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LACTATE UPTAKE BY TOADFISH HEPATOCYTES: PASSIVE DIFFUSION IS SUFFICIENT

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SUMMARY

Mechanisms of hepatic lactate uptake were studied in the gulf toadfish, *Opsanus beta* by following the accumulation of [^{14}C]lactate by isolated hepatocytes *in vitro*. Uptake rates were linear for up to 5 s at external lactate concentrations of 1 and 20 mmol l^{-1}. 5-s uptake rates for several concentrations in the physiological range (i.e. 0·2–20 mmol l^{-1}) yielded a linear double reciprocal plot (1/v *vs* 1/[lactate]) that passed through the origin. Lactate uptake was not inhibited by known inhibitors of carrier-mediated lactate transport (e.g. 5 mmol l^{-1} α-cyano-4-hydroxycinnamate; 10 mmol l^{-1} pyruvate), and was not stereospecific for L-(+)-lactate. These observations indicate that lactate uptake is by passive diffusion in toadfish hepatocytes. Lactate uptake by toadfish hepatocytes further differed from lactate uptake by mammalian tissues in that rates were not altered by changes in either pH_e (6·5–8·0) or $[Na^+]_e$ (3–151 mmol l^{-1}). Rates of lactate conversion to glucose and CO_2 were measured and compared to uptake rates, and it appears that rates of lactate metabolism are not limited by passive diffusion. These results are discussed in relation to rates of lactate uptake and metabolism in mammalian hepatocytes.

INTRODUCTION

Mechanisms of lactate transport across the plasma membrane have been reasonably well studied in several mammalian tissues and the general conclusion is that lactate is transported electroneutrally by carrier-facilitated diffusion. In renal brush-border, uptake is by lactate$^-$/Na^+ symport (Wright, 1985), whereas lactate$^-$/H^+ symport appears to be the transport mechanism in human erythrocytes (Halestrap, 1976; Dubinsky & Racker, 1978), Ehrlich ascites cells (Johnson *et al.* 1980; Spencer & Lehninger, 1976) and rat hepatocytes (Fafournoux, Demigne & Remesy, 1985). In human red cells, lactate uptake involves simultaneous release of chloride by the chloride/bicarbonate exchanger (Halestrap, 1976).

Lactate transport in tissues of ectothermic vertebrates has received considerably less attention. The present studies of lactate uptake by fish hepatocytes were undertaken for several reasons. First, preliminary studies of fish red blood cells indicate that lactate uptake is by passive diffusion, not by carrier-mediated processes

Key words: lactate transport, passive diffusion, hepatocytes, *Opsanus beta*.

(Moon, 1986). Uptake of lactate by liver, a tissue type which is known to metabolize it actively has not been examined in fish. Second, although the exact quantitative role of the liver in post-exercise lactate processing is still under study and debate, it is clear that blood lactate concentrations increase following exhaustive exercise in fish (Δ[lactate$^-$] = 2–20 mmol l^{-1} depending upon the species; Wood & Perry, 1985). If, in fish, the liver is to play a role in the post-exercise clearance of lactate from the blood (i.e. the Cori cycle), it must have sufficient uptake capacity.

A third reason for studying lactate transport in fish hepatocytes is to begin to determine the potential effects this process may have on intracellular pH regulation. Preliminary studies indicate that in fish recovering from exercise, liver pH_i *increases*, dramatically in some cases (up to 0·5 units over 12–24 h of recovery, Milligan & Wood, 1986, 1987). To begin to evaluate one possible cause of this increase, the acid–base characteristics of lactate transport into fish hepatocytes should be examined.

Lastly, in some models of the control of fluxes through metabolic pathways, the supply of substrate for the pathway, rather than a specific step within the pathway, is a potentially rate-limiting step (Kilberg, 1986; Newsholme & Paul, 1983). In the case of hepatic lactate utilization, the potential exists for the uptake step to be rate-limiting. Thus, for example, the effects of environmental temperature on rates of lactate utilization by fish hepatocytes (Walsh, Moon & Mommsen, 1985) may reflect changes in rates of lactate uptake rather than changes in pathway activity *per se*. Adaptation of methods of measurement of the rates and characteristics of lactate transport to fish hepatocytes will allow an evaluation of this possibility.

In this paper I report that in toadfish hepatocytes lactate uptake is by passive diffusion. Furthermore, although rates of uptake by this process are slow relative to rates in mammalian hepatocytes, passive diffusion does not appear to be rate-limiting for lactate oxidation and gluconeogenesis in fish hepatocytes.

MATERIALS AND METHODS

Chemicals

Radioactive chemicals were purchased from Amersham, Arlington Heights, IL (L-[U-^{14}C]lactic acid, 150 mCi $mmol^{-1}$; D-[U-^{14}C]lactic acid, 40 mCi $mmol^{-1}$) or New England Nuclear, Boston, MA ([1,2-^{14}C]polyethyleneglycol, PEG, 10·5 mCi g^{-1}). 3-Mercaptopicolinic acid (3-MPA) was generously donated by Smith, Kline & French, Philadelphia, PA. Other chemicals were purchased from Sigma Chemical, St Louis, MO, except where noted.

Experimental organism, isolation of hepatocytes and viability criteria

Specimens of the gulf toadfish, *Opsanus beta*, were obtained during January, February and March 1986 from South Biscayne Bay, FL by local shrimp trawlers. Upon return to the laboratory, fish were placed in running baywater aquaria (1000 l) and held for no longer than 1 month prior to use. Ambient temperature ranged between 17·5 and 25·0°C and salinity was approximately 29‰.

Hepatocytes were isolated by collagenase perfusion, as previously described for rainbow trout (Walsh, 1986), with slight modifications. Toadfish were anaesthetized with $0{\cdot}5\,g\,l^{-1}$ tricaine methanesulphonate (MS-222), the hepatic portal vein was cannulated and the liver was perfused with Ca^{2+}-free, Hanks' salts medium (containing in $mmol\,l^{-1}$: NaCl, 151; KCl, 5·9; $MgSO_4$, 0·88; Na_2HPO_4, 0·46; KH_2PO_4, 0·48; $NaHCO_3$, 5; Hepes, 11; pH 7·45; heparin $1{\cdot}5\,units\,ml^{-1}$) at room temperature for approximately 5 min (flow rate = $1{\cdot}8\,ml\,min^{-1}$) to clear blood from the liver. (Concentrations of all media are 10% higher than those used for rainbow trout to match slightly higher blood osmolarities in toadfish; P. J. Walsh, unpublished data.) Collagenase (Sigma C5138, $0{\cdot}3\,mg\,ml^{-1}$) and hyaluronidase (Sigma H3506, $0{\cdot}4\,mg\,ml^{-1}$) were then added and perfusion was continued for 25 min. The liver was then removed from the fish, partially chopped with a razor blade and filtered through nylon mesh (253 and 73 μm). Cells were harvested by centrifugation at 100 ***g*** for 2 min at room temperature. They were then resuspended in the Hanks' salts medium, recentrifuged, resuspended in normal suspension medium [Hanks' salts medium to which had been added $1\,mmol\,l^{-1}$ $CaCl_2$, 2% bovine serum albumin (Fraction V, Sigma A4503), and $3\,mmol\,l^{-1}$ glucose, pH 7·70] and centrifuged once again. The washing and centrifugation were repeated once more. All media were equilibrated with 0·26% CO_2 in air.

Viability was determined by two means. Cells were examined microscopically for trypan blue exclusion (0·2% solution in Hanks' salts medium). ATP content was determined by Sigma Assay Kit (366-UV) on cell suspensions (1 ml, approx. 25 mg cells) acidified with 1 ml of $0{\cdot}73\,mol\,l^{-1}$ trichloroacetic acid. After centrifugation at 3000 ***g*** for 5 min, 0·5 ml of supernatant was combined with 2·5 ml of assay mixture containing NADH (0·3 mg) and $18\,mmol\,l^{-1}$ 3-phosphoglyceric acid. Glyceraldehyde 3-phosphate dehydrogenase (32 units) and 3-phosphoglycerate phosphokinase (18 units) were added and the change in absorbance was followed for 10 min at 340 nm in an LKB Ultrospec. Preparations with more than 5% of cells staining with trypan blue or with ATP contents lower than *in vivo* values (see Results) were discarded.

Determination of lactate uptake rates

Initial rates of lactate uptake were measured by a modification of the methods used for rat hepatocytes (Fafournoux *et al.* 1985). Shortly after isolation, cells were centrifuged at 100 ***g*** and resuspended in normal suspension medium to which had been added $1\,mmol\,l^{-1}$ each of 3-mercaptopicolinic acid (3-MPA) and aminooxyacetic acid (AOA) to inhibit lactate metabolism (Fafournoux *et al.* 1985; Mommsen & Suarez, 1984; Renaud & Moon, 1980). Cells were allowed to stand for 5–10 min before centrifugation and two washes in this medium to remove any traces of lactate from glycogenolysis. Cells were then immediately used in transport measurements.

In a typical experiment, a microcentrifuge tube (1·5 ml), containing 0·8 ml of suspension medium with $1\,mmol\,l^{-1}$ each of 3-MPA and AOA, unlabelled and labelled lactate ($1{\cdot}0$–$20\,mmol\,l^{-1}$ and approx. $0{\cdot}2\,\mu Ci\,ml^{-1}$) layered onto 0·4 ml of

1-bromododecane, layered onto 0·1 ml of 1·2 mol l^{-1} perchloric acid, was equilibrated to 25°C in a water bath and then positioned in a Fisher 235B microcentrifuge. At time zero, 0·2 ml of cell suspension (containing about 25 mg of cells) was injected into the suspension medium layer with sufficient force to cause mixing, but without disturbing the layering. At 5 s after injection (unless indicated) cells were rapidly (<0·5 s) separated from the medium into the perchloric acid layer by centrifugation at 13 000 ***g***. The density of the oil layer is such that only viable cells pass through it; leaky cells and debris are left at the oil/medium interface. The supernatant layer and part of the oil layer were removed by aspiration. The bottom 0·7 cm of the tube was cut off with a razor blade, placed in a scintillation vial with 5 ml Aqualyte (Baker), and counted for ^{14}C in a TM Analytic liquid scintillation counter using an automatic external standard. A 100-μl sample of the supernatant was counted in 5 ml of Aqualyte. Cell wet mass was determined for each batch by centrifuging cells through 1-bromododecane in pre-weighed tubes, removing the suspension and bromododecane by aspiration, and reweighing.

The d.p.m. in the pellet were corrected for extracellular water content (10 % by determination with [^{14}C]PEG), and lactate uptake (nmol mg wet mass cells^{-1}) was calculated by dividing the extracellular water-corrected d.p.m. mg cell wet mass^{-1} by 3 (to correct for the uniform labelling of lactate) and then by dividing by the specific activity (d.p.m. nmol lactate^{-1}).

Determination of rates of lactate metabolism

Rates of lactate metabolism were determined by previously described methods (French, Mommsen & Hochachka, 1981; Walsh *et al.* 1985). Cell suspension medium (0·9 ml) with about 25 mg of cells, was placed in a flask with a centre well (with Whatman GF/C paper, 2·4 cm) for a CO_2 trap. The reaction was initiated by injecting 0·1 ml of suspension, containing 0·2–0·3 μCi of labelled lactate and unlabelled lactate to yield a final concentration of 1 or 10 mmol l^{-1}, through a rubber septum. After either 10 s or 60 min the reaction was terminated by addition of 0·1 ml of 70 % perchloric acid. Hyamine hydroxide (0·2 ml) was injected into the CO_2 trap, and after shaking for 1·5 h, CO_2 traps were counted in 10 ml of Econofluor (New England Nuclear) and glucose was extracted from the medium with Amberlite MB3 resin (Walsh *et al.* 1985) and counted in Aqualyte. Chemical controls (as above without cells) were performed and subtracted from d.p.m. obtained for flasks with cells. CO_2 and glucose production rates in μmol g^{-1} time^{-1} were calculated according to Walsh *et al.* (1985).

Lactate determination

Lactate contents were determined in extracts prepared for 25 mg of cells fixed in 200 μl of 1·2 mol l^{-1} perchloric acid and neutralized with 20 μl of 4 mol l^{-1} KOH. 100 μl of this extract was added to 1·9 ml of hydrazine/glycine buffer (0·5 mol l^{-1}/0·6 mol l^{-1}) pH 9·2 with 3·3 mmol l^{-1} NAD^+ and 10 mmol l^{-1} EDTA. The reaction was started by the addition of 50 units of lactate dehydrogenase, and absorbance at 340 nm was followed for 30 min.

Statistical analysis

Values are presented as means ± S.E. unless stated. Means were compared for significant differences using an unpaired Student's *t*-test. In experiments with multiple comparisons, an analysis of variance (ANOVA) was used (Zar, 1974).

RESULTS

Hepatocyte viability and methodological conditions

The isolation procedure consistently yielded viable hepatocytes as determined by trypan blue exclusion and by ATP contents, which remained near to *in vivo* values for at least 2 h (*in vivo* [ATP] = 2·632 ($N = 2$) μmol g wet mass^{-1}; *in vitro* [ATP] = 2·939 ± 0·284 at 0 min, 3·177 ± 0·277 at 60 min, 2·942 ± 0·281 μmol g^{-1} at 120 min, mean ± S.E., $N = 3$). Also, when rates of lactate metabolism were evaluated under long-term, steady-state conditions (Table 5), rates of CO_2 and glucose production were similar to those obtained for other species of fish (Moon, Walsh & Mommsen, 1985).

Lactate uptake rates were linear for approximately 5 s at 1 and 20 mmol l^{-1} lactate with some deviation at 20 mmol l^{-1} (Fig. 1). Lactate uptake rates at 5 s were routinely used in subsequent experiments. Lactate metabolism did not contribute

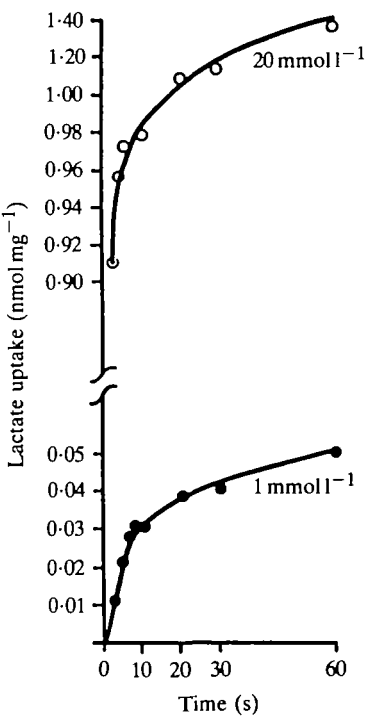

Fig. 1. Lactate uptake (nmol mg wet cell mass^{-1}) *versus* time (s) in toadfish hepatocytes at 1 (●) and 20 mmol l^{-1} (○) lactate.

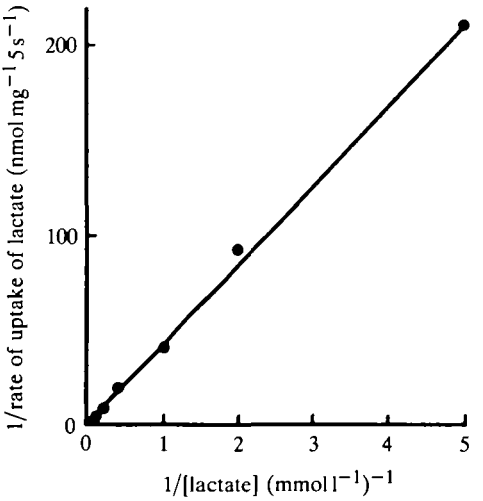

Fig. 2. Plot of 1/lactate uptake rate [1/(nmol mg wet cell mass^{-1} 5 s^{-1})] *versus* 1/[lactate]$_e$ (mmol l^{-1})$^{-1}$ in toadfish hepatocytes. Lactate concentration ranged from 0·2 to 20 mmol l^{-1}.

significantly to the disappearance of transported lactate in the short term, in that 3-MPA and AOA decreased metabolism to 6 % of normal levels (results not shown). Intracellular lactate concentration was not detectable in cells washed in normal suspension medium plus 1 mmol l^{-1} each of 3-MPA and AOA. These findings validate the method used to study initial rates of lactate transport into fish hepatocytes. After about 30 s, lactate uptake rates levelled off and then continued at this lower rate (Fig. 1). When uptake was monitored for slightly longer periods (30–180 s) at 10 mmol l^{-1}, rates were also linear (6 μmol g^{-1} h^{-1}, results not shown).

Characteristics of lactate transport

When lactate transport was measured at lactate concentrations spanning two orders of magnitude (within the physiological range for fish), typical saturation kinetics were not observed, and the double reciprocal plot passed through the origin (Fig. 2). Furthermore, several inhibitors of known lactate-transport pathways in other cell types had virtually no effect on rates of lactate transport in fish hepatocytes (Table 1). The specific inhibitor of lactate transport in mammalian tissues (α-cyano-4-hydroxycinnamate, CIN), specific inhibitors of general anion transport (4-acetamido-4′-isothiocyanostilbene-2,2′disulphonic acid, SITS and 4,4′-diisothiocyantostilbene-2,2′disulphonic acid, DIDS) and three potential competitive inhibitors (pyruvate, sulphate, D-lactate) had little effect on rates of lactate transport (Table 1). In addition, lactate uptake by toadfish hepatocytes does not depend on a Na^+ gradient (Table 2). The uptake of lactate by fish hepatocytes has no stereospecificity; D-lactate is transported at similar rates (Table 3). Lastly, variations in extracellular pH had no effect on rates of lactate uptake (Table 4).

DISCUSSION

Lactate transport in fish hepatocytes was studied by inhibition of lactate metabolism and measurement of the initial rates of accumulation of radiolabelled lactate (Fig. 1), as carried out in rat (Fafournoux *et al.* 1985). In addition, since similar uptake rates were obtained for the non-metabolized D-lactate (Table 3), it is feasible to utilize D-lactate in future transport studies with fish hepatocytes.

Table 1. *Effects of several potential inhibitors of lactate transport in toadfish hepatocytes*

[Inhibitor] (mmol l^{-1})	Percentage change
D-Lactate (10)	−6·7
Pyruvate (10)	+11·3
Na_2SO_4 (10)	−10·0
SITS (0·5)	−4·5
DIDS (0·5)	0·0*
CIN (5)	+9·3

Values are percentage change from control value (x ± S.E. = 0·010 ± 0·002 nmol mg wet mass cells^{-1} 5 s^{-1}) for triplicate determinations.

In all cases, except D-lactate, cells were incubated for 1 min with the inhibitor before uptake was initiated by addition of labelled and unlabelled L-lactate.

D-lactate rates were initiated by addition of cells to lactate-containing medium. [L-lactate] = 1 mmol l^{-1}.

* Single determination.

Table 2. *Effect of low external [Na^+] on lactate transport in toadfish hepatocytes*

Treatment	Lactate uptake rate (nmol mg cells^{-1} 5 s^{-1})
Normal suspension	0·862 ± 0·139
Low sodium suspension*	0·754 ± 0·119

Values are means ± S.E. (N = 4).

[L-lactate] = 10 mmol l^{-1}.

* NaCl was replaced with 150 mmol l^{-1} N-methyl-D-glucamine titrated with HCl, KCl was omitted, and remaining sodium salts were replaced by potassium salts. Medium contains less than 3 mmol l^{-1} Na^+ from bovine serum albumin (Walsh, 1986).

Values are not significantly different, t = 0·59.

Table 3. *Stereospecificity of lactate transport in toadfish hepatocytes*

Substrate	Lactate uptake rate (nmol mg cells^{-1} 5 s^{-1})
L-lactate*	0·367 ± 0·026
D-lactate*	0·313 ± 0·037
D-lactate†	0·382 ± 0·011

Values are mean ± S.E., N = 3; D- or L-lactate concentration = 10 mmol l^{-1}.

* Values are not significantly different, t = 1·195.

† Rate determined without 3-MPA or AOA.

Table 4. *Effect of variation in extracellular pH on lactate uptake in toadfish hepatocytes*

pH_e	Lactate uptake rate (nmol mg cells^{-1} 5 s^{-1})
6·5	0·326 ± 0·079 (5)
7·0	0·357 ± 0·062 (6)
7·5	0·292 ± 0·057 (6)
8·0	0·301 ± 0·077 (6)

Values are x ± S.E. (N).
[L-lactate] = 10 mmol l^{-1}.
There is no significant effect of pH_e (one-way ANOVA, $F = 0·83$).
Variations in pH were achieved by titration with fixed acid (HCl).
Suspension media were re-equilibrated with 0·26 % CO_2/balance air.

Lactate uptake by fish hepatocytes exhibits several differences from lactate uptake in mammalian tissues. Uptake kinetics are non-saturable (Fig. 2), uptake is not inhibited by several compounds known to inhibit the process in mammalian tissues (Table 1), and uptake is not stereospecific (Table 3). These findings indicate that lactate uptake by fish hepatocytes is by passive diffusion rather than by the carrier-facilitated diffusion pathway characteristic of rat hepatocytes (Fafournoux *et al.* 1985; Monson, Smith, Cohen & Iles, 1981) and other tissues in mammals (Halestrap, 1976; Dubinsky & Racker, 1978; Johnson *et al.* 1980; Spencer & Lehninger, 1976; Wright, 1985). In the study of rat hepatocytes by Fafournoux *et al.* (1985), their data also suggested that uptake is by lactate$^-$/H^+ symport. The lack of an effect of pH_e on lactate uptake noted for toadfish hepatocytes (Table 4) suggests that lactate$^-$/H^+ symport does not occur in this species. A second type of carrier-facilitated diffusion has been observed in renal membrane vesicles in which lactate uptake is by lactate$^-$/Na^+ symport (Wright, 1985). Lactate uptake by toadfish hepatocytes does not depend on a sodium gradient since lowering extracellular sodium concentration had no effect on uptake rates (Table 2). However, since there was some sodium present in the low-sodium medium, the presence of an uptake system that cotransports sodium without dependence on the gradient cannot be completely ruled out.

The finding of uptake of lactate by passive diffusion in toadfish hepatocytes is similar to the preliminary results of Moon (1986) for lactate uptake by fish red blood cells, and these two studies suggest that some transcellular transport processes in fish tissues may be quite different from those in their mammalian counterparts. Why should fish hepatocytes (and possibly other tissues) lack the more rapid lactate uptake rates afforded by carrier-facilitated diffusion? A possible answer emerges if rates of lactate transport and utilization are compared for fish and rat hepatocytes (Table 5). The rate of passive diffusion of lactate into fish hepatocytes is sufficient to support rates of metabolic utilization of lactate. (Note that the comparison of steady-state metabolic rates with initial rates of lactate transport is a valid one in that as transport rates began to be limiting, metabolism would decrease intracellular lactate concentrations to levels which would increase transport rates to these higher initial

Table 5. *Comparison of rates of initial uptake and metabolism of lactate in rat and toadfish hepatocytes*

Species	[Lactate] ($mmol\,l^{-1}$)	Rate of uptake	Rate of metabolism ($\mu mol\,g^{-1}\,h^{-1}$) To CO_2	To glucose	Total
Toadfish	1	7·2*	2·972	0·414	3·386
	10	216·0†	13·908	1·937	15·845
Rat	1	120‡	—	—	—
	10	720‡	70·2§	46·8§	117·0§

* Table 2.
† Table 4.
‡ Fafournoux, Demigne & Remesy (1985).
§ Cornell (1983).

rates.) In addition, in fish, uptake by passive diffusion would appear to allow for at least a 10-fold increase in metabolic rates (at $10\,mmol\,l^{-1}$, Table 5) during, for example, any potential increases in rates of gluconeogenesis from lactate or lactate oxidation following exercise. In contrast, lactate uptake rates characteristic of fish hepatocytes would be close to limiting for basal levels of lactate utilization by mammalian hepatocytes, and would certainly be limiting during any similar increases in pathway activities. Thus, it is possible that the higher rates of metabolism associated with endothermy (Schmidt-Nielsen, 1983) may have necessitated the evolution of more efficient substrate transport systems in mammals. Further studies on the mechanisms of uptake of lactate and other substrates by other tissues in different ectothermic species are called for to evaluate this hypothesis more completely.

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