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INTEGRATING METABOLIC PATHWAYS IN POST-EXERCISE RECOVERY OF WHITE MUSCLE

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Summary

Purine nucleotides (ATP, ADP, AMP, IMP), creatine, phosphocreatine, lactate, pyruvate and glycogen were measured in rainbow trout (*Oncorhynchus mykiss*) white muscle following exercise to exhaustion. Estimates of intracellular pH permitted calculation of free concentrations of nucleotides ($[\text{nucleotide}]_f$) required for most models of control of energy metabolism. Creatine charge, $[PCr]/([PCr]+[Cr])$, fell from 0.49±0.05 (mean±S.E.M.) to 0.08±0.02 with exercise but recovered completely by the first sample (2 h). Although [ATP] declined to 24 % of resting levels and recovered very slowly, R_{ATP}, $[ATP]/([ATP]+[ADP]_f+[AMP]_f)$, and energy charge, EC, $([ATP]+0.5[ADP]_f)/([ATP]+[ADP]_f+[AMP]_f)$, recovered as quickly as creatine charge. Changes in [IMP] mirrored those in [ATP], suggesting that AMP deaminase is responsible for maintaining R_{ATP} and EC. Recovery of carbon status was much slower than recovery of energy status. Lactate increased from 4 μmol g^{-1} at rest to 40 μmol g^{-1} at exhaustion and did not recover for more than 8 h. Glycogen depletion and resynthesis followed a similar time course. During the early stages of recovery, calculated $[ADP]_f$ declined by more than 10-fold relative to the resting values. The resulting high $[ATP]/[ADP]_f$ ratios may limit the rate at which white muscle mitochondria can produce ATP to fuel glycogenesis *in situ*. It is postulated that the high $[ATP]/[ADP]_f$ ratios are required to drive pyruvate kinase in the reverse direction for glyconeogenesis in recovery.

Introduction

There have been many studies addressing the metabolic changes occurring in fish white muscle during recovery from high-intensity exercise (Milligan and Wood, 1986*a*,*b*; Milligan and McDonald, 1988; Dobson and Hochachka, 1987;

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Mommsen and Hochachka, 1988; Pearson *et al.* 1990) but no single study has determined all of the variables required to understand the control of post-exercise energy metabolism. While measurement of total (i.e. free+bound) tissue levels of adenylates and phosphocreatine (PCr) can give some insight into the energy status of the tissue, the appropriate metabolite ratios and calculated free concentrations are much more informative.

During intense exercise, trout white muscle quantitatively converts as much as 90 % of its ATP pool to IMP (Mommsen and Hochachka, 1988). Because of the high activity of the purine nucleotide cycle enzyme AMP deaminase, changes in [ATP] do not adequately reflect adenylate status in white muscle. Although total tissue concentrations of adenylates have been determined (Dobson and Hochachka, 1987; Mommsen and Hochachka, 1987), large proportions of the total pools of ADP and AMP are bound. Only the free proportion of the adenylates interact with glycolytic and mitochondrial pathways. Since PCr and creatine (Cr) are assumed to be unbound, the free adenylate concentrations can be calculated by rearranging the following equilibrium expressions (Connett, 1988):

$$Cr + MgATP^{2-} \rightleftharpoons MgADP^{-} + PCr^{2-} + H^{+} \quad \text{(creatine phosphokinase)}, \quad (1)$$

$$AMP^{2-} + MgATP^{2-} \rightleftharpoons ADP^{3-} + MgADP^{-} \quad \text{(adenylate kinase)}. \quad (2)$$

As these equilibria are pH-dependent, intracellular pH must be determined to estimate free adenylate levels and interactions of the adenylates with magnesium ions must also be taken into account. The free concentrations of these metabolites cannot be calculated if PCr and Cr levels are not measured in parallel.

This study addresses changes in energy metabolism following high-intensity exercise in rainbow trout using analysis of pH (intracellular, arterial) and the substrates of carbon (lactate, pyruvate, glycogen) and energy (Cr, PCr, ATP, ADP, AMP, IMP) metabolism. Our goal was to obtain a more comprehensive appraisal of the regulation of recovery metabolism than is presently available, through the use of appropriate metabolite ratios and calculation of free metabolite concentrations.

Materials and methods

Animals

Rainbow trout [*Oncorhynchus mykiss* (Walbaum)] of both sexes (mean mass 560±89 g; S.E.M.; N=75), obtained from West Creek Trout Ponds, Aldergrove, BC, were held outdoors in a circular tank supplied with flow-through dechlorinated tap water at 8–12 °C. Fish were fed daily by hand to satiation (Ocor Pacific Salmon Feed; Moore-Clarke).

Surgical procedure

Trout (starved for one day) were anaesthetized in a buffered ($NaHCO_3$, 2 g l^{-1}) MS-222 solution at a concentration of 1:6000. Cannulations were performed

during forced ventilation with a buffered, chilled (15°C), aerated (100% oxygen) MS-222 solution (1:16000). Fish were cannulated in the dorsal aorta (Soivio *et al.* 1972) and allowed to recover for 48 h in an opaque black acrylic box (5 cm × 40 cm × 20 cm) at 8–12°C.

Exercise protocol

Fish were transferred by net to a Brett-type swim tunnel. After 15–20 min at 0.25 body lengths s^{-1}, flow was increased to the maximum the fish could attain. The speed was held constant until the fish could no longer maintain this velocity (about 3 min), at which time the speed was decreased by about 30%. This speed was held for about 5 min and then gradually increased and the cycle begun again. The flow rate was continually oscillated in this way, as the maximum attainable speed gradually decreased, until the fish could no longer maintain its position in the swim tunnel even at the slowest speeds. At this point, the fish did not respond to being grasped by the investigator. The entire exercise procedure took approximately 25–30 min to complete.

Fish were either killed immediately following exercise (at exhaustion) or transferred back to the holding box. Fish were then randomly assigned to a treatment group and killed after 2, 4, 8 or 24 h of recovery, as required. Resting (unexercised) fish were kept in the holding box for at least 48 h prior to sampling.

At the specified sampling time, 2 ml of blood was withdrawn *via* a dorsal aortic cannula and placed on ice. Fish were then killed by a 2 ml injection of Somnitol (65 mg ml^{-1} sodium pentobarbitol) to minimize struggling. Once ventilation ceased, a 1 cm thick cross-sectional slice was taken immediately posterior to the dorsal fin and freeze-clamped in aluminium tongs pre-cooled in liquid nitrogen.

Muscle homogenization, extraction and neutralization

A section of epaxial white muscle was ground to a fine powder in a mortar cooled in liquid nitrogen. Care was taken to remove any pieces of skin and bone prior to grinding. The sample was kept submerged in liquid nitrogen throughout. The sample (approximately 1 g) was then transferred to a pre-weighed chilled test tube containing 1 ml of ice-cold 7% perchloric acid (PCA) and re-weighed immediately any remaining liquid nitrogen had boiled off. 3 ml of cold PCA was added and the mixture was homogenized using an Ultra turrax tissue grinder. Throughout homogenization the tube was held in a slurry of salt water and ice at −5 to −10°C. Two samples (200 μl) of the homogenate were removed and stored at −80°C for later determination of glycogen. The remainder was centrifuged at 12000 ***g*** for 9 min (4°C). A sample of the supernatant was removed and neutralized with Tris and KOH and centrifuged at 12000 ***g*** for 3 min at 4°C. The supernatant pH was checked and adjusted with 10 mol l^{-1} KOH or HCl as necessary. The neutralized extract was stored at −80°C until needed. The extraction efficiency of this method for the labile high-energy phosphates was assessed, and both ATP and PCr were shown to undergo less than 5% hydrolysis.

Biochemical analyses

Chromatography

High performance liquid chromatography (HPLC) was used to measure ATP, ADP, AMP and IMP, each clearly separated from other purine and pyrimidine nucleotide phosphates. The procedure was carried out using an LKB 2152 HPLC controller and 2150 titanium pump coupled to a 2220 recording integrator. The separation was performed on an Aquapore AX-300 7 μm weak anion exchanger (Brownlee laboratories) eluting at 2 ml min^{-1} at 55 °C (Parkhouse *et al.* 1987, with modifications). Elution was isocratic for the first 5 min, using 60 mmol l^{-1} KH_2PO_4 (pH 3.2) followed by a linear gradient from 60 mmol l^{-1} KH_2PO_4 (pH 3.2) to 750 mmol l^{-1} KH_2PO_4 (pH 3.5) over 10 min. This concentration and pH were then maintained for 12 min. The column was re-equilibrated for 6 min with starting buffer before the next run. Adenylates were detected at 254 nm using a Bio-Rad flow-through ultraviolet monitor. Standard curves were constructed for all metabolites over the relevant concentrations by preparing a mixed solution in 60 mmol l^{-1} KH_2PO_4. The standard curves were linear throughout the range required (r^2=0.99). The coefficient of variation between duplicates was always less than 5 %.

Analytical reagent grade KH_2PO_4 was pre-purified (Reiss *et al.* 1984, with modifications) by passing a 1 mol l^{-1} stock solution through a Bio-Rad Econo column, 5 cm diameter×30 cm) packed with an anion exchange resin (AG1 X8, chloride form), a cation exchanger (chelex 100, sodium form) and activated charcoal (14–60 mesh). The solution was kept at 4 °C and constantly recirculated through the column by a peristaltic pump. Immediately prior to use, the stock buffer was diluted, brought to the appropriate pH and vacuum filtered (0.22 μm). With this technique, baseline disturbances caused by the impurities present in the high-concentration buffer were reduced by 74 %.

Spectrophotometry

All determinations were performed on a Perkin–Elmer Lambda 2 ultraviolet/visible spectrophotometer. Lactate, pyruvate, PCr and glucose were measured using routine NAD^+/NADH-linked assays, according to Bergmeyer (1974). If the coefficient of variation between duplicate determinations was greater than 5 %, a third run was performed and the outlier discarded. All assays were validated with appropriate standards.

Intracellular pH

Mean intracellular pH (pHi) was determined using 5,5 dimethyl oxazolidine 2,4 dione (DMO) distribution (Milligan and Wood, 1986*b*) and a tissue homogenate technique (Pörtner *et al.* 1991).

DMO distribution

Approximately 12 h prior to sampling, trout were injected through the dorsal

aortic cannula with 10 μCi of [^{3}H]mannitol and 2.5 μCi of [^{14}C]DMO (New England Nuclear) in Cortland's fish physiological saline (total volume 500 μl). The pH of the arterial blood sample collected just prior to Somnotol injection was measured within 4 min using a Radiometer microelectrode (type E5021) maintained at 10 °C using a recirculating water bath and linked to a Radiometer 26 pH meter.

White muscle levels of ^{3}H and ^{14}C radioactivity were measured in 1 ml of the PCA extract used for metabolite assays using Amersham ACSII aqueous fluor. All samples were counted on an LKB 1214 Rackbeta liquid scintillation counter using dual-label quench correction. Total tissue water was determined by drying a 1–2 g sample to constant mass in an oven at 75 °C. The pK for DMO was taken from Malan *et al.* (1976).

Measurement of homogenate pH

Approximately 200 mg of muscle was ground under liquid nitrogen and added to a 1.5 ml Eppendorf tube. The tube was then quickly filled with a solution of 160 mmol l^{-1} KF and 1 mmol l^{-1} disodium nitrilotriacetate, stirred briefly with a needle and capped. The insoluble fraction was spun down (3–5 s in a microcentrifuge at room temperature). Samples of the supernatant were taken for repeated measurement of pH as with the blood samples. Variation in pH between replicates from the supernatant was usually less than ±0.005 pH units and from the same muscle powder less than ±0.02 pH units.

Free adenylates

Free cytoslic concentrations of ADP and AMP were calculated while taking into account the dependence of the equilibrium conditions on pH and $[Mg^{2+}]$ (Connett, 1988, 1990). We assessed three variables that reflect the energy state of the cell: Cr charge=[PCr]/([Cr]+[PCr]), R_{ATP}=[ATP]/([ATP]+[ADP]$_f$+[AMP]$_f$); and EC=([ATP]+0.5[ADP])/([ATP]+[ADP]+[AMP]). The values of equilibrium constants (K_{eq}) selected (Table 1) were corrected to conditions of ionic strength equivalent to that *in vivo* (μ=0.17–0.2) and wherever possible to a temperature of 10 °C using the Van't Hoff equation. Implicit in these calculations is the assumption that all species involved are homogeneously distributed throughout the cell.

Total magnesium was calculated for values of $[Mg^{2+}]_f$ between 0 and 20 mmol l^{-1} using a binding site approach (Connett, 1985). Known values for total magnesium concentration of rainbow trout white muscle (approximately 19 mmol l^{-1} both at rest and at exhaustion; Parkhouse *et al.* 1987) were then used to select the most likely value for the actual intracellular $[Mg^{2+}]_f$. Since this approach yields only an estimate of $[Mg^{2+}]_f$, the effects of reasonable variations in $[Mg^{2+}]_f$ on the model calculations were assessed.

Statistical analyses

All data are reported as means±S.E.M. Metabolite differences between rest

Table 1. *Equilibrium constants*

	Constant	ΔH (kcal mol^{-1})	Reference
K_{ATP}^{Mg}	26.69 (mmol l^{-1})$^{-1}$	3.3	1
K_{ATP}^{H}	1.13×10^{7} (mol l^{-1})$^{-1}$	0.087	1, 2
K_{ATPH}^{Mg}	550.69 (mol l^{-1})$^{-1}$	1.5	1, 3
K_{ATP}^{K}	5.84 (mol l^{-1})$^{-1}$	6.0	4
K_{ADP}^{Mg}	1534 (mol l^{-1})$^{-1}$	3.3	1
K_{ADP}^{H}	6.10×10^{6} (mol l^{-1})$^{-1}$	0.522	1, 3
K_{ADPH}^{Mg}	83.99 (mol l^{-1})$^{-1}$	1.0	1
K_{ADP}^{K}	2.92 (mol l^{-1})$^{-1}$	6.0	4
K_{PCr}^{Mg}	20 (mol l^{-1})$^{-1}$	*	2
K_{PCr}^{H}	3.31×10^{4} (mol l^{-1})$^{-1}$	*	2
$K_{P_i}^{Mg}$	46.05 (mol l^{-1})$^{-1}$	5.59	1, 2
$K_{P_i}^{H}$	8.91×10^{6} (mol l^{-1})$^{-1}$	−11.59	5
$K_{P_i}^{K}$	1.76 (mol l^{-1})$^{-1}$	6.14	4
K_{AMP}^{Mg}	36.66 (mol l^{-1})$^{-1}$	3.24	1, 2
K_{AMP}^{H}	0.99 (mol l^{-1})$^{-1}$	6.0	5
K_{CPK}	4.86×10^{9} (mol l^{-1})$^{-1}$	−2.4	6, 7
K_{AdK}	8.1	†	6

All values are corrected to ionic strength μ=0.17–0.2.

Values were corrected to 10°C except where noted (* at 25°C, † at 38°C). References: 1, Phillips *et al.* (1966); 2, Smith and Alberty (1956*a*); 3, O'Sullivan and Perin (1964); 4, Smith and Alberty (1956*b*); 5, Phillips *et al.* (1965); 6, Lawson and Veech (1979); 7, Eldar and Degani (1989).

and/or the various recovery times were assessed using a one-way analysis of variance (ANOVA) followed by Tukey's HSD on those variables that showed a significant F ratio. The data were log transformed where necessary.

In the case of pH, all statistical analyses were performed using calculated $[H^+]$. The two techniques for estimating intracellular pH were compared using non-parametric tests (sign test, signed-rank test and Wilcoxon signed-rank test) because the DMO distribution technique produces pH estimates with greater variance than the homogenate technique.

Results

Arterial and intracellular pH

The homogenate technique gave estimates of pHi which were not statistically distinguishable from the estimates obtained using the DMO distribution technique (Table 2). Since the homogenate technique appeared to yield greater precision, all subsequent calculations were based on estimates of pHi derived from the homogenate technique.

Table 2. *Arterial pH (pHa) and intracellular pH (pHi) estimated by DMO and homogenate methods*

Time	pHa	pHi (homogenate)	pHi (DMO)	*N*
Rest	7.77±0.01	7.27±0.03	7.14±0.12	5
Exhaustion	7.20±0.07*	6.64±0.03*	6.78±0.11*	7
2 h	7.36±0.07*	6.59±0.03*	6.52±0.13*	5
4 h	7.31±0.07*	6.58±0.02*	6.49±0.24*	5
8 h	7.63±0.17	6.89±0.11*	7.01±0.19	4
24 h	7.77±0.05	7.22±0.02	7.12±0.11	6

Results are expressed as mean±S.E.M.
*Significantly different from resting value ($P<0.05$).

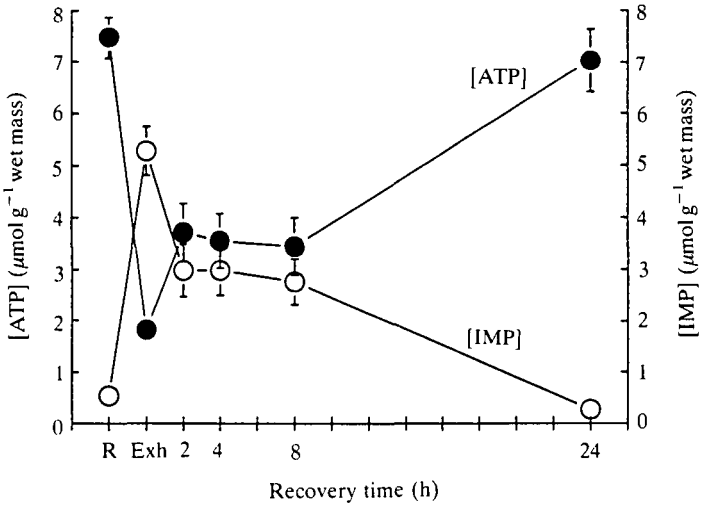

Fig. 1. ATP and IMP levels in trout white muscle during recovery. Bars are ±S.E.M.. Numbers of animals at each time are given in the legend to Table 2. R, resting fish; Exh, immediately post-exercise.

Adenylates and PCr

There was a stoichiometric relationship between [ATP] and [IMP] at all times (Fig. 1). Since neither [AMP] nor [ADP] changed significantly during exercise or recovery, total purine nucleotides (TPN) also did not change (Table 3). Similarly, [PCr]+[Cr] remained unchanged, with the decrease in [PCr] due to exercise being accounted for by the increase in [Cr]. In contrast to [ATP] (Fig. 1), [PCr] was fully recovered by 2 h post-exercise (Table 3) and did not change significantly thereafter. Changes in creatine charge paralleled the changes in [PCr] (Table 3), declining to approximately 16% of the resting value at exhaustion. By 2 h post-exercise, creatine charge had fully recovered.

Table 3. *Measured total concentrations of the adenylates, phosphocreatine and creatine*

Time	[ADP]	[AMP]	TPN	[PCr]	[Cr]	[PCr]+[Cr]	Creatine charge
Rest	0.77±0.07	0.07±0.01	8.90±0.20	22.69±2.69	19.36±2.30	41.98±2.21	0.49±0.05
Exhaustion	0.65±0.07	0.06±0.02	7.82±0.60	4.26±1.14*	40.76±0.68*	45.03±1.23	0.08±0.02*
2 h	0.61±0.07	0.06±0.02	7.37±0.52	25.37±3.26	19.82±2.10	45.19±2.22	0.51±0.06
4 h	0.60±0.05	0.05±0.02	6.78±0.26	20.44±3.49	21.04±0.96	41.49±2.69	0.43±0.06
8 h	0.49±0.02	0.07±0.02	6.78±0.26	21.97±3.76	20.80±2.76	39.87±0.62	0.50±0.10
24 h	0.71±0.05	0.05±0.01	8.06±0.63	26.23±1.01	15.74±2.13	41.97±1.26	0.59±0.04

Data are mean±S.E.M. in $\mu mol\,g^{-1}$ wet mass with number of animals (N) as in Table 2.
* Significantly different from resting value ($P<0.001$).
TPN, total purine nucleotides ([ATP]+[ADP]+[AMP]+[IMP]); Cr, creatine; PCr, phosphocreatine; Cr charge, [PCr]/([PCr]+[Cr]).

Table 4. *Tissue contents of lactate, pyruvate and glycogen, lactate/pyruvate ratio and plasma [lactate]*

Time	Tissue [lactate] ($\mu mol\,g^{-1}$)	Tissue [pyruvate] ($\mu mol\,g^{-1}$)	Tissue [glycogen] ($\mu mol\,g^{-1}$)	Lactate/pyruvate ratio	Plasma [lactate] ($\mu mol\,ml^{-1}$)
Rest	3.92±0.94	0.113±0.060	33.90±3.63	55±16	0.69±0.27
Exhaustion	41.72±2.63***	0.289±0.044***	10.48±5.47*	160±20	13.05±2.64**
2 h	36.63±4.62***	0.305±0.062*	9.43±3.52*	227±135	12.04±1.43**
4 h	34.91±1.94***	0.116±0.047	15.90±4.79	361±162*	17.12±2.85**
8 h	20.65±2.81***	0.068±0.020	19.06±3.84	359±87*	12.22±8.58**
24 h	4.85±1.29	0.069±0.022	25.81±4.08	105±45	1.11±0.38

Data are mean±S.E.M. with N as in Table 2.
Significantly different from the respective resting value: *** $P<0.0001$; ** $P<0.01$; * $P<0.05$.

Carbohydrate metabolism

[Lactate] increased more than 10-fold at exhaustion and this increase could be accounted for by the decrease in glycogen content (Table 4). Recovery of [lactate] to resting levels was relatively slow (approximately 2 μmol g^{-1} h^{-1}) but by 24 h [lactate] had returned to resting levels. Glycogen repletion was similarly slow but by 24 h [glycogen] was significantly different from the value at exhaustion. [Pyruvate] increased at exhaustion to nearly three times the resting value and remained elevated over the first 2 h of recovery, after which time it decreased rapidly, recovering entirely by 8 h post-exercise. The lactate/pyruvate ratio increased at exhaustion and over 8 h of recovery such that by 4 h post-exercise it was significantly different from the resting value (Table 4) but by 24 h it was fully recovered.

Plasma [lactate] increased nearly 20-fold at exhaustion and remained elevated through 8 h of recovery, but by 24 h was not significantly different from the resting value.

Energy metabolism

Intracellular $[Mg^{2+}]_f$ was estimated to be close to 10 mmol l^{-1} at rest, increasing to as high as 15 mmol l^{-1} at exhaustion. In general, the effects on the model calculations of changing $[Mg^{2+}]_f$ were small; in most cases less than 5%. Calculated $[ADP]_f$ showed the greatest variation. Resting $[ADP]_f$ ranged from 18.2±2.9 to 22.3±3.6 nmol g^{-1} tissue, with $[Mg^{2+}]_f$ between 1 and 20 mmol l^{-1}.

$[AMP]_f$ increased significantly at exhaustion although $[ADP]_f$ did not. By 2 h of recovery both had declined by at least an order of magnitude (Table 5). $[ADP]_f$ and $[AMP]_f$ gradually increased over the remainder of the recovery period so that by 24 h post-exercise neither was significantly different from the resting values.

Energy charge (EC) and R_{ATP} estimated using free metabolite concentrations decreased slightly at exhaustion but had recovered by 2 h post-exercise (Table 5). In contrast, EC calculated without taking into account that large fractions of the ADP and AMP pool are bound *in vivo* (EC_{total}) decreased significantly at exhaustion and remained depressed until 24 h post-exercise (Table 5).

Discussion

Recovery of fish white muscle from exercise is very slow. The exact time when a fish is deemed 'recovered' is highly dependent on which metabolite is measured. One of the most interesting observations of this study is the rapid recovery of [PCr] when [ATP] is approximately half of the resting levels. Conclusions about energy status at this time would be highly dependent on which variable was favoured. Once a metabolite is chosen, the conclusion may be erroneous if the concentration term is inappropriate (i.e. using absolute instead of relative concentrations or total instead of free concentrations). When two common indices of adenylate status (R_{ATP} and EC) are calculated using free metabolite concentrations (Table 5), it is clear that adenylate status recovers much faster than the changes in [ATP] would

Table 5. *Calculated free concentrations of ADP and AMP and indices of adenylate status*

Time	$[ADP]_f$	$[AMP]_f$	EC_{total}	EC_f	R_{ATP}
Rest	22.0±3.5	1.73±0.04	0.945±0.004	$0.9998\pm5.9\times10^{-5}$	$0.9969\pm5.1\times10^{-4}$
Exhaustion	19.0±5.6	5.75±2.41**	0.843±0.011**	$0.9968\pm1.4\times10^{-4}$	$0.9872\pm4.0\times10^{-3}$
2 h	1.9±0.5**	0.02±0.01**	0.912±0.012**	$0.9999\pm2.7\times10^{-6}$	$0.9995\pm9.0\times10^{-5}$
4 h	2.7±1.1**	0.07±0.05**	0.914±0.008**	$0.9999\pm1.5\times10^{-5}$	$0.9992\pm3.1\times10^{-4}$
8 h	4.2±1.9**	0.15±0.11**	0.919±0.008**	$0.9999\pm2.0\times10^{-5}$	$0.9989\pm3.3\times10^{-4}$
24 h	11.9±2.6	0.55±0.20	0.947±0.002	$0.9999\pm2.5\times10^{-5}$	$0.9984\pm2.9\times10^{-4}$

Calculated values are means±S.E.M. in nmol g^{-1} wet mass with N as in Table 2.

** Significantly different from resting value ($P<0.01$).

Energy charge was calculated either with the total measured metabolite concentrations or using the calculated free concentrations (assuming $[Mg^{2+}]_f=10$ mmol l^{-1}).

R_{ATP}, $[ATP]/([ATP]+[ADP]_f+[AMP]_f)$; EC_{total}, total adenylate energy charge; EC_f, free cytosolic energy charge; $[ADP]_f$, $[AMP]_f$, free cytosolic concentrations of ADP and AMP.

suggest. Comparison of energy charge calculated with free *vs* total metabolites illustrates the importance of considering the free pools only. Arguments based on [ATP] alone are less meaningful. This is particularly true in trout white muscle, which has an active purine nucleotide cycle (Mommsen and Hochachka, 1988). Indeed, calculation of free adenylate concentrations offers insight into the regulation of the purine nucleotide cycle and its temporal separation into deaminating and reaminating arms during exercise and recovery.

During exercise, a large fraction (80 %) of the ATP in white muscle is stoichiometrically converted to IMP (Fig. 1) *via* AMP deaminase. The purpose of this enzyme, the deaminating arm of the purine nucleotide cycle, is to maintain adenylate status (i.e. R_{ATP}) at high levels to support mechanical work. AMP deaminase is expected to be exquisitely sensitive to the changes in $[AMP]_f$ observed in this study (Table 5). At rest $[AMP]_f$ is less than 2 μmol l^{-1}, well below the apparent Michaelis constant ($K_{m,app}$) of rabbit muscle AMP deaminase (400 μmol l^{-1}, Smiley and Suelter, 1967). The activity of AMP deaminase in rainbow trout white muscle is approximately 40 μmol min^{-1} g^{-1} (Fijisawa and Yoshino, 1987). If, as a first approximation, we assume that the rate is linearly related to $[AMP]_f$ up to the $K_{m,app}$, then, at the $[AMP]_f$ observed at exhaustion, there would be enough AMP deaminase activity to produce about 9 μmol IMP g^{-1} muscle over the 30 min protocol (assuming 6 μmol l^{-1} $[AMP]_f$), close to the observed increase in [IMP] of about 5 μmol g^{-1}. At rest and during recovery, $[AMP]_f$ is much lower and IMP production would be expected to decrease. At the $[AMP]_f$ calculated for the 2 h post-exercise group (Table 5) at most 0.042 μmol g^{-1} IMP could be formed over the same 30 min period, less than 0.5 % of the rate expected during exercise. These changes in $[AMP]_f$ may be one of the causes of the separation of the purine nucleotide cycle into two temporally distinct arms in fish white muscle (Mommsen and Hochachka, 1988). Furthermore, the importance of changes in [AMP] is obscured if the total [AMP], rather than $[AMP]_f$, is considered.

During recovery, adenylate status is an important signal to intermediary metabolism and, again, it is the free concentration of metabolites such as ADP that influences rates of glycolysis and mitochondrial respiration. In the present study, resting $[ADP]_f$ represents less than 3 % of the measured total ADP (see Tables 3 and 5). The cost of recovery metabolism (phosphagen resynthesis, glycogen repletion, ion homeostasis) is largely met by mitochondrial metabolism. The availability of free ADP relative to ATP ($[ATP]/[ADP]_f$) is a critical determinant of mitochondrial respiration in skeletal muscle, but the exact nature of the relationship is complex (see Balaban, 1990). Although total [ADP] changes little in rest, exercise and recovery (Table 3), $[ADP]_f$ undergoes pronounced changes (Table 5), which have an important influence on white muscle metabolism. There is a strong dependence of mitochondrial oxygen uptake on ADP availability at these low concentrations (Moyes *et al.* 1992). At $[ATP]/[ADP]_f$ ratios greater than 200, as is the case in trout at rest (Fig. 1 and Table 5), white muscle respiration is less than 10 % of the maximal capacity (Moyes *et al.* 1992). In

fact, if $[ATP]/[ADP]_f$ is an important determinant of respiration *in vivo*, it is perplexing that, relative to rest, it becomes even more inhibitory during recovery when metabolic demands are expected to be greater. In a parallel study, we suggest that the added cost of recovery, as indicated by the excess post-exercise oxygen consumption, is met by mitochondria, which are stimulated primarily by changes in [phosphate] and influenced by pHi (Moyes *et al.* 1992). Instead of a signal for mitochondrial respiration, the elevated post-exercise $[ATP]/[ADP]_f$ apparent in this study may be involved in regulation of carbohydrate metabolism.

Two main questions dominate current interest in carbohydrate metabolism during recovery from high-intensity exercise in fish white muscle. What is the fate of lactate? What is the pathway of glycogen resynthesis? Glycogen could be formed from lactate *in situ* or *via* export to the liver, where hepatic gluconeogenesis produces glucose, which serves as a precursor for white muscle glycogen (Cori cycle). There is abundant evidence to suggest that Cori cycling is not necessary, at least for some skeletal muscles. In salmonids, there is little evidence for Cori cycling as an important route of lactate metabolism and glycogen resynthesis. After injection of [^{14}C]lactate into the blood of coho salmon following exhaustive exercise, more than 80 % of the total blood radioactivity was recovered as lactate (Milligan and McDonald, 1988). Less than 10 % of the glycogen resynthesized following exhaustive exercise in the trout can be accounted for by glucose uptake into white muscle, based on the uptake of 2-deoxyglucose (T. G. West, P. M. Schulte and P. W. Hochachka, unpublished results). Since the sum of [glycogen]+2[lactate] is equal in rest and throughout recovery, it is unlikely that oxidation is an important fate of lactate in recovery in this study. It appears that, in fish, the majority of the lactate formed during exhaustive exercise is converted to glycogen within the white muscle. The route for this pathway has not been established but the results of this study are consistent with a pathway involving reversal of pyruvate kinase.

In liver gluconeogenesis, phosphoenolpyruvate carboxykinase (PEPCK) and pyruvate carboxylase are required to reverse the step catalyzed by pyruvate kinase in glycolysis. Pyruvate kinase is normally thought to be an irreversible enzyme. Skeletal muscle of vertebrates lacks pyruvate carboxylase and two schemes have been suggested to bypass this step in skeletal muscle: (1) PEPCK+malic enzyme and (2) reversal of pyruvate kinase. Connett (1979) has argued that the metabolite concentrations found in recovering frog skeletal muscle suggest that pyruvate kinase is too far from equilibrium to be reversed. He suggests the PEPCK–malic enzyme route is more feasible in frog. Fish white muscle, however, also lacks PEPCK (e.g. Moon and Johnston, 1980). Consequently, glyconeogenesis in trout white muscle may require the reversal of pyruvate kinase. Reversal requires high [ATP] and [pyruvate] and low [ADP] and [PEP] (Dyson *et al.* 1975). The $[ATP]/[ADP_f]$ ratios observed in this study (500–2000) are higher than those observed in liver (20, Morikofer-Zwez and Walter, 1989) and those employed by Connett for frog skeletal muscle (30–80). They are similar to ratios reported in mammalian studies (e.g. Shoubridge and Radda, 1984). Unfortunately, PEP was

not measured in our study so we cannot assess how close to equilibrium lie the reactants of pyruvate kinase. Newsholme and Crabtree (1986) suggest that if the ratio of the mass action ratio to the equilibrium constant is between 0.2 and 5, the reaction can be assumed to be near equilibrium and reversible *in vivo*. Using this as a guideline, PEP concentrations of 10–60 μmol l^{-1} would bring this reaction near to equilibrium in recovery. [pH-independent K_{eq}=[pyruvate]×[ATP]/([PEP]×$[ADP]_f$×$[H^+]$)=2×10^{11} $(mol\,l^{-1})^{-1}$, as used by Connett (1979).] These concentrations are physiologically realistic.

The high [ATP]/$[ADP]_f$ ratios seen during recovery could be required to reverse flux through pyruvate kinase. The fact that high [ATP]/$[ADP]_f$ ratios may also limit oxidative metabolism well below its capacity (see Moyes *et al.* 1992) may be of lesser significance. Thus, there may be a trade-off involved in which the rate of glycogen synthesis is optimized relative to the rate of oxidative phosphorylation. ATP replenishment *via* the purine nucleotide cycle need not occur prior to these events.

Fish white muscle exhibits, albeit to an exaggerated degree, many of the typical characteristics of fast twitch glycolytic muscles from all animals. Energy metabolism and carbohydrate metabolism in these types of muscle must be regarded as an integrated system responding to a number of controlling factors including $[ADP]_f$ and pHi. Energy metabolism and purine nucleotide cycling, however, are less closely linked, resulting in a dissociation of [ATP] and energy status. By utilizing the calculated free concentrations of ATP, ADP and AMP, it is possible to understand more fully these relationships and the control of muscle metabolism during exercise and recovery.

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