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^{1}H- AND ^{31}P-NUCLEAR MAGNETIC RESONANCE STUDIES OF L-LACTATE TRANSPORT IN ISOLATED MUSCLE FIBERS FROM THE SPINY LOBSTER *PANULIRUS ARGUS*

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Summary

Proton (^{1}H) and phosphorus (^{31}P) nuclear magnetic resonance (NMR) spectroscopy were used to investigate the mode of transport of L-lactate across the plasma membranes of the abdominal extensor muscles of the spiny lobster *Panulirus argus*. Individual fibers or bundles of 2–3 fibers were superfused in a dual-tuned (^{1}H, ^{31}P) microsolenoid NMR probe. ^{1}H-NMR spectra were diffusion-weighted, which eliminated the signal contribution of the fast-flowing extracellular lactate but retained that of intracellular lactate. Well-resolved intracellular lactate signals could be acquired at 15 s intervals, permitting estimation of initial velocities (V_i) of influx and efflux during loading/unloading of muscle fibers. ^{31}P-NMR spectra were acquired to assess cellular energy status and intracellular pH. Transport results showed that V_i values for influx and efflux were a linear function of total lactate concentration, displaying no saturation effects. The rate of lactate influx was enhanced by increasing the concentration of the free acid by altering the superfusate pH. V_i values for influx and efflux of D- and L-lactate were identical. Finally, traditional inhibitors of monocarboxylate and/or anion transport had no effect on influx/efflux of lactate from these cells. The above results strongly suggest that the primary mode of lactate transport is by passive diffusion. These cells appear to lack a monocarboxylate transporter, which may be related to the apparent absence of organ-specific compartmentation of lactate metabolism in crustaceans.

Key words: lactate, transport, nuclear magnetic resonance, muscle, lobster, *Panulirus argus*, Crustacea.

Introduction

Lactate transport across the plasma membrane has been studied in a wide variety of vertebrate tissues and cell types. Three generalized pathways of membrane transport have been identified (Deuticke *et al.* 1982; Poole and Halestrap, 1993): (i) passive diffusion of the free acid; (ii) exchange for Cl^{-} or HCO_3^{-} as mediated by an anion transporter; and (iii) transport by a highly specific H^{+}/monocarboxylate symporter (lactate:H^{+} symporter). This latter pathway appears to be the primary mode of trans-sarcolemmal lactate transport in skeletal muscle, as has been demonstrated for frog (Seo, 1984; Mason and Thomas, 1988), rat (Watt *et al.* 1988; Roth and Brooks, 1990*a*,*b*; McDermott and Bonen, 1993; Wibrand and Juel, 1994; Allen and Brooks, 1994), mouse (Juel and Wibrand, 1989; Bonen and McCullagh, 1994) and human muscle (Juel *et al.* 1994). In terms of lower vertebrates, recent evidence has been presented which demonstrates the presence of a lactate carrier in fish skeletal muscle (Wang *et al.* 1994*b*) and red blood cells (Tiihonen and Nikinmaa, 1993), although lactate transport appears to be by passive means in fish hepatocytes (Walsh, 1987). cDNAs for several isoforms of the mammalian lactate:H^{+} symporter have recently been cloned and sequenced (Kim *et al.* 1992; Garcia *et al.* 1994; Jackson *et al.* 1995).

Lactate transport in invertebrate muscle has received minimal attention. In decapod crustaceans undergoing strenuous exercise, L-lactate is the dominant anaerobic end product and accounts for nearly all of the intracellular acidification associated with anaerobic work (Phillips *et al.* 1977; McDonald *et al.* 1979; Smatresk *et al.* 1979; Wood and Randall, 1981; Booth *et al.* 1984; Milligan *et al.* 1989). Furthermore, during environmental anoxia, decapod crustaceans produce large amounts of L-lactate (Bridges and Brand, 1980; Gäde, 1984; Albert and Ellington, 1985). Although lactate accumulates in the hemolymph during both functional and environmental anoxia in decapod crustaceans, the extent of accumulation is much greater during long-term environmental anoxia when concentrations may exceed 40 mmol l^{-1} (Gäde, 1984; Albert and Ellington, 1985). The

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mechanism(s) of sarcolemmal membrane transport of lactate in this group is unknown.

Since lactate can traverse the membrane with a proton, *via* either a specific carrier or passive diffusion of the free acid, lactate transport could play a role in regulation of intracellular pH (pHi). Pörtner (1993) suggested that these modes of lactate transport may serve as an additional mechanism of pHi regulation, which he termed non-ionic regulation. The effect of extracellular pH (pHe) on lactate transport is well documented (reviewed by Poole and Halestrap, 1993) but, in vertebrate muscle, the extent to which lactate flux influences pHi is difficult to determine owing to uncertainties in the pH gradients across the capillary walls, interstitial space and intracellular space. The capillary walls also form another barrier to lactate movement (in addition to the sarcolemma) which must be accounted for (Roth and Brooks, 1990*a*). Decapod crustaceans have an open circulatory system and large muscle cells, eliminating the capillary interface, thus creating, in effect, a two-compartment system.

In this study, a method has been developed for examining lactate influx and efflux kinetics in muscle fibers from the spiny lobster *Panulirus argus* using high-field nuclear magnetic resonance (NMR) spectroscopy. Single large cells or bundles of 2–3 cells from the abdominal extensor muscles were used. During lactate loading and wash-out experiments, intracellular lactate concentration was monitored *via* ^{1}H-NMR spectra which were diffusion-weighted to eliminate the signal contribution from flowing extracellular lactate, leaving only the signal from intracellular lactate (van Zijl *et al.* 1991). ^{31}P-NMR spectra were acquired to monitor energetic stability and to measure pHi. The use of a high-field magnet (14 T) provided the necessary sensitivity to measure initial velocities (V_i) of lactate influx and efflux. This is the first analysis of lactate transport kinetics in a crustacean muscle.

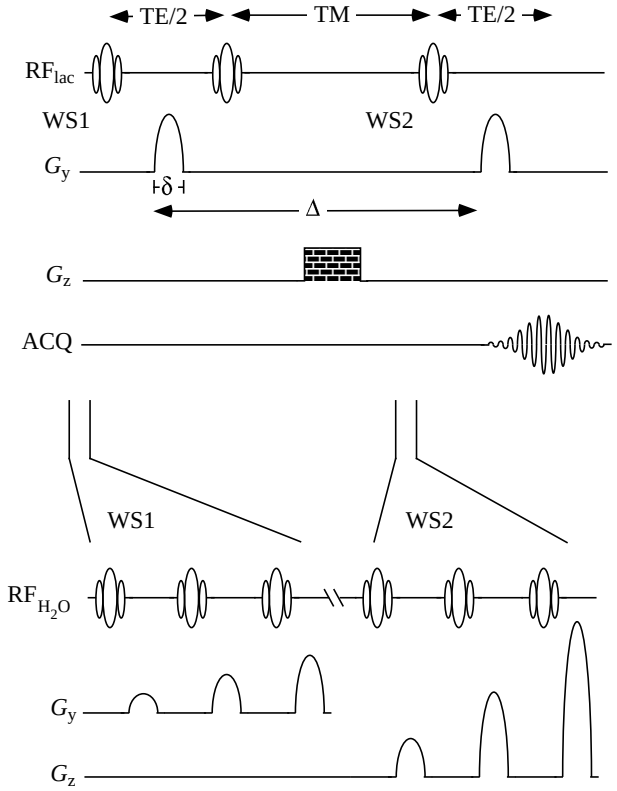

Fig. 1. Modified DRYSTEAM sequence used to collect diffusion-weighted ^{1}H-NMR spectra. The sinc-shaped radio frequency pulses selectively excited the lactate methyl protons (RF$_{lac}$). Diffusion-weighting was accomplished using *y*-axis gradient pulses (G_y) which were oriented along the longitudinal axis (axis of flow) of the superfusion chamber. For all transport experiments, δ=2 ms, Δ=100 ms and G=35 G cm^{-1}. The brick wall (G_z) is a crusher gradient used to eliminate unwanted echoes (3 ms, 15 G cm^{-1}). Data were acquired (ACQ) as half-echoes. WS1 and WS2 refer to the CHESS water suppression cycles employed during the preparation and mixing times (TM), respectively. For this additional suppression, the water resonance RF$_{H_2O}$ was selectively excited with sinc (RF$_{H_2O}$) pulses and subsequently dephased by gradient pulses which increased in amplitude for each cycle (Moonen and van Zijl, 1990). Gradient values for the dephasing gradients were 10, 20 and 40 G cm^{-1} (G_y) for WS1 and 15, 30 and 45 G cm^{-1} (G_z) for WS2. TM, mixing time; TE, echo time.

Materials and methods

Specimens and materials

Specimens of the lobster *Panulirus argus* (Latreille) were obtained from the Keys Marine Laboratory in Long Key, Florida. Specimens were maintained at 22 °C in a large recirculating seawater system, located on the Florida State University campus, and were fed shrimp on a regular basis. Biochemicals were obtained from Sigma Chemical Company (St Louis, MO, USA) and Boehringer Mannheim (Indianapolis, IN, USA). All other chemicals were of reagent-grade quality.

NMR procedures

Lobsters were placed on ice for 10–15 min prior to use. The abdomen was removed and the central portion of the dorsal exoskeleton (where the abdominal extensor muscles attach) was removed along the length of the abdomen. The exoskeleton and attached extensor muscles were placed in a solution of 457 mmol l^{-1} NaCl, 15 mmol l^{-1} KCl, 1.8 mmol l^{-1} $MgCl_2$ and 2.5 mmol l^{-1} $MgSO_4$ buffered with 10 mmol l^{-1} Hepes and 10 mmol l^{-1} Mes to pH 7.5. Individual cells or bundles of two (and very occasionally three) cells were mechanically isolated from deep extensor muscle by carefully teasing the fibers apart with fine glass probes. Individual cells were 200–300 μm in diameter and 10–15 mm long. The use of these very large individual cells or bundles of 2–3 cells resulted in the virtual elimination of the interstitial compartment. Each cell terminated with a junction to cells from each of the adjacent abdominal segments, so the adjacent cells were isolated simultaneously with the cell(s) of interest, sutured with 6-0 gauge surgical silk, and cut off near the suture point. In this manner, the cells to be observed were not in direct contact with the suture and their terminal connections with adjacent cells were intact.

The isolated cell preparations were centered in a superfusion chamber housed on a standard bore (39 mm) NMR probe (Bruker Instruments, Billerica, MA, USA). The probe contained a horizontal five-turn solenoidal radio frequency coil (i.d. 2.2 mm) that could be dually tuned to ^{31}P (242 MHz) and ^{1}H (600 MHz). The superfusion chamber consisted of a glass capillary tube (i.d. 1.9 mm) mounted within the coil and held in place by a nylon housing. The nylon housing was fed with superfusion lines which were snaked through the bottom of the probe to peristaltic pumps (total volume of superfusion system <6 ml). At the base of the probe was a glass heat exchanger connected to a recirculating water bath for temperature control (20 °C) and a superfusion bypass switch. The bypass switch allowed superfusion solutions to be changed without getting air bubbles in the lines or having to stop the flow (so the flow rate was constant), and it allowed the timing of solution changes at the sample to be precisely controlled.

Spectra were acquired using a Bruker 600 DMX wide-bore spectrometer equipped with imaging gradients. ^{1}H-spectra were acquired every 15 s using a selective stimulated echo sequence with additional water suppression and diffusion-weighting gradient pulses (Fig. 1). The sequence is analogous to a 3,3-DRYSTEAM experiment (*D*rastic *R*eduction of water signals in spectroscopy based on the *ST*imulated *E*cho *A*cquisition *M*ode; Moonen and van Zijl, 1990) except that the spectra were not localized. The number prefixes indicate that three CHESS (*CHE*mical *S*hift *S*elective) pulses were applied to the H_2O resonance in the preparation time and three in the mixing time. Each CHESS pulse selectively excites the H_2O protons and is followed by a dephasing gradient (Moonen and van Zijl, 1990). 10 ms, five-lobe sinc pulses (850 Hz bandwidth) were used to selectively irradiate both the water resonance (4.8 p.p.m.) and the region containing the lactate (1.33 p.p.m.) and alanine (1.48 p.p.m.) methyl resonances (Fig. 1). High-quality ^{1}H-spectra of isolated fibers were obtained in 15 s of acquisition (Fig. 2).

In the DRYSTEAM sequence, the signal amplitude (A) attenuation due to diffusion-weighting is described by the modified Stejskal and Tanner (1965) equation:

$$\ln(A/A_0) = \gamma^2(2/\pi)^2G^2\delta^2(\Delta - \delta/4)D = -bD\,,$$

where A_0 is the unweighted signal amplitude $(2/\pi)^2$ and $\delta/4$ (instead of $\delta/3$) arise because half-sine-shaped diffusion gradients were used, γ is the magnetogyric ratio, G is the gradient amplitude, δ is the gradient duration, Δ is the time between gradient pulses, and b is the diffusion-weighting factor. D is the diffusion coefficient.

^{31}P-spectra were acquired in as little as 15 s (32 acquisitions) with a single, 2 μs hard pulse and a 0.4 s recycle time. Fig. 3 displays a ^{31}P-spectrum of a single muscle fiber acquired in 15 s. A ^{31}P-spectrum was examined before and after each lactate influx/efflux experiment to ensure that the cell preparation was energetically stable on the basis of the relative peak amplitudes of inorganic phosphate (P_i), arginine phosphate and the ATP resonances, as well as by measuring the pHi. Intracellular pH was determined by measuring the chemical shift difference between the P_i peak (which has a pH-dependent chemical shift) and the arginine phosphate peak (which is not pH-dependent) and comparing this difference with an empirically derived titration curve (Wiseman and Ellington, 1989). Isolated fiber preparations displayed extraordinary metabolic stability in experiments displaying minimal changes in energy state for up to 18 h.

Elimination of the signal contribution from the extracellular compartment

Elimination of the non-intracellular components of the lactate signal is greatly simplified by using fiber preparations with no or minimal interstitial compartment. In cases where several fibers were used, the bundles were loosely suspended such that gaps between fibers existed. For our single-fiber and two- to three-fiber preparations, diffusion-weighted ^{1}H-spectra were acquired in series with increasing values of G. The expected attenuation curve for a system with a fast (extracellular) and slow (intracellular) moving component is bi-exponential (van Zijl *et al.* 1991). A value of b was chosen from a region of the attenuation curve that corresponded to the slow-moving component (intracellular). This value for b was used in all other experiments which examined the increase (influx) or decrease (efflux) of the intracellular lactate integral.

Lactate transport experiments

The experimental paradigm for transport measurements was as follows: (1) collect a 1 min ^{31}P-spectrum for establishing energy state and measuring pHi; (2) begin collecting diffusion-weighted ^{1}H-spectra (every 15 s); (3) switch the superfusing medium to a lactate solution where NaCl was exchanged for an equivalent concentration of sodium lactate; (4) after a short influx period, wash out the lactate with the original superfusion

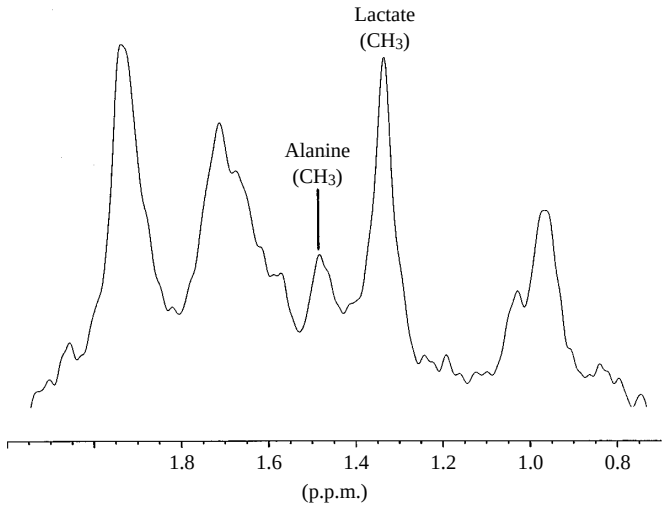

Fig. 2. ^{1}H-NMR spectrum of the selectively excited lactate methyl region of a single muscle fiber from *Panulirus argus* superfused with 70 mmol l^{-1} L-lactate. Data were acquired in 15 s. The chemical shift (p.p.m.) was referenced to the water resonance (4.8 p.p.m.).

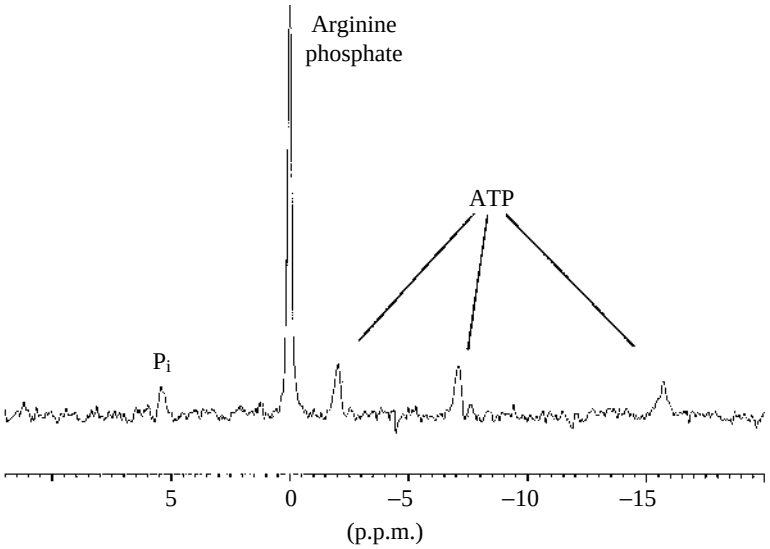

Fig. 3. ^{31}P-NMR spectrum of a single muscle fiber from *Panulirus argus*. Data were acquired in 15 s. The chemical shift (p.p.m.) was referenced to the arginine phosphate resonance (0 p.p.m.).

medium; and (5) acquire a final 1 min ^{31}P-spectrum. The lactate influx period was 2 min for most experiments, but ranged from 90 s to 6 min depending upon how much intracellular lactate loading was desired. The flow rate of 4–5 ml min^{-1} allowed the fluid in the sample chamber to be exchanged very rapidly.

For experiments with inhibitors, the above procedure was followed both with and without the inhibitor, allowing direct examination of the effect of an inhibitor on a single sample. When an inhibitor was used, it was added to both the lactate solution and the wash-out medium. The carrier-mediated transport inhibitors used were α-cyano-4-hydroxycinnamate (CIN), 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonate (SITS) and *p*-chloromercuribenzenesulfonate (PCMBS). Since NMR methods cannot distinguish between D- and L-lactate, D-lactate could not be used as a potential inhibitor. However, since all known L-lactate transporters are stereospecific (Poole and Halestrap, 1993), the rate of D-lactate transport was compared with the rate of L-lactate transport, which is useful for determining the contribution to transport of passive diffusion (McCullagh and Bonen, 1995).

Metabolite assays

Prior to each tissue being removed from the NMR probe, a final DRYSTEAM spectrum was acquired to be used as an integration reference. The fiber preparation was then immediately removed, frozen and stored at −85 °C until assays were performed. Removal and freezing of the tissue generally required less than 3 min from the time of the acquisition of the last spectrum. Frozen fiber preparations were homogenized in 1.0 ml of 80 % ethanol at 4 °C and centrifuged at full force for 5 min in a Fisher microfuge. 1 mol l^{-1} NaOH (10 μl) was added to the supernatant to form sodium lactate (non-volatile), and the supernatant was reduced to dryness in a speed-vac and stored at 5 °C. The dried samples were rehydrated in 200 μl of H_2O, centrifuged at full force for 5 min in a Fisher microfuge and assayed spectrophotometrically for lactate according to Graham and Ellington (1985).

Data analysis

Initial rates (V_i) of lactate transport were estimated by examining only the linear portion of the influx/efflux curve based on changes in the lactate integral. Lactate peaks for each experiment were integrated by assigning an integration window to the first spectrum (0.1 p.p.m. wide) and automatically integrating the remaining spectra using the Bruker peak-picking automation program. Integrals from all experiments were scaled to a single arbitrary reference, and data from metabolite assays were used to assign a lactate concentration to each integral. Lactate assay determinations yielded units of μmol g^{-1} wet mass, which were converted to approximate concentration (in mmol l^{-1}) by assuming an intracellular water content of 65 % of wet mass (no interstitial compartment). The final DRYSTEAM spectrum, taken just prior to removal of muscle fibers from the probe, was integrated and used to create conversion factors with units of μmol g^{-1} wet mass integral unit^{-1} or mmol l^{-1} integral unit^{-1}.

Student's two-tailed *t*-tests were used to test for significant differences between V_i of transport of L-lactate *versus* D-lactate as well as untreated L-lactate *versus* L-lactate with potential inhibitors. $P \leqslant 0.05$ was considered to indicate a significant difference.

Results

Separation of intra- and extracellular compartments

After superfusing *P. argus* muscle fibers with 50 mmol l^{-1} L-lactate for 15 min, a diffusion-weighted attenuation curve was generated in which the signal decays of both lactate and endogenous alanine were observed (Fig. 4). Lactate demonstrated a bi-exponential decay indicative of a fast- and a slow-moving component, while alanine showed a mono-exponential decay which indicates a single diffusional

environment. The very rapid initial decay of lactate is due to the fast flow conditions associated with the lactate in the superfusing medium, while the slowly decaying signal is from lactate which has entered the cells and is no longer influenced by the superfusate flow (van Zijl *et al.* 1991).

The similarity of the putative intracellular lactate decay slope with that of alanine (which has no extracellular component) provides additional evidence that this slow-moving component of lactate is of intracellular origin. Further, the calculated intracellular diffusion coefficients for lactate ($4.32\times10^{-6}\,cm^2\,s^{-1}$) and alanine ($4.1\times10^{-6}\,cm^2\,s^{-1}$), which are derived from the slopes of the shallow portions of the attenuation curves, are virtually identical, as expected, and fall within the expected *in vivo* range (Sotak, 1990). For all subsequent experiments, a *b* value of $1.4\times10^9\,s\,m^{-2}$ was used, which is well into the shallow portion of the attenuation curve. This *b* value should eliminate the contribution from the extracellular compartment.

Lactate transport

Results from a series of influx and efflux experiments, where the concentration of the loading medium ranged from 10 to 100 mmol l^{-1} L-lactate, are shown in Figs 5 and 6. It can be seen from these figures that the uptake/efflux of lactate is fairly linear for the first 45 s, so this time interval was used to measure V_i.

The initial rate (V_i) of lactate influx was a linear function of lactate concentration and displayed no evidence of saturation (Fig. 7). The linear regression for V_i *versus* total external lactate concentration passed through the origin (Fig. 7). Evaluation of the effect of intracellular lactate concentration on V_i of efflux was complicated by the fact that it was not possible to control the extent of lactate loading precisely prior to wash-out and efflux. Thus, the entire data set for five independent cell preparations is plotted as V_i *versus* each corresponding intracellular total lactate concentration (Fig. 8). It can also be seen that intracellular lactate loading was

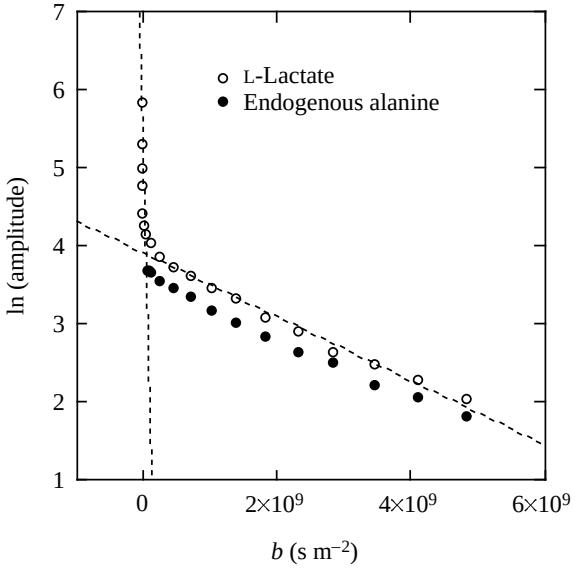

Fig. 4. Diffusion–attenuation curve of the lactate methyl (extracellular and intracellular) and alanine methyl (intracellular only) resonances for a bundle of muscle fibers from *Panulirus argus* superfused with 50 mmol l^{-1} L-lactate. Spectra were acquired every 2 min with 96 scans and a recycle delay of 1 s. For diffusion-weighting, δ was 2 ms, Δ was 100 ms and *G* was varied from 0 to 65 G cm^{-1}.

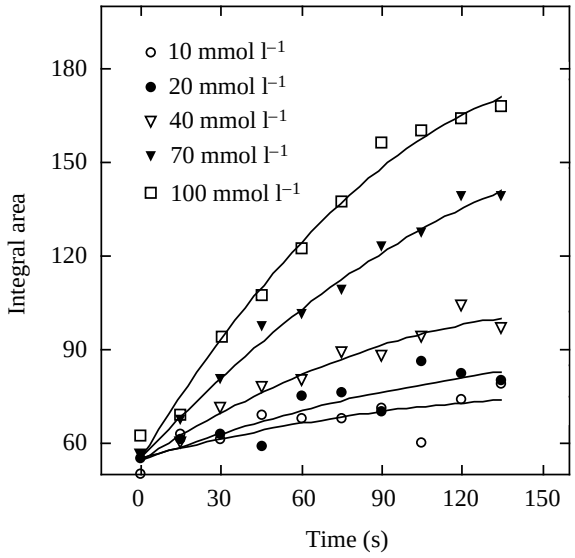

Fig. 5. Time course of influx of L-lactate into a *Panulirus argus* muscle fiber preparation. Peak integrals are specified in arbitrary units. Concentrations refer to external L-lactate concentrations. Loading pHe was 6.5 to enhance the overall rate of transport into the preparation.

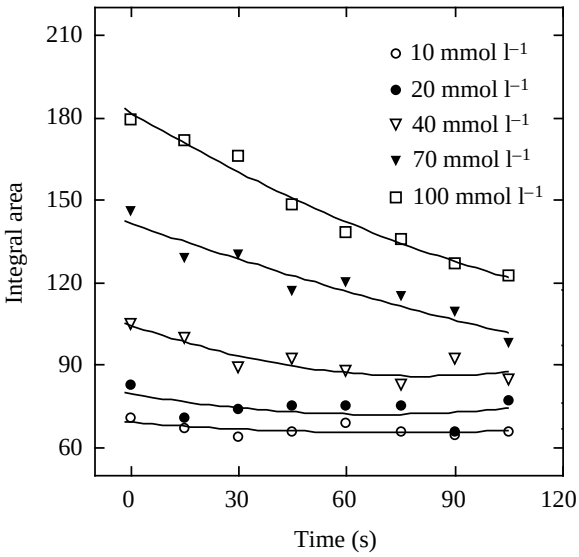

Fig. 6. Time course of efflux of L-lactate from a *Panulirus argus* muscle fiber preparation. Peak integrals are specified in arbitrary units. Concentrations refer to external concentration during loading, not intracellular concentrations (see legend of Fig. 8 for additional comments). Wash-out pHe was 7.5.

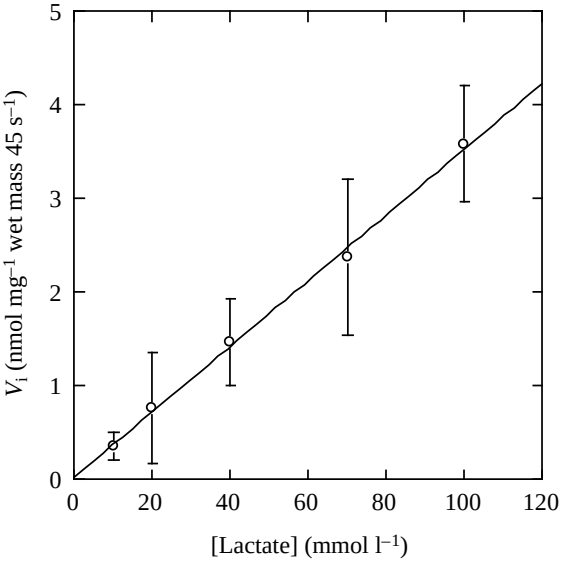

Fig. 7. The effect of extracellular L-lactate concentration on the initial velocity (V_i) of lactate influx into *Panulirus argus* muscle fiber preparations. Each value represents a mean ± 1 S.D. (N=5 independent preparations). The line corresponds to a linear regression (r^2>0.99, y=0.0350x+0.0296, P<0.0001).

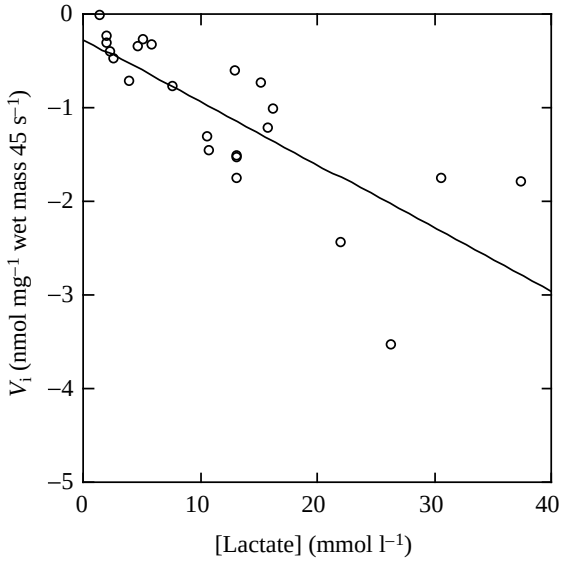

Fig. 8. The effect of intracellular lactate concentration on the initial velocity (V_i) of L-lactate efflux from *Panulirus argus* muscle fiber preparations. Wash-out pHe was 7.5. Intracellular lactate concentrations could not be precisely controlled as they represent the final lactate concentrations in the influx experiments just prior to wash-out (efflux). As a consequence, all of the individual V_i values were plotted *versus* their corresponding intracellular L-lactate concentration values (data are from N=5 independent preparations). The line corresponds to a linear regression (r^2=0.77, y=–0.0671x–0.2646, P<0.0001).

generally less than 16 mmol l^{-1} and averaged around 10 mmol l^{-1}. The efflux results display what appears to be a linear relationship between efflux V_i and total intracellular lactate concentration, with the linear regression passing close to the origin (Fig. 8).

Influx rates (Fig. 9) also appear to be a function of the free acid concentration (and hence pHe), as would be expected for both passive diffusion and carrier-mediated transport (Poole and Halestrap, 1993). In these experiments, influx V_i values were measured using a total L-lactate concentration of 40 mmol l^{-1} at pHe values of 7.5, 7.0, 6.5 and 6.0. There clearly is a trend which suggests that influx rates are accelerated by increased lactate free acid concentrations. If only the free acid form of lactate were permeable, then the line in Fig. 9 should pass closer to the origin, which is not the case here. However, the high variance of the V_i values due to exceedingly slow influx rates at low free acid concentrations (9.2–92 µmol l^{-1}) may have obscured such a relationship.

Transport of D-lactate and the effect of inhibitors

The influx and efflux rates of D-lactate were found to be the same as those for L-lactate (Table 1). Rates for L-lactate transport were unaffected by the addition of the possible monocarboxylate transporter inhibitors CIN, SITS and PCMB (Table 1). The large variance in absolute V_i values between treatments may be because animal size (and consequently fiber dimensions) varied from treatment to treatment. However, within a given treatment (for instance, D- *versus* L-lactate influx/efflux), special care was taken to use preparations with nearly identical dimensions.

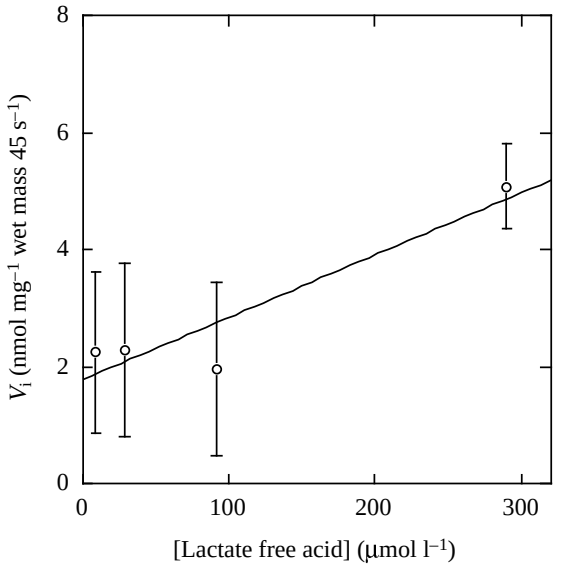

Fig. 9. Initial velocity (V_i) of L-lactate influx into *Panulirus argus* muscle fiber preparations plotted as a function of the concentration of L-lactate (free acid). Experiments were conducted with 40 mmol l^{-1} L-lactate at pHe values of 7.5, 7.0, 6.5 and 6.0. Lactate free acid concentrations were calculated on the basis of a pK_a of 3.86. Data represent a mean ± 1 S.D. (N=3 independent fiber preparations). The line represents a linear regression (r^2=0.93, y=0.0106x+1.7772, P<0.05).

Table 1. *Initial velocities of lactate influx/efflux in* Panulirus argus *muscle fibers for* D- versus L-*lactate and for* L-*lactate in the presence of well-established inhibitors of monocarboxylate transport*

Treatment	*N*	Lactate influx (nmol mg^{-1} wet mass 45 s^{-1})	Lactate efflux (nmol mg^{-1} wet mass 45 s^{-1})
L-Lactate	3	1.39±0.47	0.63±0.22
D-Lactate	3	1.35±0.57	0.79±0.25
No CIN	4	1.29±0.41	0.91±0.44
5 mmol l^{-1} CIN	4	1.17±0.44	0.91±0.25
No SITS	3	1.99±0.19	0.37±0.22
0.5 mmol l^{-1} SITS	3	1.86±0.38	0.35±0.19
No PCMB	4	2.55±1.23	1.29±0.25
0.2 mmol l^{-1} PCMB	4	2.52±0.91	1.10±0.25

Inhibitors were present in the solutions during loading and wash-out.
The data represent means ± 1 S.D. (where *N* refers to the number of independent muscle fiber preparations).
Within each treatment, no significant differences were observed.
Details of the inhibitors are given in Materials and methods.

Discussion

Validation of the experimental protocol

A critical element of our experimental protocol is the elimination of the contribution of the extracellular lactate signal to the ^{1}H-spectra. A variety of lines of evidence support our diffusion-weighting approach. In the first place, as shown in Fig. 4, the decay curves for endogenous alanine and what we presume to be intracellular lactate have virtually identical slopes. Since these two molecules should have very similar self-diffusion coefficients and the slopes of these decay curves are directly proportional to diffusivity (Steksjal and Tanner, 1965), it is highly likely that these resonances are diffusing in the same compartment, namely the cytosol. Second, the rate of exchange of superfusate in the extracellular compartment was extremely rapid (flow rate 4–5 ml min^{-1}) while the time courses for build-up of the lactate resonance during influx experiments were protracted in relative terms (see Fig. 5). If significant extracellular lactate levels were being observed, the time course would be manifested as a rapid square wave with a slower but small increase superimposed. This was clearly not the case in our experiments. Finally, in a few experiments, we followed the time course of pHi change by ^{31}P-NMR during lactate influx instead of monitoring lactate. We found that the initial decreases in pHi followed the same time course and concentration-dependence as the build-up of the lactate resonance in parallel ^{1}H-NMR experiments.

We believe our experimental methodology is entirely valid and constitutes a novel approach for the measurement of lactate transport in intact cells. In this study, unusually large single cells or bundles of 2–3 cells were utilized both to reduce the diffusive time lags associated with transport studies in tissues (Roth and Brooks, 1990*a*,*b*; Poole and Halestrap, 1993; McDermott and Bonen, 1994) and to simplify the spectral removal of the extracellular compartment. Both of these rationales imply a restriction of the technique used here to large cell samples or cell suspensions devoid of an interstitial compartment. The effect of a substantial interstitial compartment does, in fact, impose limits on kinetic studies of monocarboxylate transport, and for measuring the kinetic qualities of carriers, cell suspensions or membrane vesicles offer the best properties (for the advantages and disadvantages of these preparations, see the review by Poole and Halestrap, 1993).

However, when the interest lies more in measuring the transport process under conditions approximating those *in vivo*, rather than rigorously defining the kinetics of a carrier, multi-cell preparations are more appropriate and have been employed successfully for vertebrate skeletal muscles in many recent studies (Seo, 1984; Watt *et al.* 1988; Bonen and McCullagh, 1994; Gladden *et al.* 1994; McDermott and Bonen, 1994; McCullagh and Bonen, 1995). In fact, it has been shown in parallel studies of L-lactate transport kinetics among rat skeletal muscle sarcolemmal vesicles and skeletal muscle strips that, at least qualitatively, transport kinetic data are comparable between the two types of preparations (McDermott and Bonen, 1993, 1994). With respect to the potential limitation of our spectral editing method to preparations with minimal interstitial compartments, further work is needed to establish the general applicability of the diffusion-weighting approach to other systems.

A second area of potential concern is the impact of changing Cl^- concentrations on lactate influx rates since sodium lactate was substituted for an equivalent concentration of NaCl. We believe that a Cl^- effect is unlikely for two reasons. First, the superfusion medium contained 476 mmol l^{-1} Cl^-. In our influx experiments, Cl^- was replaced by lactate in concentration ranges from 10 to 100 mmol l^{-1}, which results in only modest reductions of Cl^- concentration of from 2 to 20 %, respectively (final concentrations ranged from 466 to 376 mmol l^{-1}). We do concede that such concentration changes may be important in vertebrate systems where prevailing Cl^- concentrations are of the order of 150 mmol l^{-1}. Second, in our efflux experiments, standard superfusion medium was used (476 mmol l^{-1} Cl^- in

wash-out) and the efflux and influx kinetics were qualitatively similar.

Passive diffusion is the predominant mode of lactate transport

The principal biological finding of this study was that *P. argus* abdominal extensor muscles do not appear to have a specific lactate transporter. V_i values for lactate influx were a linear function of total extracellular lactate concentrations (Fig. 7). It might be argued that our studies utilized an excessively high concentration range such that we were operating well above the K_m for such a transporter in a region where diffusive transport dominates. In studies of the monocarboxylate transporter in vertebrate muscle, K_m values, as determined in both vesicles and whole-muscle preparations, range from 4 to 40 mmol l^{-1} with the most common values between 10 and 20 mmol l^{-1} (Mason and Thomas, 1988; Watt *et al.* 1988; Roth and Brooks, 1990*a*,*b*; Juel *et al.* 1994; McDermott and Bonen, 1994). Our concentrations bracket this range. Furthermore, if there was significant transporter activity, the highly significant regression in Fig. 7 should have passed considerably above the origin. In addition, the intracellular concentrations of lactate for efflux experiments (Fig. 8) were generally lower than 16 mmol l^{-1}, with several values below 5 mmol l^{-1}. In this concentration range, the efflux V_i values were also a linear function of total lactate concentration, although we do concede that the scatter is high.

In addition to the observation of linearity of V_i with total lactate concentration, we found that rates of D- and L-lactate influx and efflux were also identical (Table 1). For systems with classic monocarboxylate transporters, the rate of transport of D-lactate is often used as an index of the purely passive diffusion of the free acid across the membrane (McDermott and Bonen, 1993, 1994). CIN, SITS and PCMB had no effect on the V_i values for L-lactate influx and efflux in our studies. Collectively, these data strongly support the proposal that *P. argus* muscle fibers lack a monocarboxylate transporter similar to that present in vertebrate muscles. The rates of passive transport reported here (Table 1) are also somewhat lower than those found in other studies of passive lactate transport, probably because of the decreased surface area to volume ratio in such large cells. The diffusional rate of lactate transport into rat sarcolemmal vesicles, which was based on the non-saturatable D-lactate transport rate, was 2.4 nmol mg^{-1} wet mass 45 s^{-1} for an extracellular lactate concentration of 20 mmol l^{-1} and at a pH of 7.4 (25 °C) (recalculated value from the data of Roth and Brooks, 1990*a*). Walsh (1987) found that the rate of passive diffusion of 20 mmol l^{-1} L-lactate into toadfish hepatocytes was 3.2 nmol mg^{-1} wet mass 45 s^{-1} at 25 °C (pH 7.0). In the present study, at a lactate concentration of 20 mmol l^{-1}, the influx rate for *P. argus* fibers was about 0.7 nmol mg^{-1} wet mass 45 s^{-1} (20 °C, pH 6.5).

The enhancement of uptake rates at reduced pHe is expected for both diffusive and monocarboxylate carrier-mediated transport (Poole and Halestrap, 1993). The failure of the lactate influx rates to decrease to near-zero at low levels of the free acid (Fig. 9), however, could indicate that an additional mode of transport involving dissociated lactate may be a minor contributor to total transport. An imperfect correlation of lactate free acid concentration with diffusional influx rate has been found by Mason and Thomas (1988) in frog sartorius muscle, where the uptake rate was only enhanced by 70 % of the amount expected on the basis of the increase in the lactate free acid concentration. The presence of a slow anionic pathway has also been suggested for frog sarcolemmal membranes (Woodbury and Miles, 1973) and rat diaphragm (Roos, 1975). Nagesser *et al.* (1994) found that lactate efflux in *Xenopus laevis* muscle fibers was predominantly *via* a CIN-sensitive lactate/bicarbonate exchanger (although a H^+:lactate cotransporter was also present).

If an anion exchanger such as this were at work here, transport would be expected to be saturatable and inhibited by the SITS or CIN treatments (or both). Unlike the present study, all of these results were obtained for systems having a carrier-mediated transport mechanism, which accounted for the bulk of lactate movement, and a small diffusive component. However, Walsh (1987) found, for toadfish hepatocytes, that the only mode of lactate influx was *via* passive diffusion and there was no effect of pHe on influx rates. This apparently confounding result can be explained only by diffusive transport of the lactate anion, which has been suggested to occur at slow rates (Roos, 1975; Nagesser *et al.* 1994). Recently, it has been shown that the plasma membranes of the muscle of the nematode *Ascaris suum* contain a Cl^- channel which conducts di- and monocarboxylic acids down a concentration gradient (Valkanov and Martin, 1995).

Physiological significance of diffusive transport of lactate in lobster muscle

Our results suggest that *P. argus* abdominal extensor fibers lack a monocarboxylate transporter. Membrane transport is primarily by passive diffusion, and overall rates are rather slow even in the presence of steep imposed lactate gradients. These results are consistent with previous observations of the protracted time course of lactate clearance from decapod crustacean muscles after burst contractile activity (Milligan *et al.* 1989; Henry *et al.* 1994). Milligan *et al.* (1989) found that, following anaerobic exercise in the blue crab *Callinectes sapidus*, pHi recovered well before lactate concentrations returned to resting levels, which also suggests that ionic mechanisms are the predominant means of pHi regulation under these conditions. Pörtner (1993) suggested that transport of lactate (either by passive diffusion of the free acid or by the monocarboxylate transporter) may serve to partially regulate pHi, but only during periods of low metabolic rate, when ionic pHi regulatory mechanisms are less active, and when organic acid levels are high, such as occurs in marine invertebrates during prolonged anoxia. Thus, the purely diffusive transport in *P. argus* fibers may be sufficient to allow for pH-dependent partitioning of lactate only when rates of production are low.

Considering that crustaceans generate prodigious quantities of

lactate during strenuous exercise in the laboratory, it is curious that no means of facilitating the transport of lactate is present in *P. argus* muscle fibers. In vertebrates, the role of the monocarboxylate carrier is often considered as a mechanism to facilitate the movement of lactate from sites of glycolytic (and glycogenolytic) production, such as skeletal muscle, to sites of gluconeogenesis, such as the liver and kidney (Denton and Halestrap, 1979; Brooks, 1991; Poole and Halestrap, 1993) and/or to sites of oxidative metabolism such as the brain/heart. Furthermore, transport is critical to the 'lactate shuttle' hypothesis, which views lactate as an intermediate that serves as an important source of oxidizable and gluconeogenic substrate between muscle fiber types having different metabolic potentials (Brooks, 1985; Roth and Brooks, 1990*a*). Skeletal muscle from fish represents an interesting contrast to the muscle of most higher vertebrates in that lactate is retained and metabolized in the muscle cells (reviewed in Wang *et al.* 1994*a*). There has even been speculation of active retention of lactate in fish muscle *via* an inward-directed lactate pump. This idea is based on the observation that intracellular lactate is maintained following exercise at levels much higher than predicted by the membrane potential or the pH gradient (Wang *et al.* 1994*a*). This retention of lactate occurs despite a concentration-dependent increase in the rate of passive efflux with exhaustive exercise (Milligan and McDonald, 1988). An alternative explanation to a specific inward pump for lactate in fish muscle is that diffusive rates are simply too slow to reach rapid equilibrium with the membrane potential or the pH gradient.

The physiological context in decapod crustaceans is rather different from that in higher vertebrates, but in some ways similar to that in fish. Phillips *et al.* (1977) found in the lobster (*Homarus gammarus*) and the freshwater yabby (*Cherax destructor*) that there was no evidence of gluconeogenesis occurring in the hepatopancreas, the organ typically thought to have a liver function (as shown by its name). Gäde *et al.* (1986) observed that lactate-based glyconeogenesis occurred in both muscle and hepatopancreas in the stone crab *Menippe mercenaria*. Milligan *et al.* (1989) suggested that *C. sapidus* metabolized the bulk of the lactate produced during exercise in the muscle tissue *in situ*. Subsequent studies by Henry *et al.* (1994) found only low levels of gluconeogenic enzymes in the hepatopancreas of three crustacean species and further noted that the bulk of conversion of radiolabelled lactate to glycogen occurred in the muscle. The apparent absence of monocarboxylate transporters in decapod crustaceans is probably related to this lack of organ-specific compartmentation of lactate metabolism.

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