrected for the α-cyano-4-hydroxycinnamate-insensitive component at zero time as described for Figs. 1(*b*) and 2(*b*). Parallel measurement of the transmembrane pH gradient (inside−outside) with [^{14}C]DMO gave values for control, *p*CMBS-, quercetin- and iBCLA-incubated cells of 0.42, 0.06, 0.48 and 0.33 respectively.

inhibition is also observed for lactate and pyruvate transport into human erythrocytes (Halestrap, 1976; Leeks & Halestrap, 1977), where the inhibitor works on the inner face of the membrane, and the value of the K_i for external α-cyano-4-hydroxycinnamate is dependent on the pH gradient across the membrane. For human erythrocytes incubated in citrate buffer, where ΔpH was about 0.8, K_i values of 40–90 μM were obtained, whereas in saline buffer, where ΔpH was −0.2, the K_i value was about 450 μM (Halestrap, 1976; Leeks & Halestrap, 1977). Thus the K_i value of 109 μM obtained in the present studies with hepatocytes where ΔpH was about 0.5 support the common identity of the liver and erythrocyte lactate transporter.

Effect of other inhibitors of erythrocyte lactate transport on the uptake of L-lactate into hepatocytes

4,4′-Di-isothiocyanostilbene-2,2′-disulphonate (DIDS) and 4-acetamido-4′-isothiocyanostilbene-2,2′-disulphonate (SITS) have been shown to be potent inhibitors of Cl^- exchange across the erythrocyte membrane and to bind to a specific transmembrane protein (Cabantchik & Rothstein, 1972, 1974*a*,*b*). At higher concentrations they have also been used to inhibit specific lactate transport in rabbit erythrocytes (Deuticke *et al.*, 1982; Jennings & Adams-Lackey, 1982; Donovan & Jennings, 1985). In Fig. 7 the effects of 0.5 mM-DIDS and 0.5 mM-SITS on the time course of 0.5 mM-L-lactate uptake by hepatocytes are compared with those of 5 mM-α-cyano-4-hydroxycinnamate. Both SITS and DIDS inhibited carrier-mediated uptake by about 50%. The effect of the inhibitors on pH were measured and shown to be extremely small (see Fig. 7 legend). In human erythrocytes some lactate/bicarbonate exchange can occur on the general anion exchanger (band 3), and through the operation of carbonic anhydrase this allows net uptake of lactic acid, which is inhibited by low concentrations of DIDS (Halestrap, 1976). Addition of acetazolamide, an inhibitor of carbonic anhydrase, also inhibits this process in erythrocytes, but had no effect on lactate uptake into hepatocytes (results not shown). A general anion transporter which is also capable of transporting lactate, as described by Halestrap (1976) in human erythrocytes, may exist in the liver plasma membrane, but as yet there is little evidence for this. With perfused liver, the exchange of sulphate anions was completely and irreversibly inhibited by DIDS, but sulphate did not appear to exchange with bicarbonate ions or monocarboxylates to a significant extent (Bracht *et al.*, 1981). Cheng & Levy (1980) have also provided evidence for a DIDS-sensitive sulphate exchange in isolated hepatocytes which is distinct from that found in erythrocytes.

*p*CMBS, quercetin and isobutylcarbonyl-lactyl anhydride (iBCLA) have been shown to inhibit lactate uptake into erythrocytes and Ehrlich ascites-tumour cells (Spencer & Lehninger, 1976; Leeks & Halestrap, 1977; Belt *et al.*, 1979; Deuticke *et al.*, 1978; Johnson *et al.*, 1980; Donovan & Jennings, 1985). Fig. 8 shows the effect

of these inhibitors on the time course of the uptake of 0.5 mM-L-lactate into hepatocytes; the potency of inhibition is in the order *p*CMBS > quercetin > iBCLA. The extent of inhibition by quercetin and iBCLA of lactate uptake is similar to that produced by SITS or DIDS, and that by *p*CMBS is similar to the effects of α-cyano-4-hydroxycinnamate. However, measurement of the pH gradient in the presence of the inhibitors showed that it was decreased by the presence of both iBCLA and *p*CMBS, from a control value of 0.42 to 0.33 and 0.06 respectively. The rate of carrier-dependent lactate efflux from and influx into erythrocytes is very sensitive to the transmembrane pH gradient between pH values of 7 and 9 (Leeks & Halestrap, 1977; Deuticke *et al.*, 1982), and the data of Table 1 suggest that the transport of lactate into the hepatocyte might also be sensitive to the pH gradient. Thus some of the inhibition observed in the presence of these reagents might be indirect. The concentrations of both quercetin and iBCLA (approx. 10 μg/mg of protein for both inhibitors) used in these experiments were much higher than those used by Belt *et al.* (1979) and Johnson *et al.* (1980) in Ehrlich ascites-tumour cells. In these cells lactate efflux was inhibited by 50% by quercetin at a concentration of 0.1 μg/mg of protein (Belt *et al.*, 1979), and inhibition of lactate uptake was 50% at 0.5 μg of iBCLA/mg of protein (Johnson *et al.*, 1980). Thus neither quercetin nor iBCLA is as potent an inhibitor of lactate uptake into isolated hepatocytes as they are of transport of lactate across the plasma membrane of Ehrlich ascites-tumour cells.

General conclusion

The results presented here suggest that lactate and pyruvate enter the liver by a carrier-mediated process at low physiological concentrations and that, at concentrations of 5 mM and above, non-ionic diffusion becomes an increasingly important means of lactate entry. This is in agreement with Monson *et al.* (1982) and Fafournoux *et al.* (1985). The kinetic characteristics of transport and the effects of various inhibitors strongly support a common identity for the liver and erythrocyte transporters. A common identity for the lactate transporter of erythrocytes and that of striated muscle has also been suggested as a result of kinetic measurements using intracellular pH electrodes (Mason, 1986) and by the identification of a patient with an apparent deficiency in both transport mechanisms (Fishbein, 1986). The K_m value for L-lactate transport (2.42 mM) is similar to that obtained by Fafournoux *et al.* (1985) at 37 °C (1.8 mM). These workers used hepatocytes isolated from fed rats incubated in the presence of inhibitors of transaminases and gluconeogenesis. However, the $V_{max.}$ obtained by these workers was only 8 nmol/min per μl of intracellular space, which, as argued in the Introduction, would be insufficient to account for observed rates of gluconeogenesis from L-lactate and would not allow equilibration of lactate and pyruvate across the liver cell membrane, as has been observed (Tischler *et al.*, 1977; Groen *et al.*, 1983, 1986). In agreement with Monson *et al.* (1982), we believe that it is not possible to make accurate measurements of initial transport rates at 37 °C, and therefore the kinetic analysis by Fafournoux *et al.* (1985) must be regarded as questionable. By assuming that the activation energy of the carrier in liver cells is similar to that obtained from measurements in the human erythrocyte (see Halestrap, 1976), it can be calculated from the present data that the $V_{max.}$ at 37 °C is about 550 nmol/min per mg of protein. Even if this value is an overestimate, as a result of the pH gradient used in the present studies, the maximal rate of lactate transport under physiological conditions is likely to be considerably greater than the maximal rate required for gluconeogenesis [about 10 nmol of glucose (20 nmol of lactate)/min per mg of protein]. There is thus no evidence to support a regulatory role for this transporter in the metabolism of lactate by the liver.

G.L.E. was in receipt of a research studentship from the Medical Research Council. We thank Dr. J. D. McGivan for helpful discussions.

REFERENCES

Addanki, S., Cahill, F. D. & Sotos, J. F. (1986) J. Biol. Chem. **243**, 2337–2348
Belt, J. A., Thomas, J. A., Buchsbaum, R. N. & Racker, E. (1979) Biochemistry **19**, 3506–3511
Berry, M. N. & Friend, D. S. (1969) J. Cell Biol. **43**, 506–520
Bracht, A., Bracht, A. K., Schwab, A. J. & Scholz, R. (1981) Eur. J. Biochem. **114**, 471–479
Cabantchik, Z. I. & Rothstein, A. (1972) J. Membr. Biol. **10**, 311–330
Cabantchik, Z. I. & Rothstein, A. (1974*a*) J. Membr. Biol. **15**, 207–226
Cabantchik, Z. I. & Rothstein, A. (1974*b*) J. Membr. Biol. **15**, 227–248
Cheng, S. & Levy, D. (1980) J. Biol. Chem. **255**, 2637–2640
Cohen, N. S., Cheung, C.-W. & Raijman, L. (1987) Biochem. J. **245**, 375–379
Cremer, J. E., Cunningham, V. J., Pardridge, W. M., Braun, L. D. & Oldendorf, W. H. (1979) J. Neurochem. **33**, 439–445
Cremer, J. E. Teal, H. M. & Cunningham, V. J. (1982) J. Neurochem. **39**, 674–677
De Hemptinne, A., Marrannes, R. & Vanheel, B. (1983) Am. J. Physiol. **245**, C178–C183
Deuticke, B. (1982) J. Membr. Biol. **70**, 89–103
Deuticke, B., Rickert, I. & Beyer, E. (1978) Biochim. Biophys. Acta **507**, 137–155
Deuticke, B., Beyer, E. & Frost, B. (1982) Biochim. Biophys. Acta **684**, 96–110
Donovan, J. A. & Jennings, M. L. (1985) Biochemistry **24**, 561–564
Dubinsky, W. P. & Racker, E. (1978) J. Membr. Biol. **44**, 25–36
Elliot, K. R. F., Pogson, C. I. & Smith, S. A. (1977) Biochem. J. **164**, 283–286
Fafournoux, P., Demigne, C. & Remesy, C. (1985) J. Biol. Chem. **260**, 292–299
Fishbein, W. N. (1986) Science **234**, 1254–1256
Gornall, H. G., Bardawill, C. J. & David, M. M. (1949) J. Biol. Chem. **177**, 751–756
Groen, A. K., Vervoorn, R. C., van der Meer, R. & Tager, J. M. (1983) J. Biol. Chem. **258**, 14346–14363
Groen, A. K., van Roermund, C. W. T., Vervoorn, R. C. & Tager, J. M. (1986) Biochem. J. **237**, 379–389
Halestrap, A. P. (1975) Biochem. J. **148**, 85–96
Halestrap, A. P. (1976) Biochem. J. **156**, 193–207
Halestrap, A. P. (1978) Biochem. J. **172**, 377–387
Halestrap, A. P. & Denton, R. M. (1974) Biochem. J. **138**, 313–316
Halestrap, A. P. & McGivan, J. D. (1979) in Techniques in Metabolic Research (Kornberg, H. L., Metcalfe, J. C., Northcote, D. H., Pogson, C. I. & Tipton, K. F., eds.), vol. B206, pp. 1–23, Elsevier/North-Holland, Amsterdam

Illsley, N. P., Wootton, R., Penfold, P., Hall, S. & Duffy, S. (1986) Placenta **7**, 200–220
Jennings, M. L. & Adams-Lackey, M. (1982) J. Biol. Chem. **257**, 12866–12871
Johnson, J. H., Belt, J. A., Dubinsky, W. P., Zimmiak, A. & Racker, E. (1980) Biochemistry **19**, 3836–3840
Jorgensen, K. E. & Sheikh, M. I. (1984) Biochem. J. **223**, 803–807
Kastendieck, E. & Moll, W. (1977) Pflugers Arch. **370**, 165–171
Koch, A., Webster, B. & Lowell, S. (1981) Biophys. J. **36**, 775–796
Krebs, H. A. & Henseleit, K. (1932) Hoppe-Seyler's Z. Physiol. Chem. **210**, 33–36
Kutchai, H., Geddis, L. M. & Martin, M. S. (1978) Biochim. Biophys. Acta **541**, 312–320
Lamers, J. M. J. (1975) Biochim. Biophys. Acta **413**, 265–276
Leeks, D. R. & Halestrap, A. P. (1977) Biochem. Soc. Trans. **6**, 1363–1366
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
Leichtweiss, H. P. & Schroder, H. (1981) Pflugers Arch. **390**, 80–85
Mann, G. E. & Yudilevich, D. L. (1981) J. Physiol. (London) **328**, 19*P*–20*P*
Mann, G. E., Zlokovic, B. V. & Yudilevich, D. L. (1985) Biochim. Biophys. Acta **819**, 241–248
Mason, M. J. (1986) Ph.D. Thesis, University of Bristol
Mason, M. J. & Thomas, R. C. (1985) J. Physiol. (London) **361**, 23*P*
McDaniel, H. G., Reddy, W. J. & Boshell, B. R. (1972) Biochim. Biophys. Acta **276**, 543–550
McGivan, J. D., Bradford, N. M. & Beavis, A. D. (1977) Biochem. J. **162**, 147–156
Mengual, R. & Sudaka, P. (1983) J. Membr. Biol. **71**, 163–171
Mengual, R., Leblanc, G. & Sudaka, P. (1983) J. Biol. Chem. **258**, 15071–15078
Moll, W., Giraird, H. & Gross, G. (1980) Pflugers Arch. **385**, 229–239
Monson, J. P., Smith, J. A., Cohen, R. D. & Iles, R. A. (1982) Clin. Sci. **62**, 411–420
Mowbray, J. & Ottaway, J. H. (1973*a*) Eur. J. Biochem. **36**, 363–368
Mowbray, J. & Ottaway, J. H. (1973*b*) Eur. J. Biochem. **36**, 369–379
Oldendorf, W. H. (1972) Eur. Neurol, **6**, 49–55
Oldendorf, W. H. (1973) Am. J. Physiol. **224**, 1450–1453
Partridge, W. M. & Oldendorf, W. H. (1977) J. Neurochem. **28**, 5–12
Quinlan, P. T., Thomas, A. P., Armston, A. E. & Halestrap, A. P. (1983) Biochem. J. **214**, 395–404
Rice, W. R. & Steck, T. L. (1976) Biochim. Biophys. Acta **601**, 500–508
Schwab, A. J., Bracht, A. & Scholtz, R. (1979) Eur. J. Biochem. **102**, 537–547
Spencer, T. L. & Lehninger, A. L. (1976) Biochem. J. **154**, 405–414
Thomas, A. P. & Halestrap, A. P. (1981) Biochem. J. **196**, 471–479
Tischler, M. E., Friedrichs, D., Coll, K. & Williamson, J. R. (1977) Arch. Biochem. Biophys. **184**, 222–236
Watts, D. J. & Randle, P. J. (1967) Biochem. J. **104**, 51P
Welch, S. G., Metcalfe, H. K., Monson, J. P., Cohen, R. D., Henderson, R. M. & Iles, R. A. (1984) J. Biol. Chem. **259**, 15264–15271
Williamson, J. R., Browning, E. T. & Scholtz, R. (1969) J. Biol. Chem. **244**, 4607–4616

Received 8 April 1987/3 August 1987; accepted 8 September 1987