MEMBRANE PERMEABILITY AND UTILIZATION OF L-LACTATE AND PYRUVATE IN CARP RED BLOOD CELLS

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Summary

L-Lactate and pyruvate permeability and utilization in carp (Cyprinus carpio) red blood cells was studied in vitro with tracer methods. Transport of lactate and pyruvate across the carp red blood cell membrane is rapid. At low plasma concentrations, lactate and pyruvate are transported into carp red blood cells mainly via a specific monocarboxylate carrier. This is shown by a study of the saturation kinetics and by inhibition using α-cyano-4-hydroxycinnamic acid and, more powerfully, p-chloromercuriphenylsulphonic acid. At higher plasma concentrations both simple diffusion and, apparently, the band 3 anion exchange system become increasingly important transport pathways. Carbon dioxide production rates from lactate and pyruvate as a function of their extracellular concentrations showed saturation kinetics. The transport rates of lactate and pyruvate are considerably higher than those required for their maximal rate of oxidation. The rapid transport of lactate and pyruvate into carp red blood cells thus guarantees that substrate availability is not the rate-limiting factor for the oxidation of these substrates.

Introduction

Transport studies of l-lactate and pyruvate in vertebrate cells have been limited mainly to red blood cells of mammalian origin. l-Lactate and pyruvate may enter the mammalian erythrocyte by three mechanisms: by free diffusion of the undissociated acid, by the band 3 anion exchanger or via a specific monocarboxylate carrier. The latter mechanism dominates at physiological concentrations in most species studied, although the carrier is absent in sheep and ox erythrocytes (Deuticke et al. 1978). Data are available on the transport of lactate into the red blood cells of only one non-mammalian species: it appears that the transport of lactate into the erythrocytes of the tuna, Katsuwonus pelamis, occurs by simple diffusion (Moon et al. 1987).

The component of transport mediated by the monocarboxylate carrier can be distinguished from others by its sensitivity to certain sulphhydryl reagents, particularly p-chloromercuriphenylsulphonic acid (PCMBS) (Deuticke et al. 1978). α-Cyanocinnamates have also been used for this purpose (Halestrap and Denton, 1974;
Halestrap, 1976), although they are also known to inhibit the band-3-mediated transport (Halestrap, 1976; Deuticke, 1982). Monocarboxylate transport via the band 3 anion exchange system is difficult to study, because most band 3 anion exchange inhibitors also affect the specific monocarboxylate carrier (Deuticke et al. 1982; Jennings and Adams-Lackey, 1982; Poole and Halestrap, 1991).

Fish erythrocytes appear to produce energy by mainly aerobic means (e.g. Eddy, 1977; Ferguson and Boutilier, 1988; Ferguson et al. 1989; Sephton et al. 1991). L-Lactate and pyruvate appear to be the most important oxidative energy substrates of carp erythrocytes (Tiihonen and Nikinmaa, 1991). Thus, monocarboxylate transport may be one of the control steps in energy production. For this reason we have investigated the mechanisms of lactate and pyruvate transport into carp erythrocytes. We have also investigated the concentration dependence of L-lactate and pyruvate oxidation rates and compared these to the transport rates of L-lactate and pyruvate. Thus, we have been able to evaluate the role of monocarboxylate transport in the control of metabolic energy production.

**Materials and methods**

**Animals and handling of blood**

The carp, *Cyprinus carpio* L., were obtained from Porla Fisheries Station and maintained under laboratory conditions (in running, dechlorinated Helsinki tapwater at 12–17°C) for a minimum of 10 days before experimentation. One group of carp used in the lactate and pyruvate utilization experiments had been maintained in laboratory conditions for a year. Fish were fed on a commercial diet, but were fasted for 24–96 h before use. Animals were anaesthetized with MS-222 (0.1 gl⁻¹, 5min) and the blood samples were taken by venipuncture.

Red blood cells (RBCs) and plasma were separated by a short centrifugation (10000 g). Cells were prepared for transport experiments by washing three times with an iso-osmotic incubation medium containing (in mmol l⁻¹): 128 NaCl, 3 KCl, 1.5 CaCl₂, 1.5 MgCl₂, 15 Tris. Earlier studies (e.g. Bushnell et al. 1985; Salama and Nikinmaa, 1988) have shown that cellular nucleotide triphosphate concentrations remain stable in such incubation media for several hours. Washing the red cells removes intracellular pyruvate and lactate. The buffy coat was discarded and the washed red cells were resuspended to a haematocrit (Hct) of 20% in incubation medium or 10% in medium containing inhibitor. The pH of the incubation medium was initially adjusted to 7.8. During the experiments, the pH of the medium remained quite stable, except in the highest PCMBS and α-cyanocinnamate concentrations tested in dose–response experiments, where the pH decreased by approximately 0.1–0.2 units. All the experiments were carried out, under air, at room temperature (21±1°C) unless otherwise stated.

**Transport studies**

Uptake of L-lactate and pyruvate was measured using the modified oil-stop procedure (Young and Ellory, 1982). Uptake of ¹⁴C-labelled L-lactate and pyruvate was initiated by mixing the cell suspension (Hct 20%) with an equal volume of incubation medium containing labelled substrate. In dose–response studies with inhibitors, the ratio of cell
suspension (Hct 10%) to medium containing labelled substrate was 9:1 (v:v), but final incubations, in both cases, contained $^{14}$C-labelled substrate at an activity of approximately 18.5kBqml$^{-1}$. In the inhibition studies, a preincubation period of at least 1h with the inhibitor (PCMBS or $\alpha$-cyanocinnamate) preceded the uptake experiment, which was carried out as described above. Fresh stock solutions of PCMBS and $\alpha$-cyanocinnamate were prepared daily.

Incubations were stopped at pre-determined times by transferring 0.2ml of the cell suspension (10% Hct) to an Eppendorf tube containing 0.8ml of stop medium layered on top of 0.5ml of dibutyl phthalate. Initial rates of lactate and pyruvate uptake were determined from the first 30s of incubation, during which the uptake was linear. The stopping medium, containing 0.5mmol$^{-1}$ PCMBS in incubation medium, was prepared daily. The tube was centrifuged immediately (10000g for 10s at 4˚C) and stored on ice to minimize metabolism. The medium and oil layers were removed by suction, leaving the cell pellet at the bottom of the tube. After carefully wiping the inside of the tube, the cell pellet was lysed with 0.2ml of 0.6mmol$^{-1}$ perchloric acid. The radioactivity taken up by the red blood cells was measured by liquid scintillation counting (LKB-Wallac 1211 MiniBeta). A correction factor for the radioactivity trapped in the extracellular space was estimated using ice-cold cell samples which were mixed with ice-cold labelled substrate and centrifuged immediately. Uptake values were calculated after subtraction of these ‘blank’ estimates. Intracellular substrate concentrations are presented as mmoll$^{-1}$RBC.

$[\text{U-14C}]$lactic acid sodium salt (5.69GBqmmol$^{-1}$) and $[1\text{-14C}]$pyruvic acid sodium salt (1.07GBqmmol$^{-1}$) were obtained from Amersham International. Non-radioactive pyruvate was from Merck and non-radioactive L-lactate from Sigma, as were $\alpha$-cyano-4-hydroxycinnamic acid and $p$-chloromercuriphenylsulphonic acid (PCMBS).

Kinetic analyses

The rate constants were calculated using the equation:

$$U_t = U_{tot}(1 - e^{-kt}),$$

where $U_t$ is the substrate uptake at time $t$, $U_{tot}$ is the total uptake of substrate at equilibrium and $k$ is the rate constant.

Kinetic variables are determined using the Michaelis–Menten equation modified to include a diffusional component:

$$V = V_{max}s/(K_m + s) + Ds,$$

where $V$ is the initial uptake rate, $V_{max}$ is the maximum influx through the transport system, $s$ is the substrate concentration, $K_m$ is the apparent half-saturation constant of the transport system and $D$ is the diffusional constant. To ensure that the initial rate of substrate entry was being measured, preliminary investigations of the time course of uptake were performed.

Measurement of plasma and RBC lactate, pyruvate and Cl$^{-}$ concentrations

Blood samples for plasma and RBC lactate, pyruvate and Cl$^{-}$ concentration measurements were taken by venipuncture as previously described. Plasma and RBCs
were separated by centrifugation at 10000g for 1min. RBC fractions were lysed with 0.6mmoll−1 perchloric acid. Both fractions were immediately frozen prior to measurements. Pyruvate and lactate concentration measurements were based on absorbance changes at 340nm by means of LDH and NAD+/NADH conversions. One mole of NAD+ is reduced to one mole of NADH for each mole of lactate present in assay tube and correspondingly NADH is oxidized to NAD+ in pyruvate measurements. The fluorescence of NADH was measured using a Transcon 102 FN analyzer. Cl− concentrations were measured with a Radiometer CMT10 chloride titrator. Intracellular substrate concentrations are presented as mmoll−1 RBCwater.

Measurement of CO2 production
Carbon dioxide production from L-[U-14C]lactic acid sodium salt (5.69GBqmmol−1) and [1-14C]pyruvic acid (1.18GBqmmol−1) was measured as described previously (Tiihonen and Nikinmaa, 1991). Instead of a single substrate concentration, we measured a wide range of concentrations while maintaining the amount of labelled substrate at a constant level by adding additional unlabelled substrate to the incubation medium. During the 1h incubation, the pH of the medium remained quite stable at pH7.6. As the carbon dioxide production from these substrates showed saturation kinetics, we estimated the kinetic variables using the Michaelis–Menten equation.

Results and discussion
The time courses of lactate and pyruvate uptake by carp RBCs are shown in Figs 1 and 2. The rate constant of 1mmoll−1 lactate uptake was 0.038±0.011min−1 and that for 0.05mmoll−1 pyruvate was 0.232±0.048min−1 (mean ± S.E.M., N=4). As can be seen from the insets, the uptake for both substrates remained linear for approximately 30s, and this incubation period was used to measure the initial rates of uptake. The tracer fluxes observed in these time course studies, especially in the case of pyruvate, may be affected by metabolic modification of the labelled substrates inside the red blood cell.

In physiological situations, the lactate concentration in the plasma, as well as in RBCs, exceeds the pyruvate concentration. The mean plasma lactate concentration of the carp in this study was 4.27±0.48mmoll−1 while that of the RBCs was 3.02±0.37mmoll−1 RBCwater (mean ± S.E.M., N=6). For pyruvate, corresponding concentrations were 0.082±0.003mmoll−1 for plasma and 0.101±0.003mmoll−1 RBCwater for RBCs (N=6). Because of the sampling procedure, these concentrations cannot be considered as resting plasma concentrations. The ratio of intracellular to plasma lactate concentration was 0.71, whereas the same ratio for pyruvate was 1.23. These RBC/plasma ratios are higher than the measured chloride distribution ratio (0.61; Cl− concentration: red blood cell 74.0±1.55mmoll−1 RBCwater and plasma 121±0.58mmoll−1, mean ± S.E.M., N=4). Thus, either the uptake of pyruvate and, to a smaller extent, lactate into the carp red cells is concentrative or these anions are, to a significant extent, bound to intracellular proteins such as haemoglobin.

Figs 3 and 4 show full dose–response curves for the inhibition of lactate and pyruvate uptake by PCMBS and α-cyanocinnamate, respectively. The extent of inhibition of the
initial rate of 1mmol$^{-1}$ lactate and 0.05mmol$^{-1}$ pyruvate uptake depended strongly on inhibitor concentration. The sensitivity of pyruvate and lactate transport to inhibition by PCMBS is quite similar. Half-maximal inhibition was achieved at a concentration of $2.3 \times 10^{-5}$ mol$^{-1}$ in both cases. PCMBS sensitivity of lactate transport in carp red blood cells is similar to that in human red blood cells (Deuticke et al. 1978). Monocarboxylate transport was also inhibited by $\alpha$-cyanocinnamate, although the inhibition was weaker.
than that caused by PCMBS: the concentrations required for 50% inhibition of pyruvate and lactate uptake by α-cyanocinnamate were 0.47 and 0.93 mmol l\(^{-1}\), respectively.

Fig. 3. Effects of PCMBS on uptake of 0.05 mmol l\(^{-1}\) pyruvate (filled circles) and 1 mmol l\(^{-1}\) L-lactate (open circles) by carp red blood cells. Results are given as a percentage of the control uptake rate in the absence of inhibitor. Values are means of four pyruvate experiments and 3–6 L-lactate experiments.

Fig. 4. Effects of α-cyano-4-hydroxycinnamate on uptake of 0.05 mmol l\(^{-1}\) pyruvate (filled circles) and 1 mmol l\(^{-1}\) L-lactate (open circles) by carp red blood cells. Results are given as a percentage of the control uptake rate in the absence of inhibitor. Values are means of four experiments, except for pyruvate uptake at 0.5 mmol l\(^{-1}\) inhibitor concentration, which is the mean of duplicate determinations.
Essentially complete (90%) inhibition of lactate and pyruvate uptake were caused by 2 mmol l\(^{-1}\) α-cyanocinnamate and 0.25 mmol l\(^{-1}\) PCMBS. Since the inhibition characteristics for lactate and pyruvate were similar, and since pyruvate uptake is inhibited by excess lactate (a 20-fold concentration of cold lactate inhibits pyruvate transport by 28%; K. Tiihonen, unpublished results), it is likely that the monocarboxylates share a common carrier molecule.

The concentration dependence of lactate and pyruvate uptake in the presence and absence of PCMBS is shown in Figs 5 and 6, respectively. Transport can be dissected into inhibitor-sensitive and inhibitor-insensitive components. The inhibitor-sensitive component of lactate and pyruvate uptake represents transport via the monocarboxylate carrier. The carrier tends to favour pyruvate over lactate, since the apparent half-saturation constant (\(K_m\)) for lactate was slightly higher (2.76 mmol l\(^{-1}\)) than that for pyruvate (2.14 mmol l\(^{-1}\)). The \(K_m\) value for lactate is within the physiological range for the lactate concentration in carp plasma. The maximum influxes through the transport system (\(V_{\text{max}}\)) for lactate (0.377 mmol l\(^{-1}\) RBC min\(^{-1}\)) and pyruvate (0.627 mmol l\(^{-1}\) RBC min\(^{-1}\)) by the carp RBC are quite similar, as would be expected if they share a common carrier. In the presence of the inhibitor, the relationship between the initial uptake rate and substrate concentration is linear, showing the diffusional

![Graph](image)

Fig. 5. Concentration dependence of l-lactate uptake by carp red blood cells in the absence (filled circles) and in the presence (open circles) of 0.5 mmol l\(^{-1}\) PCMBS. Each value is the mean ± S.E.M. of four experiments, except for the values at 40 mmol l\(^{-1}\) l-lactate concentration, which are the means of three experiments. Lactate uptake during the first 30 s of incubation was measured as described in Materials and methods. In the absence of inhibitor, the apparent \(K_m\) and \(V_{\text{max}}\) values (determined by the Michaelis–Menten equation including diffusional component) are 2.76 mmol l\(^{-1}\) and 0.377 mmol l\(^{-1}\) RBC min\(^{-1}\). The slope (i.e. influx/extracellular substrate concentration) for the linear part of the uptake is 0.025 mmol l\(^{-1}\) RBC min\(^{-1}\) (mmol l\(^{-1}\))\(^{-1}\). In the presence of inhibitor, results were fitted by linear regression, yielding the slope 0.034 mmol l\(^{-1}\) RBC min\(^{-1}\) (mmol l\(^{-1}\))\(^{-1}\).
The rates of this PCMBS-insensitive transport for both lactate and pyruvate as a function of external substrate concentrations are almost equal [0.034 and 0.046 mmol l\(^{-1}\) RBC min\(^{-1}\)], respectively. It is obvious from Figs 5 and 6 that, under resting physiological conditions, lactate and pyruvate are mainly transported into the red blood cell via the monocarboxylate carrier. At higher extracellular concentrations, such as occur after exercise, at least for lactate (e.g. Jensen et al. 1983), the ‘diffusional’ component becomes increasingly important in relative terms. The diffusional component may include a contribution from the anion exchange pathway, as in other vertebrate red blood cells (Deuticke et al. 1978, 1982; Deuticke, 1989), since pyruvate transport in PCMBS-containing medium is further inhibited by approximately 10\% by 0.1 mmol l\(^{-1}\) DIDS at a pyruvate concentration of 0.05 mmol l\(^{-1}\) (K. Tiihonen, unpublished results).

The present results differ from those reported for lactate uptake in tuna red blood cells (Moon et al. 1987), which suggested that tuna red blood cells lack a lactate-transporting system. The difference may, however, be mainly methodological. If there were a carrier-mediated lactate transport system in tuna red blood cells with properties similar to that found in carp, its contribution to total flux of lactate would be difficult to detect using the relatively high concentration range (1–30 mmol l\(^{-1}\)) or long incubation times (3 min) of Moon et al. (1987), which preclude the estimation of a true initial uptake rate.
Lactate and pyruvate transport in carp erythrocytes

Fig. 7. Effects of substrate concentration on carbon dioxide production rates from L-lactate (squares; mean ± s.e.m., N=4–5) and pyruvate (circles) by carp red blood cells. Filled circles (mean ± s.e.m., N=4) describe the pyruvate oxidation in a group of carp that had been maintained in laboratory conditions for 1 year. Open circles (mean ± s.e.m., N=2–7) are from a group taken from the fisheries station 10 days to 2 months before experimentation. Apparent $K_m$ and $V_{max}$ values (determined from the Michaelis–Menten equation) are: lactate, 1.71 mmol l$^{-1}$ and 0.276 mmol CO$_2$ l$^{-1}$ RBC h$^{-1}$; pyruvate (filled circles), 0.29 mmol l$^{-1}$ and 0.848 mmol CO$_2$ l$^{-1}$ RBC h$^{-1}$; pyruvate (open circles), 0.62 mmol l$^{-1}$ and 0.275 mmol CO$_2$ l$^{-1}$ RBC h$^{-1}$.

Table 1. Comparison between the uptake and oxidation rates of L-lactate and pyruvate at various extracellular concentrations by carp red blood cells

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Extracellular concentration (mmol l$^{-1}$)</th>
<th>Uptake rate (mmol l$^{-1}$ RBC h$^{-1}$)</th>
<th>Oxidation rate (mmol l$^{-1}$ RBC h$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-lactate</td>
<td>0.5</td>
<td>3.90</td>
<td>0.028</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>6.97</td>
<td>0.034</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>21.76</td>
<td>0.065</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>36.22</td>
<td>0.072</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>46.98</td>
<td>0.107</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.05</td>
<td>2.05</td>
<td>0.003–0.027</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>3.54</td>
<td>0.012–0.064</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>10.36</td>
<td>0.032–0.175</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>15.22</td>
<td>0.069–0.246</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>47.62</td>
<td>0.078–0.251</td>
</tr>
</tbody>
</table>

Data are taken from the experiments described in Figs 5, 6 (uptake) and 7 (oxidation).

Oxidation rates were calculated from carbon dioxide production rates, assuming that 1 mol of lactate or pyruvate produces 3 mol of carbon dioxide.

The lower and upper values for pyruvate oxidation are taken from the two groups of carp.
Transport of monocarboxylates by two parallel transport systems, a monocarboxylate carrier and a band 3 anion exchanger, and also by non-ionic diffusion (see Deuticke, 1982, 1989), makes a detailed analysis difficult. These three pathways contribute to a varying extent depending on the experimental conditions, especially temperature, pH and, as the present study also shows, substrate concentration. Since most of the inhibitors of the band 3 anion exchange system also inhibit the monocarboxylate carrier (Deuticke et al. 1982; Jennings and Adams-Lackey, 1982; Poole and Halestrap, 1991), the contribution of the band 3 system of monocarboxylate transport is difficult to study. The nominally bicarbonate-free medium (Tris-buffered) used in the present experiments may have the effect of exaggerating the amount of monocarboxylate uptake via the band 3 system because of the decreased probability of bicarbonate ions entering the transport site.

The dependence of oxidation rates on L-lactate and pyruvate concentrations yields saturation kinetics, which are described in Fig. 7 by a Michaelis–Menten analysis. The utilization of lactate and pyruvate was much slower than their rate of transport into the erythrocytes at the same extracellular concentrations (Table 1). This indicates that if energy production from lactate and pyruvate is regulated to respond to varying metabolic needs, the controlling steps occur within the metabolic pathways. The $K_m$ values for lactate and pyruvate (1.71 mmol l$^{-1}$ and 0.29–0.62 mmol l$^{-1}$, respectively) oxidation are lower than those for transport.

For pyruvate, apparent $K_m$ values (0.29 and 0.62 mmol l$^{-1}$) as well as $V_{max}$ values (0.848 and 0.275 mmol CO$_2$ l$^{-1}$ RBC h$^{-1}$) depended on the carp group used. The variation between the carp groups may be explained by their acclimatization to different environments. The group that had been held in laboratory conditions for 1 year could utilize pyruvate more effectively than the group taken from the fisheries station 10 days to 2 months before experimentation. Omnivorous fish, such as carp, have considerable flexibility of feeding habit and possibly are also able to utilize different nutrients. For example, carp are able to adapt to the quantity, and apparently also to the type, of digestive carbohydrate in the diet (Buddington, 1987).

Since pyruvate concentrations in fish plasma do not exceed 0.5 mmol l$^{-1}$ (Milligan and Wood, 1986), carp RBCs do not reach their maximal pyruvate oxidation rates in vivo. However, energy production from pyruvate may exceed that from lactate utilization at high physiological substrate concentrations (above 0.1 mmol l$^{-1}$ for pyruvate and 4 mmol l$^{-1}$ for lactate), although lactate appears to be the most important substrate for energy metabolism at low physiological concentrations (0.05 mmol l$^{-1}$ for pyruvate and 1 mmol l$^{-1}$ for lactate).

Despite the similarity between the human and carp monocarboxylate carriers, there is a significant difference in their physiological role. Monocarboxylates taken up by carp RBCs can be used for aerobic energy production inside the cells (Wood et al. 1990; Walsh et al. 1990; Tiihonen and Nikinmaa, 1991), but in mammalian red blood cells the monocarboxylate carrier (Halestrap, 1976; Deuticke et al. 1978; Dubinsky and Racker, 1978) allows a net loss of lactate, produced by anaerobic glycolysis, to occur.

In conclusion, the present findings show that carp red blood cells have a specific transport system for lactate and pyruvate. At resting plasma concentrations, about 90% of
Lactate and pyruvate transport in carp red cell membrane proceeds via the specific monocarboxylate carrier. Only 10% of the total transport under the conditions of this study occurs by free diffusion or via the anion exchanger. The results also show that the transport rates of lactate and pyruvate are considerably higher than those required for their maximal rate of oxidation. In salmonids, catecholamines may double the oxygen consumption/energy production of red blood cells (Ferguson and Boutilier, 1988). Even if such activation of metabolism occurred in carp red blood cells, lactate and pyruvate transport would provide the cells with a saturating amount of substrate. The rapid transport of lactate and pyruvate into carp red blood cells thus guarantees that substrate availability is not the rate-limiting factor for the oxidation of these substrates.

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References


