

**LIPID AND CARBOHYDRATE METABOLISM IN MUSCLE
OF RAINBOW TROUT (*Oncorhynchus mykiss*) DURING
EXERCISE AND RECOVERY**

by

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**LIPID AND CARBOHYDRATE METABOLISM IN
TROUT MUSCLE**

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ABSTRACT

A biochemical approach was employed to examine the oxidative utilization of lipid and carbohydrate in red and white muscle of rainbow trout (*Oncorhynchus mykiss*) during high- and low- intensity exercise and during recovery from high-intensity exercise. Measurements of the activation state of the rate-limiting enzymes, glycogen phosphorylase (Phos) and pyruvate dehydrogenase (PDH), and their allosteric regulators (e.g. acetyl-CoA, free ADP) have yielded information on the interaction and regulation of pathways involved in lipid and carbohydrate oxidation in fish muscle.

High-intensity exercise in trout is powered predominately by white muscle. During the first 10 s of exercise, high ATP turnover rates ($3.7 \mu\text{mol}\cdot\text{g}^{-1} \text{ wet tissue}\cdot\text{s}^{-1}$) are achieved by rapid creatine phosphate (CrP) hydrolysis and glycolysis. Activation of glycolysis is achieved by a large transformation of Phos into its active form. Exercise performed from 10 s to exhaustion (52 s) occurs at a lower ATP turnover rate (0.5 to $1.2 \mu\text{mol}\cdot\text{g}^{-1} \text{ wet tissue}\cdot\text{s}^{-1}$) and is primarily supported by glycolysis. A gradual transformation of PDH into its active form points to a minor contribution of oxidative phosphorylation of carbohydrate to support ATP turnover. The gradual activation of PDH and an increasing mitochondria redox ($[\text{NAD}^+]/[\text{NADH}]$) in white muscle during exercise suggests that O_2 is not limiting during high-intensity exercise and thus anaerobiosis may not be responsible for lactate production in trout white muscle.

During recovery from high-intensity exercise, there is a rapid activation of pathways involved in lipid oxidation to provide ATP for CrP, ATP, and glycogen synthesis. A rapid transformation of PDH into its inactive form spares lactate from an oxidative fate, likely to serve as the substrate for *in situ* glycogen re-synthesis. Decreases in free carnitine and increases in long-chain fatty acyl (LCFA) carnitine, acetyl-CoA, and acetyl-carnitine indicate an activation of pathways involved in lipid oxidation. Increases in white muscle malonyl-CoA during recovery suggest that malonyl-CoA may not regulate carnitine palmitoyltransferase-1 in trout white muscle.

Sustainable, “aerobic” exercise at 30 and 60% U_{crit} is characterized by an initial (2 min) oxidative utilization of carbohydrate for ATP production. Within 15 min swimming at 30 and 60% U_{crit} , PDH activation returns to resting values, and at 240 min increases in LCFA-carnitine and decreases in malonyl-CoA indicate an overall enhancement of lipid oxidation. Non-sustainable swimming at 90% U_{crit} is characterized by a sustained activation of red muscle PDH. Overall, sustained swimming at 30 and 60% U_{crit} is supported by approximately equal contributions of carbohydrate (~45%) and lipid (~35%) oxidation, whereas non-sustainable swimming is supported primarily by carbohydrate oxidation.

Palmitate uptake by red and white muscle membrane vesicles is facilitated by a saturable transport mechanism which is sensitive to phloretin, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid, (DIDS), and mercury. Red and white muscle transporters have similar affinities for palmitate ($K_m=26\pm6$ and 33 ± 8 nM free palmitate, respectively), but red muscle exhibits a significantly higher maximal uptake

rate compared to white muscle ($V_{\max} = 476 \pm 41$ and 229 ± 23 pmol·mg⁻¹ protein·s⁻¹, respectively). Prolonged swimming and chronic cortisol elevations do not stimulate palmitate uptake by muscle, indicating that the mechanisms involved in LCFA uptake by muscle cells may not limit lipid oxidation in trout muscle.

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THESIS ORGANIZATION AND FORMAT

The present thesis is presented in a “closed-faced” format approved by McMaster University. Therefore, this dissertation consists of a total of 6 chapters. Chapter 1 provides a general introduction and statements on the objectives of the thesis. Chapters 2, 3, 4, and 5 are manuscripts that have been published, accepted for publication, or submitted for publication in peer-reviewed journals. Chapter 6 provides a summary of the findings and conclusions. Chapter 7 contains the references cited throughout the thesis.

Chapter 1: **General introduction and thesis objectives.**

Chapter 2: **Lipid oxidation fuels recovery from exhaustive exercise in white muscle of rainbow trout.**

Authors: Jeff G. Richards, George J.F. Heigenhauser, and Chris M. Wood
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Chapter 5: Palmitate movement across red and white muscle membranes of rainbow trout.

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Chapter 6: Summary of findings and conclusions.

Chapter 7: References

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LIST OF ABBREVIATIONS

ACC	acetyl-CoA carboxylase	DA	dorsal aorta
Acetyl-CoA	acetyl coenzyme A	DIDS	4,4'-diisothiocyanatostilbene
ADP	adenosine diphosphate		-2,2'-disulfonic acid
ADP _f	free adenosine diphosphate	DMSO	dimethyl sulfoxide
AMP	adenosine monophosphate	EDTA	ethylenediaminetetraacetic
AMP _f	free adenosine		acid
	monophosphate	EGTA	ethylene glycol- <i>bis</i>
ANOVA	analysis of variance		(β -aminoethyl ether)-
ATP	adenosine triphosphate		N,N,N'N'-tetraacetic acid
BSA	bovine serum albumin	EPOC	excess post-exercise
CoA-SH	free coenzyme A		oxygen consumption
CPT-1	carnitine	FABP _{pm}	plasma membrane fatty
	palmitoyltransferase-1		acid binding protein
CPT-2	carnitine	FAT	fatty acid translocase
	palmitoyltransferase-2	FATP	fatty acid transport protein
Cr	free creatine	FFA	free fatty acid
CrP	creatine phosphate	GC	gas chromatography
cytFABP	cytoplasmic fatty acid	Glu 1-P	glucose 1-phosphate
	binding protein	Glu 6-P	glucose 6-phosphate

Gly 3-P	glycerol 3-phosphate	PDH _b	inactive fraction of PDH
GS	glycogen synthase	PDH _t	total PDH activity
GS _a	active fraction of GS	PFK-1	phosphofructokinase-1
GS _b	inactive fraction of GS	Phos	glycogen phosphorylase
HDL	high density lipoprotein	Phos _a	active fraction of Phos
HSL	hormone sensitive lipase	Phos _b	inactive fraction of Phos
IMP	inosine monophosphate	Phos _{tot}	total Phos activity
IMTG	intramuscular Triacylglycerol	P _i	inorganic phosphate
K _m	Michealis-Menten constant	PMSF	phenylmethyl sulphonyl fluoride
LCFA	long chain fatty acid	P _{O₂}	oxygen partial pressure
M _{O₂}	oxygen consumption	SCFA	short chain fatty acid
NAD ⁺	β-nicotinamide adenine dinucleotide, oxidized form	RP-HPLC	reverse phase high performance liquid chromatography
NADH	β-nicotinamide adenine dinucleotide, reduced form	TAG	triacylglycerol
NEFA	nonesterified fatty acid	TCA	tricarboxylic acid
NH ₃	ammonia	U _{crit}	critical swimming speed
ODS	octadecyl silane	UDP	uridine diphosphate
PDH	pyruvate dehydrogenase	V _{O₂Max}	maximum oxygen consumption
PDH _a	active fraction of PDH		

CHAPTER 1

GENERAL INTRODUCTION AND OBJECTIVES

Over the past several decades the rainbow trout has become a popular research model in the study of exercise physiology (Black *et al.*, 1962; Moyes and West, 1995; Kieffer, 2000). This popularity is due, in part, to the unique spatial separation of red, Type I, oxidative muscle fibres from white, Type II, glycolytic fibres within the body musculature, and their known recruitment patterns (Hudson, 1973; Johnson, 1981; Wilson and Egginton, 1994; Burgetz *et al.*, 1998). Furthermore, human activities (*e.g.* angling and water-way damming) impact upon fish causing them to undergo forced exercise. An understanding of the metabolic consequences of exercise and recovery in fish muscle is essential to the developing aquaculture industry in order to sustain fish populations for sport and food. To this end, research has focused on determining the substrates utilized by white muscle during high-intensity exercise and the pattern of substrate recovery during post-exercise recovery (Wood, 1991; Milligan and Girard, 1993; Wang *et al.*, 1994a; Milligan, 1996; Milligan *et al.*, 2000).

High-Intensity Exercise in Trout White Muscle

White muscle of trout has a low oxidative capacity ($3.5 \mu\text{mol ATP}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ white muscle; Moyes *et al.*, 1992) reflecting a low mitochondrial density. Thus, high ATP

turnover rates during intense exercise are primarily supported by creatine phosphate (CrP) hydrolysis and glycolysis leading to lactate production. Exhaustion in trout is characterized by a 40 to 60% decrease in white muscle CrP and ATP and up to a 90% decrease in muscle glycogen concentrations with reciprocal and stoichiometric increases in free creatine (Cr), inosine monophosphate (IMP), and lactate, respectively (Wood, 1991; Schulte *et al.*, 1992; Wang *et al.*, 1994a; Kieffer, 2000).

Recovery from High-Intensity Exercise

During recovery from high-intensity exercise, pathways must be activated to provide ATP for the re-synthesis of ATP, CrP, and glycogen in preparation for another possible bout of exercise. To this end, trout exhibit an excess post-exercise O₂ consumption (EPOC; Scarabello *et al.*, 1991a,b, 1992), in part, representing a stimulation of O₂ uptake for ATP production during recovery. Debate surrounds the question of what substrate supports oxidative phosphorylation during recovery. Lactate is retained in trout white muscle during the post-exercise period at concentrations exceeding those found in the plasma (Wang *et al.*, 1994a, 1996, 1997; Labree and Milligan, 1999). Classically, it was believed that a portion of the accumulated lactate (4 to 6 $\mu\text{mol}\cdot\text{g}^{-1}$ wet tissue) was oxidized to supply ATP to support the metabolic cost of recovery. However, there is accumulating evidence that lactate is retained in trout white muscle during the post-exercise period to act as the substrate for *in situ* glycogen synthesis (Moyes *et al.*, 1992; Schulte *et al.*, 1992; Milligan and Girard, 1993; Moyes and West, 1995; Milligan, 1996). Contrary to the classical theory of lactate oxidation during recovery, indirect evidence

also suggests that lipid oxidation may supply the ATP required for recovery metabolism in trout white muscle (Moyes *et al.*, 1992; Moyes and West, 1995).

Sustained Exercise

Considerable debate surrounds the issue of what substrates are oxidized during sustained “aerobic” exercise in fish. Traditionally, protein and lipids were considered to be the major fuels oxidized during sustained swimming while carbohydrate oxidation was considered minimal (Dreidzic and Hochachka, 1987; Jobling, 1994; Wilson and Egginton, 1994; Burgetz *et al.*, 1998). However, Lauff and Wood (1996), using respirometry, demonstrated that juvenile rainbow trout primarily oxidize lipid during sustained swimming at 55% and 85% of the critical swimming speed (U_{crit}), that carbohydrate oxidation is the next most important substrate oxidized, and that protein oxidation is minimal. White muscle glycolytic utilization of carbohydrate has been demonstrated to contribute to ATP turnover during the initial fast-start of swimming (Wokoma and Johnson, 1981) and at swimming speeds $\geq 70\% U_{crit}$ (Burgetz *et al.*, 1998). In addition, whole body oxidative utilization of carbohydrate increases as swimming speed approaches U_{crit} (Lauff and Wood, 1996). To date, no study has examined substrate selection in red muscle of trout during graded “aerobic” exercise.

Integration of Metabolic Pathways

Based on available literature of substrate selection during exercise and recovery, there is an emerging consensus that carbohydrate and lipid are the primary substrates oxidized to support muscle ATP turnover in trout. However, little information is

available on coordination and regulation of the pathways involved in carbohydrate and lipid oxidation. Protein oxidation by trout does not change during aerobic exercise (~20% of oxygen consumption; \dot{M}_{O_2}) and decreases upon training suggesting that protein oxidation does not contribute to ATP turnover during exercise (Lauff and Wood, 1996, 1997; Weber, 1997).

From rest to exercise, ATP turnover rates in fish muscle, especially white muscle, can increase by 200 fold (Dobson *et al.*, 1987; Moyes and West, 1995). Adenosine triphosphate stores within the muscle are low (3 to 8 $\mu\text{mol}\cdot\text{g}^{-1}$ wet tissue), thus at the onset of exercise, metabolic pathways must be activated to balance ATP production with demand. In mammals, large and rapid changes in substrate flux through metabolic pathways is achieved by the activation of non-equilibrium, rate-limiting enzymes (*e.g.* pyruvate dehydrogenase, PDH; Newsholme and Crabtree, 1979). Rate-limiting enzymes set the direction of metabolic flux and the reactions they catalyse are irreversible. Furthermore, rate-limiting enzymes have a low K_m (*i.e.* high affinity), thus they are usually saturated with their substrate. Large changes in substrate flux are achieved by covalent modification (*e.g.* phosphorylation or dephosphorylation), which activates or deactivates the enzyme. The catalytic rate of these enzymes is further tuned by allosteric regulation and product inhibition. Equilibrium enzymes, which constitute the majority of metabolic enzymes, are regulated by substrate concentration and cannot affect large changes in metabolic flux or ATP production (Newsholme and Crabtree, 1979).

In mammals, glycogen phosphorylase (Phos), glycogen synthase (GS), and PDH are non-equilibrium, rate-limiting enzymes that play important roles in regulating

carbohydrate metabolism. Glycogen phosphorylase catalyzes the flux-generating step of glycogen entry into glycolysis. Glycogen synthase catalyses the rate-limiting transfer of glucose from uridine diphosphate (UDP)-glucose to glycogen. Pyruvate dehydrogenase catalyzes the decarboxylation of pyruvate into acetyl-CoA for entry into the tricarboxylic acid (TCA) cycle, regulates the rate of carbohydrate oxidation, and sets the maximal rate of TCA cycle activity from carbohydrate.

Glycogen phosphorylase is regulated at the transformational level by reversible phosphorylation and at the post-transformational level through substrate availability (inorganic phosphate; P_i), product inhibition (glucose 1-phosphate; glu 1-P), and changes in allosteric modulators [free ADP (ADP_f) and free AMP (AMP_f); Fig. 1-1; Ren and Hultman, 1988; Heigenhauser and Parolin, 1999a]. Phosphorylation of Phos by Phos kinase transforms the low activity form of Phos ($Phos_b$) into the high-activity form ($Phos_a$), whereas the dephosphorylation by Phos phosphatase converts $Phos_a$ into $Phos_b$ (Krebs *et al.*, 1964). The transformation between $Phos_b$ and $Phos_a$ is regulated at the contractile level through Ca^{2+} release from the sarcoplasmic reticulum and at the hormonal level through epinephrine mediated changes in cyclic AMP (Johnson, 1992). Decreases in intracellular pH (pH_i) affect the transformation of Phos by inhibiting Phos kinase (Connett and Sahlin, 1996) and affect substrate availability by shifting the speciation of P_i (Kasvinski and Meyer, 1977).

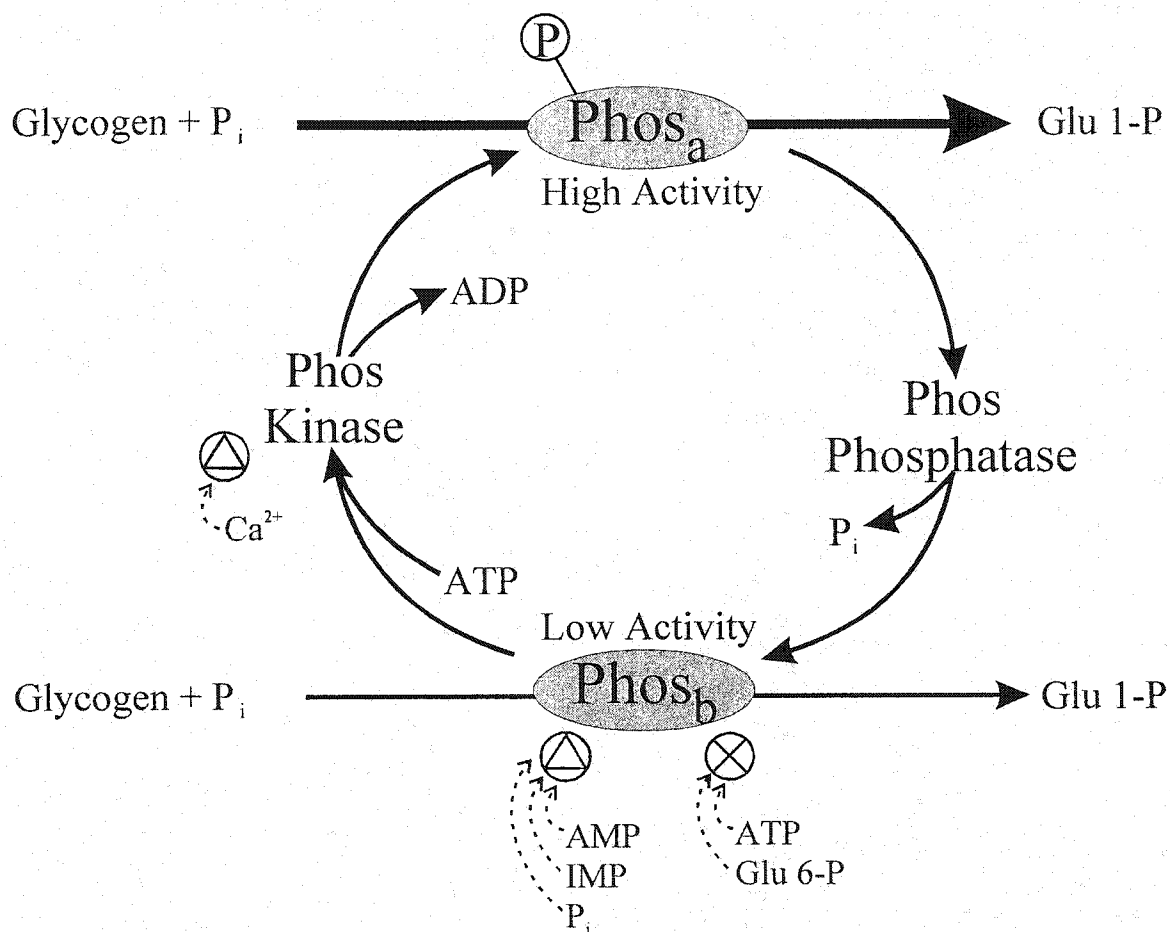


Figure 1-1:

Schematic illustration of the factors that regulate glycogen phosphorylase (Phos) activity.

Wider arrows indicate greater metabolic flux. AMP, adenosine monophosphate; ATP, adenosine triphosphate; glu 1-P, glucose 1-phosphate; glu 6-P, glucose 6-phosphate;

IMP, inosine monophosphate; Phos_a, high activity form of glycogen phosphorylase;

Phos_b, low activity form of glycogen phosphorylase; P_i, inorganic phosphate. Illustration

modified from Heigenhauser and Parolin (1999a).

Pyruvate dehydrogenase is the rate-limiting enzyme that sets the rate of entry of glycolytically derived pyruvate into the TCA cycle and oxidative phosphorylation. The catalytic rate of PDH is regulated by covalent modification (phosphorylation and dephosphorylation) and by product inhibition (Fig. 1-2; Weiland, 1983).

Dephosphorylation of PDH by PDH phosphatase transforms the inactive PDH_b into the active PDH_a while phosphorylation of PDH by PDH kinase transforms the active PDH_a into the inactive PDH_b. PDH kinase is allosterically stimulated by acetyl-CoA, NADH, and ATP and is inhibited by Ca²⁺, ADP, free coenzyme A (CoA-SH), NAD⁺, and pyruvate. In mammalian muscle, PDH phosphatase is stimulated by Ca²⁺ release from the sarcoplasmic reticulum and hormonally by insulin (Randle, 1995). Elevations in mitochondrial NADH and acetyl-CoA inhibit PDH phosphatase and reduce its transformation. The relative transformation patterns and catalytic rates of Phos and PDH have recently been implicated in lactate production in human muscle (Parolin *et al.*, 1999; Spriet *et al.*, 2000).

At the onset of exercise, the transformation states and catalytic rates of Phos and PDH are coordinated through a shift in cellular phosphorylation status. Classically, substrate use during exercise involves the temporally separate utilization of CrP followed by activation of glycolysis and then, in oxidative tissues, an activation of oxidative phosphorylation (Meyer and Foley, 1996). In this scheme, the initial depletion of CrP at the onset of exercise results in the accumulation of P_i, ADP_f, AMP_f, IMP, and NH₃, all of which are thought to activate glycolysis for further ATP production. Subsequently, the accumulation of pyruvate and lactate from glycolysis stimulate PDH for ATP production

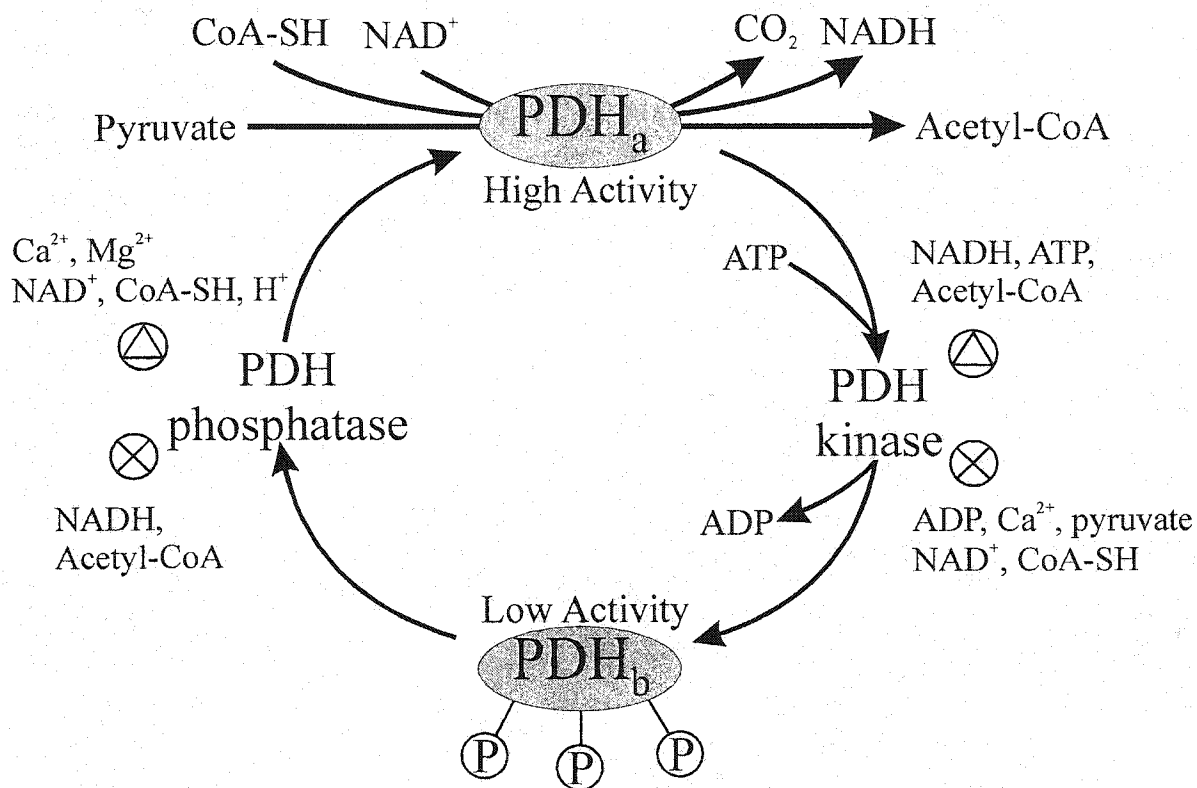


Figure 1-2:

Schematic illustration of the factors that regulate pyruvate dehydrogenase (PDH) activity.

Acetyl-CoA, acetyl-coenzyme A; AMP, adenosine monophosphate; ATP, adenosine triphosphate; CoA-SH; free coenzyme A; NAD⁺, nicotine adenine dinucleotide (oxidized form); NADH, nicotine adenine dinucleotide (reduced form); PDH_a, high activity form of PDH; PDH_b, low activity form of PDH. Illustration modified from Heigenhauser and Parolin (1999a).

via oxidative phosphorylation.

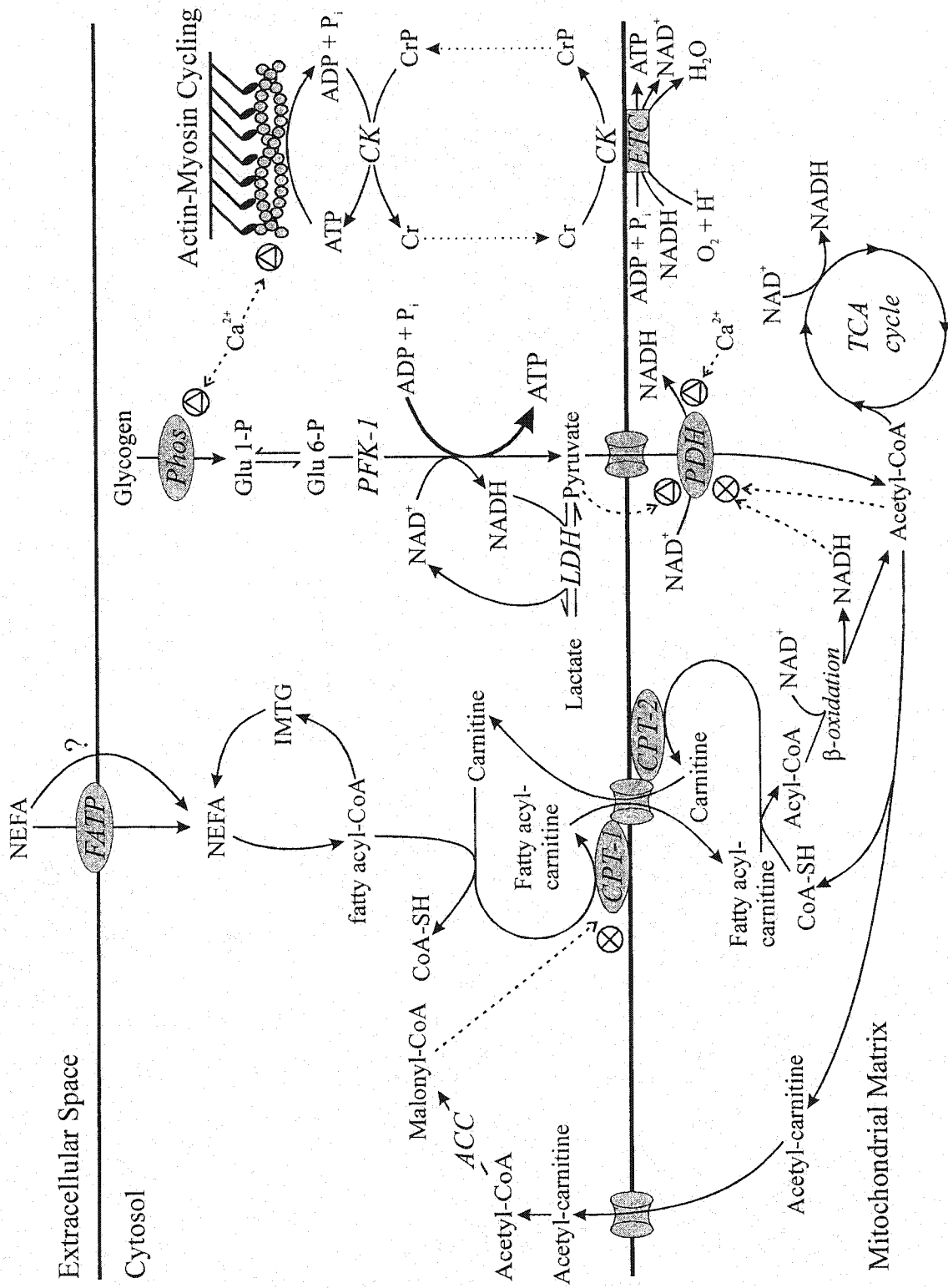
In general, much less is known about the control of lipid oxidation in muscle. Hormone sensitive lipase (HSL) is a rate-limiting enzyme that is thought to be involved in the hydrolysis of intramuscular triacylglycerol (IMTG) for fatty acid release for oxidation (van der Vusse and Reneman, 1996). However, based on mammalian research, the rate of lipid oxidation appears to be primarily regulated by the activity of carnitine palmitoyltransferase-1 (CPT-1), which catalyzes the transfer of fatty acyl moieties from CoA to carnitine for transport into the mitochondria for oxidation (Rasmussen and Wolfe, 1999). Carnitine palmitoyltransferase-1 is thought to be regulated *in vivo* by malonyl-CoA production (Ruderman *et al.*, 1999). Malonyl-CoA is the first committed step in fatty acid synthesis and is formed by the carboxylation of acetyl-CoA by acetyl-CoA carboxylase (ACC). High concentrations of malonyl-CoA allosterically inhibit CPT-1 and reduce the rate of fatty acid oxidation.

The rate of lipid oxidation may also be regulated at the site of fatty acid transport into muscle cells. In rats, the insertion of transport proteins associated with fatty acid uptake into the muscle membrane has been implicated in contributing to the enhanced fatty acid oxidation during 30 min of electrical stimulation (Bonen *et al.*, 1999, 2000). Furthermore, Turcotte *et al.* (1998) demonstrated that electrically induced contraction increased the V_{\max} of palmitate uptake in an isolated, perfused, rat muscle preparation. However, no information is available on the mechanisms of fatty acid movement across fish red and white muscle cell membranes and whether such mechanisms play a role in regulating lipid metabolism in fish.

Glucose Fatty Acid Cycle

The coordinated regulation of lipid and carbohydrate oxidation in mammalian muscle is commonly referred to as the glucose fatty acid cycle (Fig. 1-3; Randle, 1995, 1998). This proposed mechanism explains reduced carbohydrate utilization in the presence of excess fatty acid. During periods of enhanced fatty acid oxidation, carbohydrate oxidation is reduced through covalent and allosteric inhibition of PDH and Phos. Increased β -oxidation results in sustained increases in [acetyl-CoA]/[CoA-SH] and [NADH]/[NAD⁺]. These increases in acetyl-CoA and NADH are thought to override the stimulatory effects of Ca²⁺ on PDH phosphatase and cause a reduction in carbohydrate oxidation (Fig. 1-3). Elevations in acetyl-CoA and NADH stimulate PDH kinase resulting in a phosphorylation of PDH and inactivation. Acetyl-CoA also inhibits PDH_a *in vivo* through product inhibition. Increases in PDH kinase activity during periods of enhanced lipid oxidation have been proposed to explain reduced carbohydrate oxidation in rat red quadriceps muscle during sustained exercise (Denyer *et al.*, 1991). It has also been postulated that elevations in citrate, a product of high TCA cycle flux, may allosterically inhibit phosphofructokinase-1 (PFK-1) and reduce glycolytic flux (Saha *et al.*, 1999). Furthermore, changes in PFK-1 activity are known to affect the catalytic rate of Phos, through an accumulation of glucose 6-phosphate (glu 6-P), thus coordinating the rate of glycolytic flux at Phos and the rate of pyruvate entry into oxidative phosphorylation at PDH.

Based on mammalian research, changes in muscle malonyl-CoA have also been implicated in the coordinated regulation of lipid and carbohydrate oxidation in muscle



(Saha *et al.*, 1997; Odland *et al.*, 1998; Ruderman *et al.*, 1999; Bavenholm *et al.*, 2000). During periods of high ATP turnover, carbohydrate oxidation predominates in muscle and malonyl-CoA concentrations are elevated due to a high rate of acetyl-CoA production (Fig. 1-3). High concentrations of malonyl-CoA allosterically inhibit CPT-1 and limit the rate of lipid oxidation. Decreases in carbohydrate oxidation decrease malonyl-CoA production and relieve the resting inhibition of CPT-1. As a result, the rate of lipid oxidation is enhanced by a greater transport of fatty acids into the mitochondria for β -oxidation. In rat muscle, submaximal exercise reduces the concentration of malonyl-CoA and relieves the resting inhibition of CPT-1 (Saha *et al.*, 1995; Odland *et al.*, 1996; Chien *et al.*, 2000). However, the response of CPT-1 to malonyl-CoA is not consistent between species. In the human, submaximal exercise at 60% $V_{O_{2max}}$ does not cause a decrease in muscle malonyl-CoA despite the fact that this exercise intensity is characterized by enhanced lipid oxidation (Romijn *et al.*, 1993; Odland *et al.*, 1996).

OBJECTIVES

The overall objectives of the present thesis were to employ a biochemical approach to identify the rate-limiting processes involved in lipid and carbohydrate metabolism in red and white muscle of the rainbow trout during exercise and recovery from high-intensity exercise, and determine the factors that regulate them. By measuring changes in the transformation state of non-equilibrium, rate-limiting enzymes (PDH and Phos) and changes in their allosteric modulators, conclusions about relative fuel utilization can be made. Specifically, the objectives of this thesis were:

1. To determine the relative contributions of carbohydrate and lipid oxidation in providing ATP to support synthesis of CrP, ATP, and glycogen during post-exercise recovery in trout white muscle. The transformation states of PDH and GS were measured to examine the metabolic fate of accumulated lactate (Chapter 2).
2. To determine the role of Phos and PDH in regulating white muscle metabolism during a continuous bout of high-intensity exercise. Insights into cellular O₂ limitations during high-intensity exercise were made through estimates of cytoplasmic and mitochondrial redox state ($[NAD^+]/[NADH]$; Chapter 3).
3. To examine the biochemical pathways involved in carbohydrate and lipid utilization by red and white muscle of rainbow trout during swimming at sustainable “aerobic” swimming speeds. Insights into the regulation of lipid and carbohydrate oxidation were gained through measurements of PDH activation and malonyl-CoA concentrations (Chapter 4).
4. To determine if long-chain fatty acid (LCFA) uptake by red and white muscle of trout occurs *via* a carrier-mediated process and assess whether this transport process contributes to the regulation of lipid oxidation in fish red and white muscle (Chapter 5).

CHAPTER 2

LIPID OXIDATION FUELS RECOVERY FROM EXHAUSTIVE EXERCISE IN WHITE MUSCLE OF RAINBOW TROUT

ABSTRACT

The oxidative utilization of lipid and carbohydrate was examined in white muscle of rainbow trout (*Oncorhynchus mykiss*) at rest, immediately after exhaustive exercise, and for 32 h recovery. In addition to CrP and glycolysis fuelling exhaustive exercise, near maximal activation of PDH at the end of exercise points to oxidative phosphorylation of carbohydrate as an additional source of ATP during exercise. Within 15 min post exercise, PDH activation returned to resting values, thus sparing accumulated lactate from oxidation. Glycogen synthase activity matched the rate of glycogen resynthesis and represented near maximal activation. Decreases in white muscle free carnitine, increases in LCFA-carnitine, and sustained elevations of acetyl-CoA and acetyl carnitine indicate a rapid utilization of lipid to supply ATP for recovery. Increases in malonyl-CoA during recovery suggest that malonyl-CoA may not regulate CPT-1 in trout

muscle during recovery, but instead may act to elongate short-chain fatty acids (SCFA) for mitochondrial oxidation. In addition, decreases in IMTG and plasma nonesterified fatty acids (NEFA) indicate that both endogenous and exogenous lipid fuels may be oxidized during recovery.

INTRODUCTION

Over the past several decades many studies have examined the metabolic responses of fish white muscle to high-intensity, exhaustive exercise together with the pattern of metabolite recovery (Kieffer, 2000). These studies have led to the development of a model of fuel selection during exhaustive exercise based on hydrolysis of high-energy phosphates (*i.e.* CrP and ATP) and “anaerobic” glycolysis leading to lactate accumulation. Furthermore, it has been demonstrated that there is a temporal shift in fuel selection during exhaustive exercise from an initial hydrolysis of CrP (Dobson and Hochachka, 1987; Dobson *et al.*, 1987) to an activation of glycogenolysis and glycolysis (Moyes and West, 1995). As a result, exhaustion in rainbow trout is characterized by a 40 to 60% decrease in white muscle ATP and CrP concentrations and up to a 90% decrease in muscle glycogen concentrations with reciprocal and stoichiometric increases in IMP, Cr, and lactate, respectively (*e.g.* Wang *et al.*, 1994a).

During recovery, pathways must be activated to resynthesize ATP, CrP, and glycogen in preparation for another possible bout of exercise. To this end, trout experience EPOC (Scarabello *et al.*, 1991a), in part, representing a stimulation of oxidative phosphorylation for ATP production during recovery. The TCA cycle supplies reducing equivalents for mitochondrial oxidative phosphorylation through the utilization of acetyl-CoA. Acetyl-CoA can be produced either from the decarboxylation of pyruvate *via* PDH or through β -oxidation of lipid fuels. Amino acids can also supply substrate for the TCA cycle and support ATP production, but it is believed that the contributions of

protein oxidation to metabolism are low and can be ignored, particularly during exercise (Weber, 1997). Therefore, the two major fuel sources available to trout white muscle during recovery are the accumulated lactate from glycolysis and lipid fuels. The complete oxidation of a small amount of lactate (4 to 6 $\mu\text{mol}\cdot\text{g}^{-1}$ wet tissue), through the activation of PDH, could yield adequate ATP to support recovery in white muscle. However, there is accumulating circumstantial evidence that suggests the majority of accumulated lactate in trout white muscle is spared from an oxidative fate (Wood, 1991; Milligan and Girard, 1993) and retained as the substrate for *in situ* glyconeogenesis (Schulte *et al.*, 1992; Moyes and West, 1995).

Traditionally, lipids have not been considered an important fuel during exhaustive exercise and recovery; however, there is mounting evidence that suggests many species rely on lipid oxidation in muscle to fuel recovery (van der Vusse and Reneman, 1996; Keins and Richter, 1998). In the rainbow trout, Wang *et al.* (1994a) showed that immediately after exhaustive exercise there were decreases in free carnitine and increases in acetyl-carnitine and acetyl-CoA concentrations in white muscle. Accumulation of acetyl groups during recovery points to an activation of oxidative phosphorylation during recovery. Decreases in free carnitine, accompanied by the accumulation of SCFA-carnitine, suggested that lipid was the source of acetyl-groups. Decreases in white muscle total lipid concentrations (Milligan and Girard, 1993) and decreases in plasma NEFA (Dobson and Hochachka, 1987) during post-exercise recovery in trout further support the use of lipids in fuelling recovery from exhaustive exercise.

If we accept this scenario that β -oxidation may contribute to ATP production during post-exercise resynthesis of CrP, ATP, and glycogen, then the question arises as to the underlying mechanism that regulates fuel selection during recovery. Moyes *et al.* (1992) demonstrated that NEFA oxidation inhibited pyruvate oxidation when isolated trout white muscle mitochondria were incubated simultaneously with pyruvate and NEFA. These workers speculated that this inhibition of carbohydrate oxidation in the presence of NEFA was due to allosteric inhibition of PDH providing a mechanism by which carbohydrate can be spared at the expense of lipid oxidation. Furthermore, in higher vertebrates, recent evidence has implicated malonyl-CoA in regulating lipid oxidation in skeletal muscle (Ruderman *et al.*, 1999). Malonyl-CoA is the first committed step in the *de novo* synthesis of fatty acids and has been shown in muscle to allosterically regulate CPT-1, the enzyme responsible for catalyzing the rate-limiting transfer of fatty acids to carnitine for uptake by mitochondria (Awan and Saggerson, 1993). There remains considerable debate surrounding the regulatory role of malonyl-CoA in fuel selection in muscle of different species (Winder *et al.*, 1989; Ruderman *et al.*, 1999).

The objectives of the present research were to determine the metabolic fuels oxidized during recovery in trout white muscle to support synthesis of CrP, ATP, and glycogen. Specifically, we measured the activation state of PDH and GS and changes in oxidative metabolites (*e.g.* acetyl-CoA) and glycolytic intermediates in an attempt to isolate whether lipid or carbohydrate was oxidized during recovery in trout white muscle. Furthermore, we measured changes in IMTG and plasma NEFA in an attempt to

determine whether endogenous or exogenous lipids were oxidized during recovery.

Insights into the control of lipid and carbohydrate oxidation were gained through measurements of malonyl-CoA and estimates of ADP_f and AMP_f .

METHODS

Animal Care

Adult rainbow trout (*Oncorhynchus mykiss*, Walbaum; 240 - 350 g) were purchased from Humber Springs Trout Hatchery, Orangeville, Ontario, Canada, and held under flow through conditions in 800 L tanks supplied with aerated, dechlorinated city of Hamilton tapwater [composition as in Milligan and Wood (1986); 10°C] for at least one month before experimentation. During holding, fish were fed daily with commercial trout pellets. Three days before an experiment fish were transferred into a separate tank and feeding ceased.

Experimental Protocol

Fish were anaesthetized with 0.08 g·L⁻¹ 3-aminobenzoic acid ethyl ester (methanesulfonate salt; neutralized to pH 8.0 with KOH) and fitted with a dorsal aortic (DA) catheters using Clay-Adams PE-50 polyethylene tubing while their gills were irrigated with water containing anaesthetic (Soivio *et al.*, 1972). Heparin was not used during surgery or blood sampling due to its stimulation of lipoprotein lipase (Rennie *et al.*, 1976). Once surgery was complete, trout were revived in fresh water containing no anaesthetic and allowed to recover for ~48 hr in dark, aerated, 2.5 L, acrylic boxes supplied with ~100 ml·min⁻¹ freshwater at 10°C. During recovery, catheters were flushed daily with Cortland saline (Wolf, 1963).

Arterial blood and white muscle were terminally sampled at rest, immediately after exhaustive exercise, and at 0.25, 0.5, 1, 2, 4, 8, 16, and 32 hr recovery. Resting fish were kept in the acrylic boxes for at least 48 h before sampling. For exhaustive exercise, individual fish were transferred from their acrylic box to a 150 L circular tank filled with water at experimental temperature and manually chased to exhaustion [5 min; similar protocol to Wang *et al.* (1994a)]. Upon exhaustion, identified by no further response to manual stimulation, trout were returned to their individual boxes and sampled at the pre-assigned recovery times. At sampling, trout were terminally anaesthetized by adding 0.5 g·L⁻¹ MS-222 to their surrounding water from a neutralized stock solution. During the onset of anaesthesia, 3 mL arterial blood was drawn into an ice-cold gas-tight Hamilton syringe through the DA catheter. Plasma was immediately separated from blood cells by centrifugation at 16,000 g for 10 s. A portion (300 µL) of the plasma was deproteinized in 600 µL of 1 M HClO₄ and the remaining plasma (~1.5 mL) was frozen in liquid nitrogen.

At complete anaesthesia (~ 1 min), the fish was removed from water, and a white muscle sample was excised from between the lateral line and dorsal fin with a scalpel. The muscle samples were immediately freeze-clamped between two aluminium blocks cooled in liquid N₂ and all samples were stored under liquid N₂ for later analysis. White muscle sampling took less than 10 s.

Analytical Techniques

An aliquot of frozen white muscle was broken into small pieces (50 to 100 mg) in an insulated mortar and pestle cooled with liquid N₂. Several pieces of white muscle were stored separately in liquid N₂ for determination of PDH activity. The remaining broken muscle was lyophilized for 72 hr, dissected free of connective tissue, powdered, and stored dry at -80°C for subsequent analysis.

The active fraction of PDH (PDH_a) was measured in muscle homogenates using a modified technique of Putman *et al.* (1995). Briefly, muscle (30 to 50 mg) was homogenized in 15 times its wet weight in a buffer containing 200 mM sucrose, 50 mM KCl, 5 mM MgCl₂, 5 mM EGTA, 50 mM TRIS·HCl, 50 mM NaF, 5 mM dichloroacetic acid, 0.1% Triton X-100, at pH 7.5. Homogenates were immediately frozen in liquid N₂ until analysis on the same day. To assay for PDH activity, homogenates were thawed on ice, and 60 µL aliquots of homogenate were incubated in duplicate at 10°C in an assay buffer containing 144 mM TRIS·HCl, 0.72 mM EDTA, 1.44 mM MgCl₂, 3 mM NAD⁺, 1 mM CoA-SH, 1 mM thiamine pyrophosphate, at pH 7.5. The reaction was initiated by the addition of 1 mM pyruvate and 200 µL aliquots of the incubation media were sampled at 2, 4, and 6 min, except those tissues from the exhausted fish that were sampled at 1, 2, and 3 min because of the high PDH activity. Tissue blanks were also run with homogenates incubated in the same buffer, but with the addition of deionized water instead of pyruvate. The reaction was stopped by addition of each aliquot to 40 µL of 0.5 M HClO₄. After 5 min at room temperature, each aliquot was neutralized with 1 M K₂CO₃, centrifuged for 3 min at 16,000 g, and stored at -80°C until analysis of acetyl-

CoA (see below). Pyruvate dehydrogenase activity determined in the presence of pyruvate was corrected for PDH activity in the blank, and a regression between acetyl-CoA production and time was used to calculate reaction rates.

Total PDH activities (PDH_{tot}) were assayed on a separate group of fish taken from the same stock. Briefly, muscle was homogenized in a similar buffer as described for PDH_a with the addition of 10 mM D-glucose, 10 mM $CaCl_2$, and 4 $U \cdot mL^{-1}$ sulphate free hexokinase. Homogenates were immediately frozen in liquid N_2 , thawed on ice, and incubated at 10°C for 30 min before samples were incubated in assay buffer as described above for PDH_a . The percent mole fraction of PDH transformation was determined by dividing PDH_a by PDH_{tot} .

An aliquot of lyophilized muscle was used for the determination of GS activity. Briefly, 5 to 10 mg of dry muscle was homogenized at -25°C in 200 μL of buffer containing 50 mM imidazole-HCl, 100 mM KF, 10 mM EDTA, 60% (vol/vol) glycerol, at pH 7.5. Homogenates were then diluted with 800 μL of the above buffer without glycerol and homogenized further at 0°C. Total glycogen synthase (GS_{tot}) and the active fraction (GS_a) were determined at saturating and physiological concentrations of glu 6-P, respectively. The GS assay measured the incorporation of glucose from UDP-glucose into glycogen with the subsequent analysis of liberated UDP. For GS_{tot} activity (high glu 6-P), 100 μL aliquots of homogenate were incubated with 450 μL of buffer containing 50 mM imidazole-HCl, 2 mM EDTA, 0.2 % (wt/vol) glycogen, 0.02 % (wt/vol) bovine serum albumin (BSA), and 0.5 mM dithiothreitol, 10 mM glu 6-P, at pH 7.5. For GS_a , 100 μL aliquots of homogenate were incubated with 450 μL of buffers of the same

composition as for GS_{tot} , except glu 6-P concentrations were adjusted to reflect those measured *in vivo* in white muscle (see Table 2-2 in results). The reactions for GS_a and GS_{tot} were initiated by the addition of 8 mM UDP-glucose and incubated at 10°C for 45 min. The reaction was stopped by addition of 60 μ L 0.5 M HCl, and after 10 min on ice, samples were neutralized with 60 μ L 0.5 M KOH, centrifuged at 20,000 g for 5 min at 4°C, and the supernatant assayed for free UDP. Free UDP was assayed in a buffer containing 20 mM TRIS·HCl, 30 mM KCl, 4 mM $MgCl_2$, 0.02 % (wt/vol) BSA, 0.4 mM phospho(*enol*)pyruvate, 0.2 mM NADH, and 5 U/mL lactate dehydrogenase following the oxidation of NADH after the addition of 3 U/mL pyruvate kinase. The percent mole fraction of GS activation was determined by dividing GS_a by GS_{tot} .

For the determination of muscle glycogen, ~20 mg of lyophilized muscle was digested in 1 mL 30% KOH at 100°C. Glycogen was isolated as described by Hassid and Abraham (1957) and free glucose was determined after digestion with amyloglucosidase (Bergmeyer, 1983). IMTG was determined by measuring total glycerol spectrophotometrically after transmethylation with tetraethylammonium hydroxide (20% aqueous solution; Keins and Richter, 1998).

For the extraction of metabolites from white muscle, aliquots of lyophilized muscle (~20 mg) were weighed into borosilicate tubes; 1 mL of 1 M $HClO_4$ was added and homogenized for 20 s at 0°C using a Virtis handishear homogenizer at the highest speed. Homogenates were transferred to 1.5 mL centrifuge tubes, centrifuged for 5 min at 20,000 g at 4°C, and the supernatant neutralized with 3 M K_2CO_3 . These extracts were assayed for ATP, CrP, Cr, pyruvate, lactate, glu 6-P, fructose 6-phosphate (fru 6-P),

glycerol 3-phosphate (gly 3-P), and glycerol spectrophotometrically (Bergmeyer, 1983). Acetyl-CoA, CoA-SH, acetyl-, free-, and total-carnitine were assayed on neutralized extracts according to radiometric methods (Cederblad *et al.*, 1990). Short chain fatty acyl carnitine was estimated by subtracting acetyl-carnitine and free carnitine from total carnitine. Long chain fatty acyl carnitines were determined on digested white muscle pellets after HClO₄ extraction. Briefly, white muscle pellets were suspended in 1 M HClO₄, vortexed, and centrifuged at 4,800 g for 5 min at 4°C. The washed pellet was then digested in 0.5 mL 0.5 M KOH for 1.5 h at 50°C, neutralized with 0.25 mL 1 M HCl, and centrifuged for 10 min at 20,000 g at 4°C. The supernatant was then assayed for free carnitine as described above.

Malonyl-CoA was extracted from lyophilized, white muscle in 15 times its weight of 0.5 M HClO₄ containing 50 µM dithioerythritol and 10 µg/mL iso-butyl-CoA as an internal standard. After homogenization for 20 s at 0°C at the highest speed of the Virtis homogenizer, homogenates were centrifuged at 20,000 g for 10 min at 4°C, and 200 µL of supernatant was transferred to a borosilicate vial. Extract pH was adjusted to 4 – 5 with 17.5 µL 4 M NaOH while being vortexed. Supernatants were transferred to autosample vials containing 20 µL of 1 M MOPS (pH 6.8) and final pH of the sample determined using pH paper: pH was always < 5. Autosample vials containing the tissue extracts were immediately placed into a refrigerated autosampler (4°C; WISP 601; Waters, Mississauga, Ontario, Canada). Malonyl-CoA was separated by reverse-phase high performance liquid chromatography (RP-HPLC) using a modified method originally described by Demoz *et al.* (1995). Briefly, 50 µL aliquots of extract were automatically

injected onto a Kromasil-octadecyl silane (ODS) column (25 x 0.46 cm; 100Å ODS, 5µm; Chromatography (CSC) Sciences, Montreal, QC, Canada) fitted with a guard column packed with the same material. An elution gradient, set up by a Waters 660 controller, was used to separate the CoA esters. Solvent A was 100 mM sodium phosphate and 75 mM sodium acetate in ultrapure deionized water (pH 4.2) and solvent B was the same as A except in 30% CH₃CN. The gradient was as follows: 0 min, 90% A; 10 min 60% A; 17.6 min, 10% A. Baseline condition was established again after 8 min of washing with 90% A. The elution was carried out at ambient temperature and the flow rate was 1.5 ml·min⁻¹. Absorbance measurements were made at 254 nm on a photodiode array detector (Waters). Resulting peaks were manually identified by comparison of retention times to standards of known composition and peaks were quantified by comparison with the internal standard.

Plasma lactate and glycerol were analyzed on deproteinized plasma and triacylglycerol (TAG) was analyzed on plasma using spectrophotometric methods (Bergmeyer, 1983). Plasma total NEFA was analyzed using a Wako NEFA C assay kit (WAKO Chemicals, Osaka, Japan). In order to determine the fatty acid profiles, plasma NEFA samples were methylated using a modification of the methods of Lepage and Roy (1988) and separated using gas chromatography. Briefly, 150 µL of frozen plasma, along with 15 µg of heptadecanoic acid (internal standard) were added to 5 mL methanol-acetyl chloride mixture (50:1) in siliconized vials with tight fitting teflon lined caps. Vials were maintained at 25-26°C in a Reacti-Therm dry block, placed on a rotating stir plate, for 45 min. Methylation was stopped by the addition of 3 mL 6% K₂CO₃ followed by the

addition of 400 μL of hexane. Tubes were shaken and centrifuged at 2,000 g for 10 min and 300 μL of the upper hexane layer, containing the methyl esters, was removed and placed into 2 mL borosilicate vials with tight fitting teflon lined caps. Hexane was then evaporated under N_2 gas and the methyl esters were re-dissolved in 50 μL CS_2 . Methyl esters dissolved in CS_2 were stored under N_2 at -25°C until analysis. One μL CS_2 containing methyl esters was injected into a gas chromatograph (3400 star gas chromatograph, Varian, Mississauga, Canada) fitted with a flame ionization detector. Fatty acid methyl esters were separated on a DB-1 capillary column (30 m x 0.25 mm ID, 0.25 μm film; Chromatographic Specialities Inc., Brockville, Ontario, Canada) using a temperature gradient from 100°C to 300°C increasing at a rate of $5^\circ\text{C}\cdot\text{min}^{-1}$ and H_2 carrier gas. Unknown fatty acid methyl esters were identified by comparing their retention times with those of a known standard, and the fatty acid methyl esters were quantified by comparison with the internal standard.

Calculations

The concentrations of ADP_f and AMP_f were calculated from the near-equilibrium reactions of creatine kinase and adenylate kinase, respectively (Dudley *et al.*, 1987) and constants calculated from Schulte *et al.* (1992). Intracellular pH values used for the calculation of ADP_f and AMP_f were taken from a similar study from our laboratory (Wang *et al.*, 1994a) that demonstrated similar changes in muscle CrP and lactate immediately after exhaustive exercise and during 4 hr recovery. Changes in muscle pH_i

after manual exhaustive exercise are very similar across different studies (e.g. Milligan and Wood, 1986; Wang *et al.*, 1994a).

Data Presentation and Statistical Analysis

All data are presented as means \pm SE (n). All muscle metabolite concentrations determined on lyophilized tissues were converted back to wet weights by taking into account a wet:dry ratio of 4:1 (Wang *et al.*, 1994a). Exercise and recovery data were tested against resting data by a one-way analysis of variance (ANOVA). Significance was set at $\alpha = 0.05$, and, when obtained, the Tukey's honestly significant difference *post hoc* test was used to identify where significant differences occurred.

RESULTS

In response to manual chasing, trout swam vigorously for the first 1-2 min, thereafter swimming slowed for the remaining 5 min of exercise. Complete exhaustion was characterized by the lack of an avoidance response to > 20 s of handling.

Muscle Metabolites

Adenylates and Creatine Phosphate

Muscle [ATP] decreased by about 65% due to the exercise regime and remained lower than resting values for greater than 2 h post-exercise (Fig. 2-1A). Exhaustive exercise caused a 75% decrease in [CrP] that was restored to resting concentrations within 15 min (Fig. 2-1B). Decreases in [CrP] were mirrored by stoichiometric increases in [Cr] which remained lower than resting values for > 1 h (Table 2-1). The calculated [ADP_f] and [AMP_f] increased immediately after exhaustive exercise, but returned to resting values or lower by 15 min post-exercise. The ATP/ADP_f ratio followed the same pattern as [ADP_f] and [AMP_f], decreasing immediately after exhaustive exercise then recovering to resting values by 15 min (Table 2-1).

Glycogen Synthase

The maximal GS_{tot} activity was similar at rest and throughout the recovery period at $15.1 \pm 0.3 \text{ nmol} \cdot \text{g}^{-1} \text{ wet tissue} \cdot \text{min}^{-1}$ (n = 72) except at time zero where GS_{tot} was significantly lower at $11.8 \pm 0.6 \text{ nmol} \cdot \text{g}^{-1} \text{ wet tissue} \cdot \text{min}^{-1}$ (n = 7). The activation state of

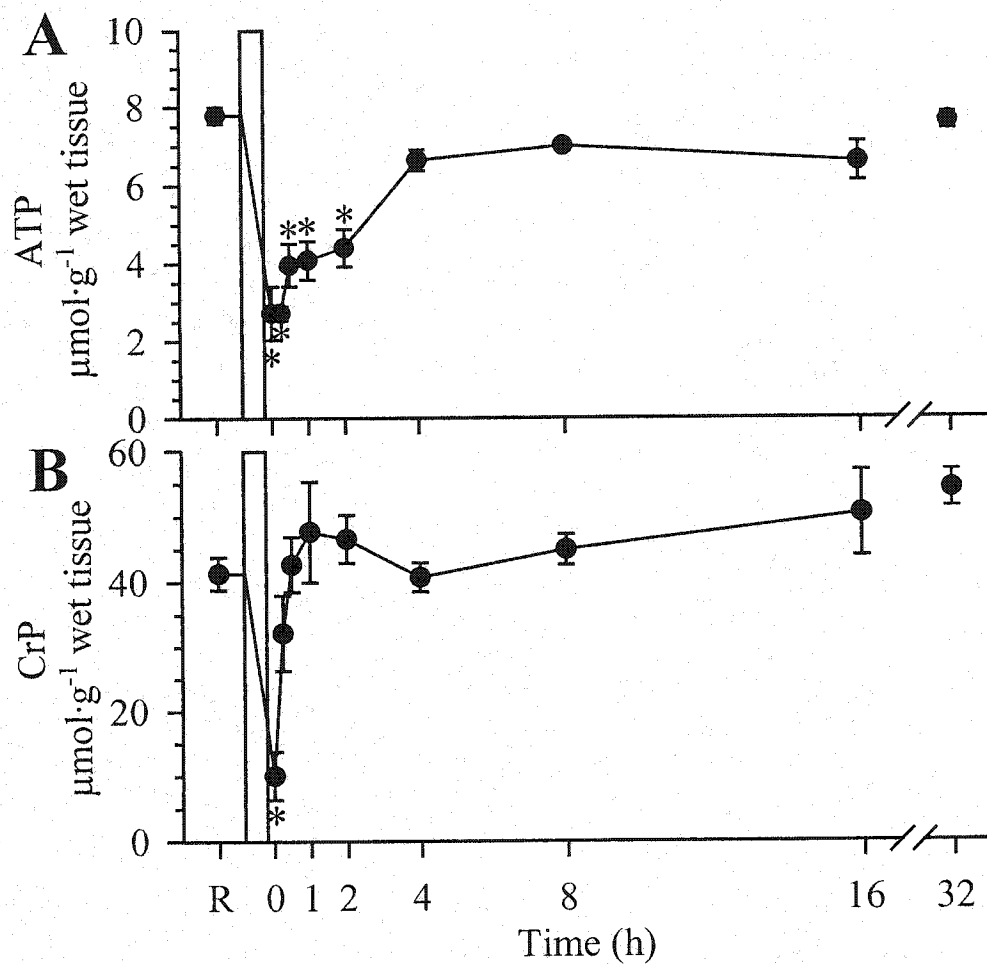


Figure 2-1:

White muscle ATP (A) and CrP (B) concentrations at rest (R) and during 32 hr recovery from exhaustive exercise. Vertical bar represents 5 min of exhaustive exercise. Data are means \pm SE, $n = 8$ for each point except at time zero where $n = 7$. Asterisks represent significant differences from rest.

Table 2-1: White muscle creatine concentrations and adenylate status at rest and during 32 h recovery from exhaustive

exercise.

Measure	Rest	Exhausted	Time									
			0.25 hr	0.5 hr	1 hr	2 hr	4 hr	8 hr	16 hr	32 hr		
Cr	19.8±2.3	57.6±4.2*	44.8±5.3*	33.1±2.9*	29.9±2.5	17.6±2.5	20.8±3.6	16.3±1.6	18.4±3.9	18.6±2.3		
pH _i [†]	7.20	6.80	6.75	6.79	6.82	7.05	7.05	7.2 [§]	7.2 [§]	7.2 [§]	7.2 [§]	7.2 [§]
ADP _f	7.9±1.2	21.1±5.6*	4.0±1.4	2.5±0.4	2.5±0.5	2.8±0.7	5.2±1.2	5.2±0.6	5.6±1.4	5.5±0.8		
AMP _f	0.23±0.06	9.31±4.61*	0.30±0.22	0.04±0.01	0.04±0.01	0.05±0.02	0.15±0.08	0.10±0.02	0.21±0.13	0.11±0.03		
ATP/ADP _f	1201±248	237±100	1165±268	1720±208	1942±259	2554±774	1710±323	1469±161	2051±716	1960±330		

Data are means ± SE (n=8 for each, except exhausted where n = 7). Cr, free creatine; pH_i intracellular pH; ADP_f free ADP;

AMP_f free AMP. Cr measurements are in μmol·g⁻¹ wet tissue, ADP_f and AMP_f are in nmol·g⁻¹ wet tissue. [†]Data are taken

from a similar study by Wang *et al.* (1994). Asterisks represent a significant difference from rest. [§]These time points were not

measured by Wang *et al.* (1994), thus they are assumed to equal resting values.

GS (% of GS in the a form) increased from about 40% at rest to almost 90% during the bout of exhaustive exercise and remained transformed for >8 hr recovery (Fig. 2-2).

Glycogen, Glycolytic Intermediates, and IMTG

White muscle [glycogen] decreased by 85% during exhaustive exercise and this decrease was mirrored by stoichiometric (1:2) increases in muscle [lactate] (Fig. 2-3). Muscle glycogen took between 8 and 16 h to return to values that were not statistically different from resting concentrations, although they were still non-significantly lower at 32 h. [Lactate] recovered to resting values within 4 h.

Exhaustive exercise in trout caused large increases in the glycolytic intermediates, glu 6-P and fru 6-P, which remained elevated compared to resting values for 2 h and 15 min, respectively, thereafter returning to resting values (Table 2-2). Muscle [gly 3-P] increased by 85% due to the exercise and remained elevated for >2 h; these changes in [gly 3-P] were matched by non-significant decreases in glycerol (Table 2-2). White muscle [pyruvate] increased due to exhaustive exercise and returned to resting values by 30 min (Table 2-2). [IMTG] did not change during exercise, but decreased to a value that was significantly lower than resting concentrations at 1 hr recovery (Table 2-2).

Pyruvate Dehydrogenase

Total PDH activity was $167.6 \pm 8.9 \text{ nmol} \cdot \text{g}^{-1} \text{ wet tissue} \cdot \text{min}^{-1}$ ($n = 7$). Exhaustive exercise caused a 50-fold increase in PDH_a in trout white muscle and fully transformed PDH into the active state (Fig. 2-4). Following the activation of PDH at exhaustion, there

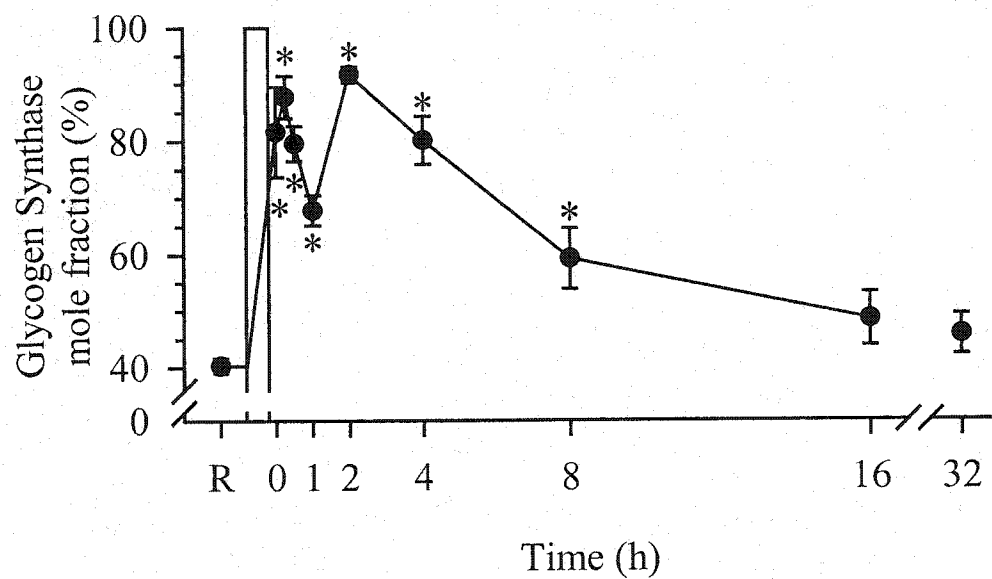


Figure 2-2:

White muscle glycogen synthase activation state at rest (R) and during 32 h recovery from exhaustive exercise. See figure 2-1 caption for other details.

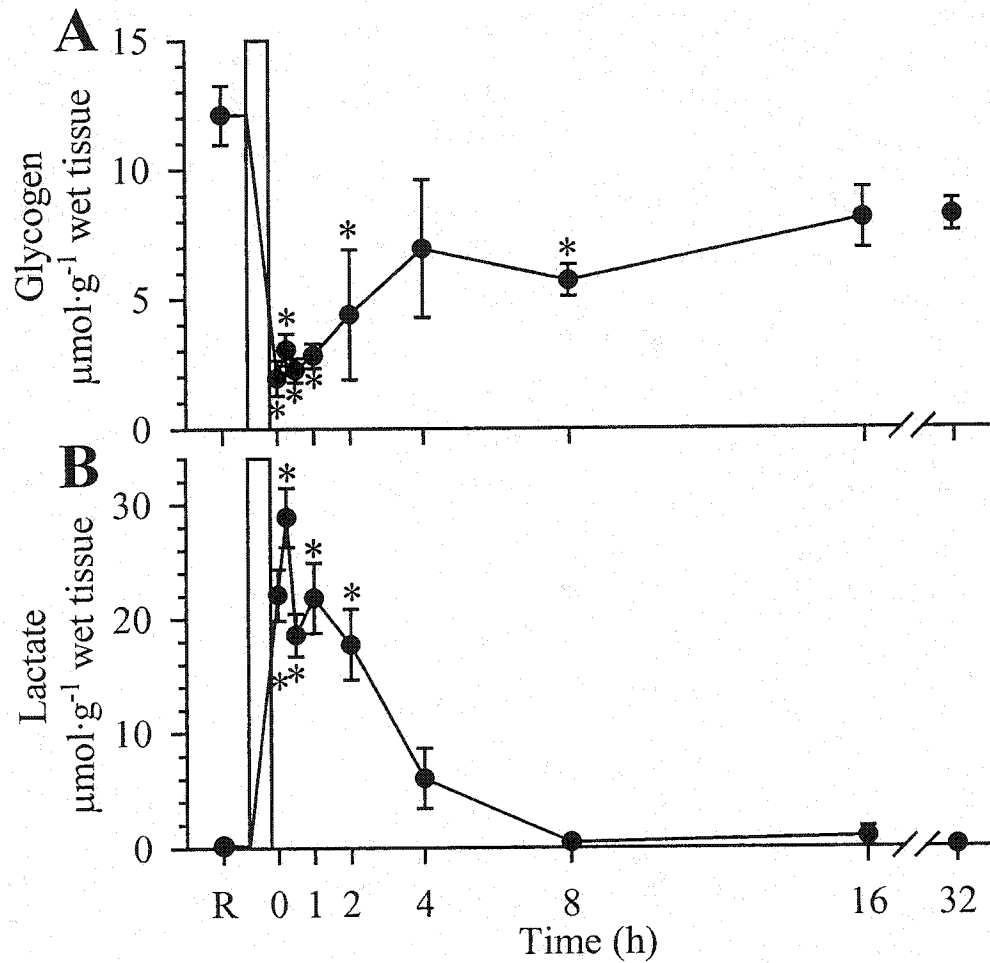


Figure 2-3:

White muscle glycogen (A) and lactate (B) concentrations at rest (R) and during 32 h recovery from exhaustive exercise. See figure 2-1 caption for other details.

Table 2-2: White muscle metabolite content at rest and during 32 h recovery from exhaustive exercise.

Measure	Rest	Exhausted	Time									
			0.25 hr	0.5 hr	1 hr	2 hr	4 hr	8 hr	16 hr	32 hr		
Glu 6-P	0.02±0.01	0.51±0.24*	0.57±0.16*	0.23±0.09	0.11±0.04*	0.49±0.13*	0.28±0.10	0.10±0.05	0.05±0.03	0.12±0.09		
Fru 6-P	<0.01	0.19±0.12*	0.24±0.10*	0.05±0.04	0.03±0.01	0.22±0.10	0.09±0.07	0.07±0.03	0.03±0.02	<0.01		
Gly 3-P	0.13±0.02	0.82±0.09*	0.94±0.05*	0.81±0.66*	0.85±0.15*	0.84±0.12*	0.39±0.13	0.07±0.02	0.21±0.11	0.08±0.02		
Pyruvate	0.05±0.02	0.43±0.07*	0.30±0.04*	0.13±0.03	0.25±0.05	0.18±0.04	0.04±0.01	0.03±0.01	0.14±0.12	0.03±0.01		
Glycerol	1.42±0.21	0.51±0.21	0.61±0.31	0.54±0.69	0.71±0.27	0.65±0.18	1.00±0.17	1.34±0.18	3.51±1.93	2.04±0.51		
TAG	19.2±1.1	16.5±0.9	15.0±2.6	18.0±2.5	12±1.2*	15.7±1.2	16.9±2.4	14.6±1.3	15.0±0.8	18.4±1.2		

Data are means ± SE in $\mu\text{mol}\cdot\text{g}^{-1}$ wet tissue ($n=8$ for each, except exhausted where $n = 7$). Glu 6-P, glucose 6-phosphate; Fru 6-P, fructose 6-phosphate; Gly 3-P, glycerol 3-phosphate; TAG, triacylglycerol. Asterisks represent a significant difference from rest.

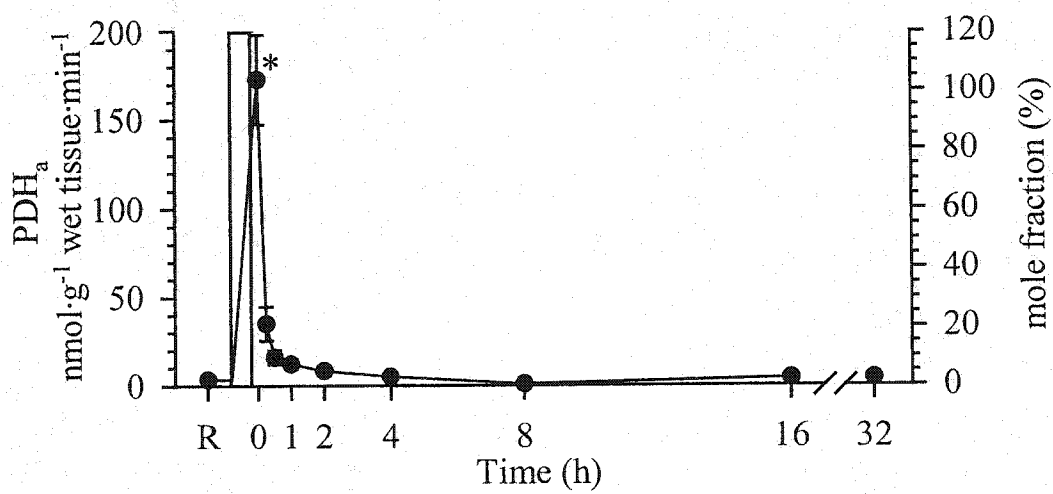


Figure 2-4:

White muscle pyruvate dehydrogenase activity and PDH_a mole fraction at rest (R) and during 32 h recovery from exhaustive exercise. See figure 2-1 caption for other details.

was a dramatic decrease in PDH_a transformation and activity, back to resting values, within the first 15 min post-exercise.

Acetyl Group Accumulation and Carnitine

Muscle [CoA-SH] did not change significantly after exercise and throughout the post-exercise period (Fig. 2-5) and constituted ~90% of the total CoA pool within the muscle. Muscle [acetyl-CoA] increased by 1.6 fold at 15 min recovery and remained elevated compared to resting values for >2 h (Fig. 2-5).

[Acetyl-carnitine] increased by five fold during exhaustive exercise and continued to increase by another 1.3 times the resting value during the first 15 min of the post-exercise period (Fig. 2-6A). Acetyl-carnitine concentrations remained elevated for up to 4 h and then returned to resting values.

Total and SCFA-carnitine concentrations remained constant throughout the exercise regime and during recovery (Fig. 2-6A). Muscle [free carnitine] was not affected by the exercise regime, but decreased by 35% during the first 15 min of the post-exercise period. Free carnitine concentrations remained low for 1 hr recovery then returned to resting values. Long chain fatty acyl carnitine concentrations increased 1.4 fold over the first 15 min (Fig. 2-6B) then returned to resting values.

Malonyl-CoA

Muscle [malonyl-CoA] did not change due to the exhaustive exercise, but it increased gradually to approximately twice the resting levels at 2 and 4 h (Fig. 2-7). Subsequently, [malonyl-CoA] returned to resting values by 8 h.

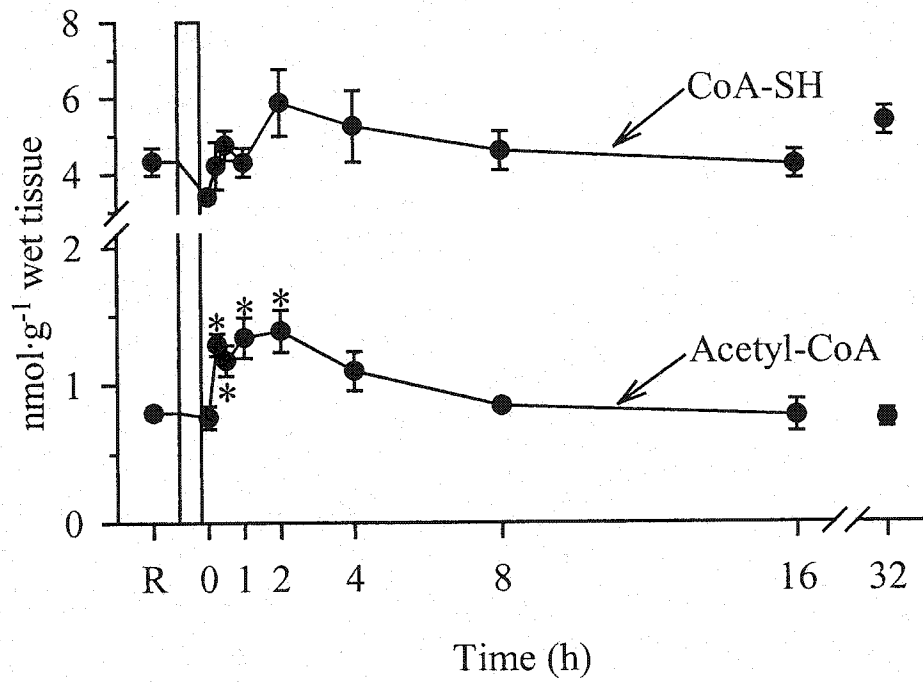


Figure 2-5:

White muscle acetyl-CoA and free CoA (CoA-SH) concentrations at rest (R) and during 32 h recovery from exhaustive exercise. See figure 2-1 caption for other details.

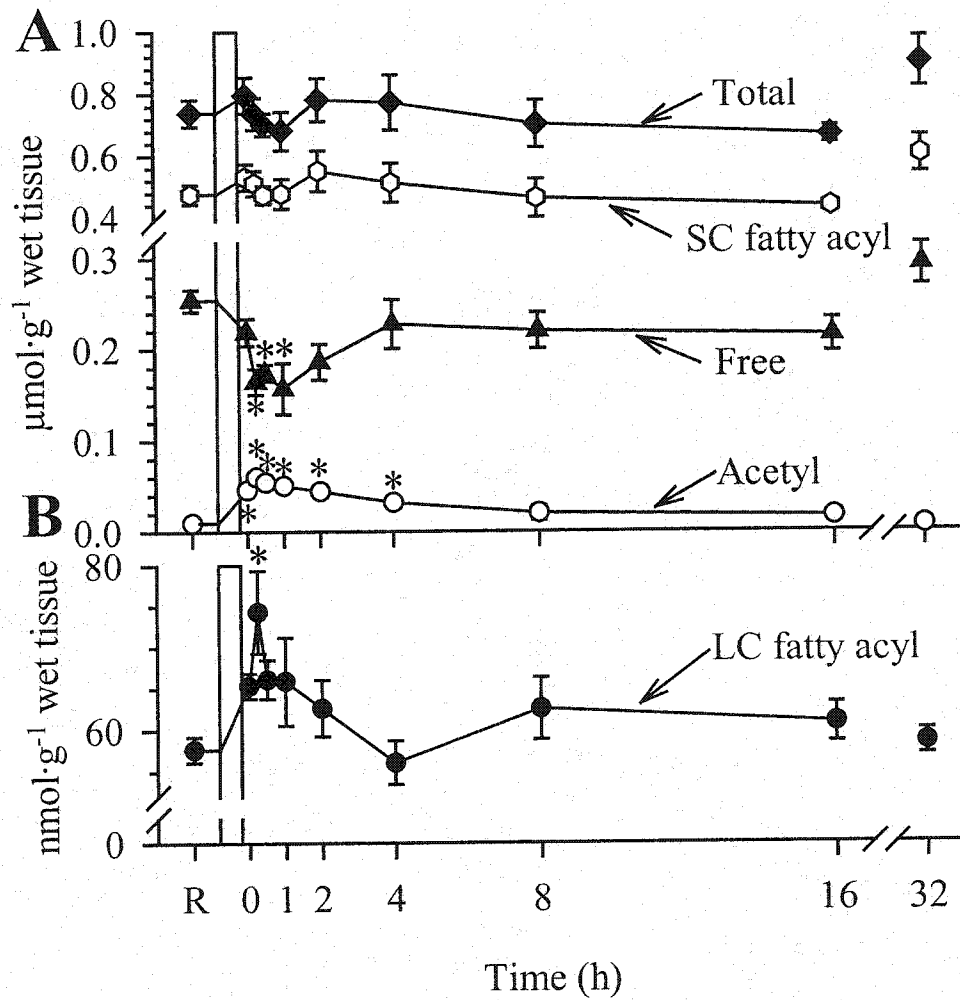


Figure 2-6:

White muscle acetyl-carnitine (Acetyl), free carnitine (Free), short-chain (SC) fatty acyl carnitine, and total carnitine (Total) concentrations (A) and long chain (LC) fatty acyl carnitine (B) at rest (R) and during 32 h recovery from exhaustive exercise. See figure 2-1 caption for other details.

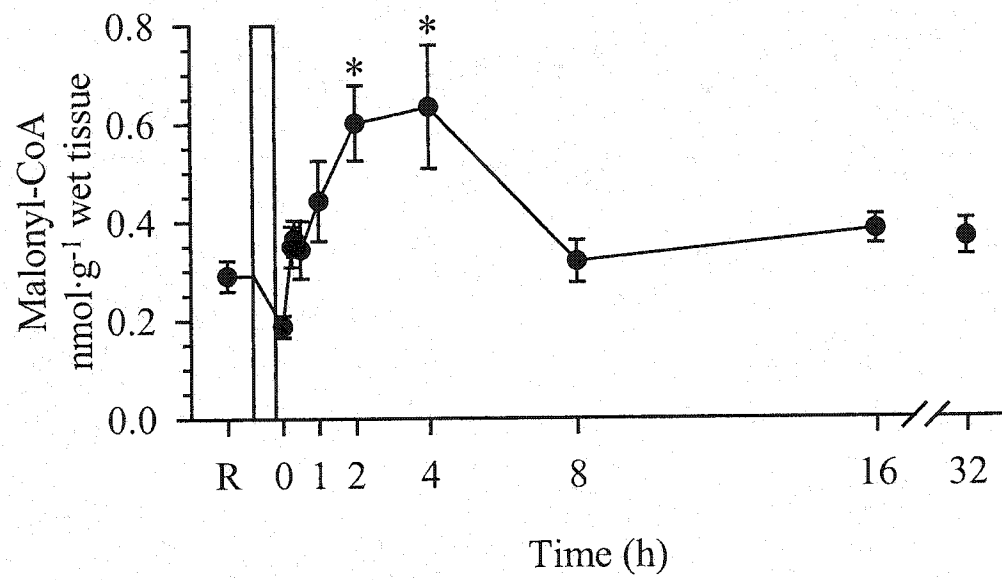


Figure 2-7:

White muscle malonyl-CoA concentrations at rest (R) and during 32 h recovery from exhaustive exercise. See figure 2-1 caption for other details.

Plasma Metabolites

Plasma [lactate] increased ~5 fold due to exhaustive exercise, and the level reached 10-fold during the first 1 h of the post-exercise period (Table 2-3). Plasma lactate concentrations returned to resting values by 8 h. Plasma [glycerol] increased due to the exercise regime, but returned to resting concentrations within 15 min (Table 2-3). Plasma TAG remained constant compared to resting concentrations throughout the exercise regime and during the post-exercise period (Table 2-3).

Total [NEFA], measured by enzymatic analysis, was not affected by exhaustive exercise, but decreased within the first 15 min post-exercise and remained depressed for 1 h then returned to resting values (Table 2-4). Analysis of plasma NEFA by gas chromatography (GC; Table 2-4) yielded changes in total [NEFA] that followed a similar trend to that observed by enzymatic analysis, but the concentrations were 4 to 6 fold higher by GC. At rest, palmitic acid (16:0) accounted for ~ 24% of the total [NEFA] while unsaturated 18, 20, and 22 carbon NEFA comprised 20, 15, and 26%, respectively, of the total [NEFA]. The decreases in total [NEFA] observed by enzymatic and GC analysis during recovery were made up of significant decreases in palmitoleic acid (16:1) and unsaturated 18 carbon fatty acids, plus non-significant decreases in many of the others (Table 2-4).

Table 2-3: Plasma metabolite content at rest and during 32 h recovery from exhaustive exercise.

Measure	Time											
	Rest	Exhausted	0.25 hr	0.5 hr	1 hr	2 hr	4 hr	8 hr	16 hr	32 hr		
Lactate	0.6±0.2	3.1±0.3*	4.4±0.5*	3.9±0.6*	6.6±0.6*	5.6±0.7*	5.5±1.1*	1.4±0.4	0.5±0.2	0.43±0.12		
Glycerol	0.03±0.03	0.16±0.02*	0.06±0.03	0.11±0.03	0.09±0.01	0.06±0.01	0.05±0.02	0.06±0.02	0.11±0.02	0.04±0.02		
TAG	18.1±3.0	22.9±4	15.0±2.9	21.7±4.5	18.8±3.4	17.8±3.4	16.4±4.0	13.8±3.1	18.2±2.0	18.6±4.4		

Data are means ± SE in $\mu\text{mol}\cdot\text{mL}^{-1}$, n = 8 for each except at rest where n = 7. TAG, triacylglycerol. Asterisks represent a significant difference from rest.

Table 2-4: Plasma nonesterified fatty acid content at rest and during 32 h recovery from exhaustive exercise.

Measure	Time										
	Rest	Exhausted	0.25 hr	0.5 hr	1 hr	2 hr	4 hr	8 hr	16 hr	32 hr	
14:0	39±9	39±13	17±10	25±9	21±13	43±1	56±25	17±13	48±20	4±4	
16:0	359±64	306±39	241±40	246±21	256±21	317±40	276±40	229±46	277±20	254±45	
16:1	67±16	41±14	14±7*	36±11	39±4	57±1	46±11	19±10	49±19	28±6	
18:0	100±14	120±37	81±17	115±34	96±12	118±32	93±21	66±27	81±26	79±30	
18 Unsaturated	304±76	168±23	91±36*	169±32	149±10*	187±40	194±32	152±18	178±17	197±64	
20:0	8±5	28±15	12±8	18±12	11±6	19±19	9±7	N/D	N/D	4±4	
20 Unsaturated	185±24	178±59	129±26	141±33	123±16	258±96	145±25	72±25	112±24	121±5	
22:0	N/D	2±2	N/D	4±4	N/D	19±19	N/D	N/D	N/D	N/D	
22 Unsaturated	406±66	392±110	210±79	326±50	303±37	510±214	34324160	260±108	298±52	297±36	
24:0	11±5	17±8	6±6	6±4	13±7	37±2	13±7	9±5	11±142	19±8	
Total GC	1479±221	1292±296	802±187	1087±152	1010±84	1565±458	1174±189	860±145	1053±142	1000±140	
Total Enz.	320±30	260±50	140±40*	130±20*	110±20*	220±70	240±90	200±40	200±30	260±50	

Data are mean±SE in $\text{nmol}\cdot\text{mL}^{-1}$. From left n = 8, 7, 5, 7, 8, 2, 8, 6, 6, and 4. All plasma NEFA data were determined by gas chromatography (GC) except Total Enz. which was determined by enzymatic analysis (See Methods). N/D, non-detectable. Asterisks represent a significant difference from rest.

DISCUSSION

The present study examined the effects of 5 min exhaustive exercise on white muscle metabolism in trout and monitored the recovery of muscle metabolites for up to 32 hr. Based on substrate depletion and enzyme activities, we have demonstrated that CrP hydrolysis, glycolysis, and oxidative phosphorylation of carbohydrate fuels are responsible for ATP production during exhaustive exercise in trout white muscle. Immediately post-exercise, there is a dramatic shift in substrate utilization from phosphagen and carbohydrate during exercise to lipid during recovery. Furthermore, this substrate shift from carbohydrate to lipid oxidation during recovery occurs in the presence of a high concentration of carbohydrate substrate (lactate). Our data argue against lactate oxidation during recovery (classical O₂ debt hypothesis; Hill and Lupton, 1923) and add further evidence to the growing idea that recovery metabolism is supported by lipid oxidation (Moyes *et al.*, 1992; Wang *et al.*, 1994a; Keins and Richter, 1998).

ATP Production during Exercise

It has been well established in the literature [reviewed by Kieffer (2000)] that the high-ATP turnover rates observed during exhaustive exercise in fish are sustained primarily by substrate level phosphorylation (CrP) and “anaerobic” glycolysis yielding lactate production. The large changes in white muscle ATP and CrP concentrations (Fig. 2-1) and the large decreases in white muscle glycogen and accumulation of lactate (Fig.

2-3) observed immediately after exercise in the present study further adds credence to the notion of substrate phosphorylation and glycolytic supply of ATP to support exercise. However, the maximal transformation of PDH to PDH_a observed at the end of exercise (Fig. 2-4) also clearly implicates oxidative phosphorylation of carbohydrate-based fuels as an additional pathway supplying ATP for muscle contraction during exercise.

Pyruvate dehydrogenase is the rate-limiting enzyme that regulates the entry of glycolytically derived pyruvate into the TCA cycle and oxidative metabolism (Weiland, 1983); therefore, PDH controls the oxidative utilization of carbohydrate fuels. Pyruvate dehydrogenase activity is regulated by both product inhibition (NADH and acetyl-CoA) and by reversible covalent modification (phosphorylation and dephosphorylation). The transformation of PDH between active PDH_a and inactive PDH_b is regulated by the relative activities of PDH kinase, which phosphorylates and thus deactivates PDH, and the activity of PDH phosphatase, which dephosphorylates and thus activates PDH (Weiland, 1983). PDH kinase is stimulated by elevated ratios of acetyl-CoA to CoA-SH, ATP to ADP_f, and NADH to NAD⁺ and is inhibited by elevated pyruvate concentrations (van der Vusse and Reneman, 1996). PDH phosphatase is stimulated by elevated Ca²⁺ concentration (Weiland, 1983).

At the onset of exercise, Ca²⁺ release from the sarcoplasmic reticulum probably acted as the initial cue to activate PDH in the trout white muscle. Subsequently, an accumulation of pyruvate (Table 2-2) from high glycolytic flux and a decrease in ATP/ADP_f (Table 2-1) likely acted to maximally stimulate PDH during exhaustive exercise. There was no change in acetyl-CoA/CoA-SH ratio at exhaustion (Fig. 2-5), and

in a very similar study in our laboratory the redox potential (NADH/NAD⁺) of white muscle cytoplasm did not change during a comparable exercise regime (Wang *et al.*, 1994a). The constant acetyl-CoA/CoA-SH ratio and redox state at exhaustion indicate that there were no strong inhibitory forces acting upon PDH kinase and thus PDH transformation. If PDH was fully transformed into PDH_a for the 5 min of exhaustive exercise, oxidative phosphorylation of pyruvate could contribute up to 13 $\mu\text{mol ATP}\cdot\text{g}^{-1}$ wet tissue in addition to the $\sim 80 \mu\text{mol ATP}\cdot\text{g}^{-1}$ wet tissue supplied by ATP, CrP, and glycogen.

The activation of PDH at exhaustion also challenges the dogma that lactate accumulation during high intensity exercise is due to “anaerobiosis” (Dobson *et al.*, 1987). In fact, accumulating evidence in human muscle suggests that lactate accumulation is due to a mismatched balance between the activities of glycogen phosphorylase, which sets the upper limit for glycogen entry into glycolysis, and the activity of PDH (Parolin *et al.*, 1999). The same, yet accentuated, explanation of lactate production can be applied to trout white muscle. White muscle of fish has a very low mitochondrial content (<2% volume density; Johnson, 1981) and very likely has a low copy number of PDH. Maximal activation of PDH in trout white muscle is not sufficient to accommodate the rate of pyruvate production by glycolysis during exhaustive exercise, thus resulting in lactate accumulation. Clearly, more research is needed to clarify the role of PDH in ATP and lactate production in trout white muscle during exhaustive exercise.

Recovery Metabolism in White Muscle

The pattern of white muscle metabolite recovery observed in the present study is in general agreement with many previously published studies (Wood, 1991; Schulte *et al.*, 1992; Wang *et al.*, 1994a). In general, trout confined in acrylic black boxes during recovery show a rapid recovery of CrP (within 15 min), slower recovery of ATP and lactate (2-4 h), and very slow recovery of glycogen (>8 h). The rapid recovery of CrP indicates an immediate activation of ATP generating pathways, which results in the phosphorylation of accumulated Cr. The reason for the slower recovery of ATP seems elusive, but several possibilities exist. First, decreases in white muscle ATP concentrations are mirrored by a stoichiometric increase in IMP (Schulte *et al.*, 1992; Wang *et al.*, 1994a). For the resynthesis of ATP from IMP, there must be activation of the IMP-AMP conversion arm of the purine-nucleotide cycle that requires the input of nitrogen and guanosine 5'-triphosphate (Mommensen and Hochachka, 1988). In addition, utilization of ATP during recovery of CrP and glycogen may delay the recovery of endogenous ATP.

Glycogen synthesis is regulated, in part, by the activation of the rate-limiting enzyme GS which catalyses the addition of glucose from UDP-glucose to glycogen (Soderling *et al.*, 1977). The transformation of GS between active GS_a and inactive GS_b is regulated by the relative activities of a GS kinase and a GS phosphatase. Phosphorylation by a kinase inactivates GS, and dephosphorylation by a phosphatase activates GS. In human muscle, GS phosphatase is primarily stimulated by an increase in glu 6-P and inhibited by high ATP and glycogen concentrations (Okubo *et al.*, 1988).

The large decreases in white muscle glycogen observed at the end of exercise (Fig. 2-3A) and the accumulation of glu 6-P during the first 4 hr of recovery were probably the main allosteric regulators for the stimulation of GS phosphatase activity and therefore activation of GS. As a result, within 30 min post-exercise, ~90% of the GS was transformed into GS_a (relative to a resting level of ~40%) and remained elevated compared to resting values, for > 8 h post-exercise (Fig. 2-2). During this period, there was a 53% increase in white muscle glycogen and a return to resting values for white muscle lactate (Fig. 2-3B). However, our data suggests further that the maximal activity of GS_a (14.4 nmol·g⁻¹ wet tissue·min⁻¹) may limit the rate of recovery in trout white muscle. At the maximal GS activities measured during recovery in trout muscle, it would take >12 h for glycogen to be resynthesized; similar to the pattern of glycogen recovery observed (Fig. 2-3A).

The slow recovery observed in the present study is probably due to the confinement of the trout immediately after exercise. Milligan *et al.* (2000) have shown that rainbow trout swum at 0.9 body lengths·s⁻¹ during recovery restore white muscle glycogen and lactate concentrations to resting values within 2 h post-exercise *versus* >4 h needed in the present study and others (Dobson and Hochachka, 1987; Milligan and Girard, 1993; Wang *et al.*, 1994a). There is mounting evidence that cortisol release during the post-exercise period in confined trout may be responsible for prolonged recovery times. If cortisol levels are kept low, either by allowing the fish to swim slowly during the post-exercise period (Milligan *et al.*, 2000), or by pharmacological blockade of cortisol synthesis or release (Eros and Milligan, 1996), trout white muscle carbohydrate

and acid-base status recovers at an accelerated rate. However, the precise mechanism behind the action of cortisol during recovery remains elusive. Given the apparent limiting activity of GS in trout white muscle demonstrated in the present study, there might be a yet uninvestigated link between cortisol release and GS activity.

At the onset of recovery, the main purpose of ATP production shifts from providing energy for actin-myosin cycling during the exercise to providing energy for the resynthesis of metabolites. During recovery, ATP must be rapidly generated to resynthesize CrP ($\sim 31 \mu\text{mol ATP}\cdot\text{g}^{-1}$ wet tissue needed within 15 min recovery) followed by a slower synthesis of ATP and glycogen (>2 h and >8 h, respectively). From the present study, ATP synthesis from IMP (Wang *et al.*, 1994a) would require $\sim 10 \mu\text{mol ATP}\cdot\text{g}^{-1}$ wet tissue, and glycogen synthesis from lactate would require between 19 – 30 $\mu\text{mol ATP}\cdot\text{g}^{-1}$ wet tissue. The total ATP required to fuel recovery in the present study was calculated to be between 60 and 70 $\mu\text{mol ATP}\cdot\text{g}^{-1}$ wet tissue.

Fate of Lactate during Recovery

Over the past several decades, numerous studies have aimed to determine the fate of accumulated lactate during recovery. Although it is generally well accepted that lactate is retained in trout white muscle during recovery for *in situ* glycogen synthesis (Schulte *et al.*, 1992), no study has been able to conclusively rule out oxidation as a minor end-point for the accumulated lactate. In fact, the complete oxidation of only 4 to 6 $\mu\text{mol lactate}\cdot\text{g}^{-1}$ wet tissue could supply enough ATP (60 to 70 $\mu\text{mol}\cdot\text{g}^{-1}$ wet tissue) to support the complete recovery of white muscle CrP, ATP, and glycogen and represents

only 15 to 20% of the accumulated lactate. Lactate disappearance in trout white muscle is faster than glycogen resynthesis (Fig. 2-3) and this discrepancy has been taken as evidence to support the contention that a portion of lactate is oxidized by muscle during recovery to supply the ATP for glycogen synthesis (Hill and Lupton, 1923). However, the discrepancy between lactate and glycogen recovery can, in part, be explained by lactate appearance in the plasma (see Table 2-3) and slow oxidation or carboxylation of pyruvate by pyruvate carboxylase in other tissues such as the liver (Milligan and Girard, 1993).

Lactate oxidation during recovery would require the sustained transformation of PDH into PDH_a as well as a maintained catalytic rate. Within 15 min post exercise, PDH is nearly fully transformed into its inactive form (PDH_b). This rapid transformation into PDH_b is probably due to increases in acetyl-CoA/CoA-SH (see Fig. 2-5) and ATP/ADP_r (Table 2-1) ratios acting to increase PDH kinase activity, resulting in greater PDH phosphorylation and inactivation of PDH. On the sole basis of the transformation state of PDH, $\sim 6 \mu\text{mol}\cdot\text{g}^{-1}\text{ wet tissue}\cdot\text{min}^{-1}$ of pyruvate could be decarboxylated by PDH during the first 4 hr of recovery, allowing enough lactate oxidation to provide ATP for recovery. Under most exercise conditions (*e.g.* Putman *et al.*, 1995; Parolin *et al.*, 1999) the catalytic rate of PDH is equal to the transformation state. However, in the present study, it seems likely that the catalytic rate of PDH would be far lower than indicated by transformation because of significant product inhibition (acetyl-CoA from lipid oxidation; Fig. 2-5) and the low substrate concentration (pyruvate) observed during recovery (Table 2-2). Therefore the rate of lactate oxidation *in vivo* would be far less

than required to supply ATP for recovery. However, these regulatory effects of low pyruvate and elevated acetyl-CoA on PDH have only been documented in mammalian muscle (Weiland, 1983) and await confirmation in fish muscle.

Lipid Oxidation during Recovery

The present research contributes significantly to the proposed idea that the majority of ATP needed for recovery is generated by an activation of β -oxidation utilizing lipid as a substrate (Moyes *et al.*, 1992; Wang *et al.*, 1994a). Fish maintain large labile lipid stores both within their muscle, as IMTG, and in adipose tissue, also as TAG (Moyes and West, 1995), both of which can release NEFA for oxidation. For long chain NEFA to be oxidized by β -oxidation, they must first be bound to carnitine by CPT-1 for transport into the mitochondria (van der Vusse and Reneman, 1996). During the first hour post-exercise, there was a rapid binding of long chain NEFA to carnitine resulting in a decrease in muscle free carnitine (Fig. 2-6). The subsequent action of β -oxidation yielded acetyl-CoA which contributed to the significant elevation in white muscle acetyl-CoA concentrations for >2 h (Fig. 2-5). However, to sustain flux through β -oxidation during the initial portion of recovery, intramitochondrial acetyl-CoA concentrations were kept relatively low through the formation of acetyl-carnitine (Fig. 2-6A). These results are in general agreement with those of Wang *et al.* (1994a), except the decreases in free carnitine in that study were due to an increase in binding of short chain NEFA to carnitine, rather than long chain NEFA alone as observed in the present study. This is a subtle difference, and the preferential binding of long chain NEFA to carnitine as

observed in the present study makes sense in that short chain fatty acids can pass freely through mitochondrial membranes and do not necessarily require carnitine for mitochondrial transport (van der Vusse and Reneman, 1996).

The rate-limiting step in muscle lipid oxidation is the CPT-1 catalyzed binding of NEFA, especially long chain NEFA, to carnitine for the subsequent transfer of fatty acyl carnitines into the mitochondria. Recent evidence implicates CPT-1 as the main point of regulation of lipid oxidation through the interactions with malonyl-CoA. Malonyl-CoA is an intermediate in the *de novo* synthesis of fatty acids and has been demonstrated in rat muscle to negatively regulate CPT-1 and thus lipid metabolism (Saha *et al.*, 1997) and further contribute to the regulation of the glucose-fatty acid cycle (Randle *et al.*, 1963). However, malonyl-CoA does not equally regulate CPT-1 in all organisms. In human muscle, malonyl-CoA participates in the regulation of fuel selection at rest, but does not appear to be important for fuel selection during exercise (Odland *et al.*, 2000). In the trout white muscle, malonyl-CoA concentrations were low at rest and increased between 2 and 4 h post-exercise (Fig. 2-7). It is paradoxical that there were increases in malonyl-CoA during a period characterized by an increase in lipid oxidation. Two possibilities exist to explain these increases in malonyl-CoA during recovery. First, malonyl-CoA may not be an important modulator of CPT-1 in trout white muscle during recovery. Elevated malonyl-CoA may represent an increased elongation of short chain fatty acids in an attempt to maintain elevated concentrations of long chain fatty acids for mitochondrial oxidation (Ruderman *et al.*, 1999). Second, the delayed increase in malonyl-CoA may indicate that the majority of the costs of recovery are met within the first 2 h of recovery

and subsequently lipid oxidation is allosterically inhibited by increasing malonyl-CoA. Further research is needed to clarify the role of malonyl-CoA in trout muscle during recovery.

Further support for lipid oxidation during the early stages of recovery is provided by the general decreases in IMTG that were significant at 1 h post-exercise (38% reduction; Table 2-2). IMTG hydrolysis yields three NEFA for β -oxidation and one glycerol. If the decrease in IMTG represents complete oxidation of TAG containing palmitic acid (16:0) it could supply $1.8 \text{ mmol ATP}\cdot\text{g}^{-1}$ wet tissue, 21 fold more ATP than required for recovery metabolism. Thus, it is likely that in addition to increased oxidation of fatty acids during recovery there is probably an increase in TAG – NEFA cycling between the muscle and other tissues, both contributing to the EPOC observed in juvenile rainbow trout (Scarabello *et al.*, 1991a). Utilization of endogenous lipid during recovery is supported further by the results of Milligan and Girard (1993) who showed large, highly variable decreases in trout white muscle total lipid concentrations after exhaustive exercise that persisted through 6 h recovery.

Nonesterified fatty acids released into the bloodstream from adipose tissue may also be utilized during recovery for ATP synthesis. Plasma total NEFA concentration, determined using enzymatic analysis, decreased during the first 15 min and remained depressed for up to 1 h post-exercise. These decreases in plasma NEFA concentration were primarily due to significant decreases in palmitoleic (16:1) and unsaturated 18 C fatty acids, though many of the others also tended to decrease (Table 2-4). Furthermore, these decreases in plasma NEFA were not associated with a change in plasma TAG

(Table 2-3) indicating that esterification of NEFA into TAG does not occur in the extracellular fluid during recovery. The decrease in plasma NEFA observed during recovery was probably due to the combined effects of increased NEFA uptake from the plasma in addition to decreased NEFA release from adipose tissue.

The mobilization of NEFA from adipose tissue is determined by the relative activities of two opposing non-equilibrium reactions: lipolysis of stored TAG and re-esterification of NEFA into TAG. This substrate cycling between NEFA and TAG constitutes a means of rapidly adjusting substrate flux without extreme activation or inactivation of any one reaction (Wasserman and Cherrington, 1996). Stimulation of hormone sensitive lipase (HSL), by the characteristic mobilization of catecholamines into trout plasma after exhaustive exercise (*e.g.* Milligan *et al.*, 2000), would be expected to shift NEFA-TAG cycling toward NEFA production, thus resulting in a release of NEFA into circulation (Wasserman and Cherrington, 1996). However, high plasma lactate concentrations, such as those observed during the post-exercise period in trout (Table 2-3), have been demonstrated to inhibit HSL in human adipose tissue (Boyd *et al.*, 1974; Wasserman and Cherrington, 1996). Inhibition of HSL would result in reduced NEFA release from adipose tissue. Increased muscle uptake of NEFA coupled with decreased NEFA release from adipose tissue may explain the reduction in plasma NEFA concentrations.

The distribution of NEFA within plasma, as determined by gas chromatography, is similar to distributions observed by other researchers who employed the same methylation technique (Harrington *et al.*, 1991); however, there are unresolved

differences in plasma NEFA concentrations when analyzed using enzymatic analysis *versus* GC. Trout, like many teleost fish, are unique in that they have high concentrations of high-density lipoprotein in their plasma [HDL; 15 mg·mL⁻¹ (Chapman *et al.*, 1978) *versus* ~2 mg·mL⁻¹ in mammals (Metcalf *et al.*, 1999)], and in addition to albumin, they utilize HDL as a fatty acid transport protein. The chemical methylation process involved in GC may liberate NEFA from HDL and therefore yield higher plasma [NEFA] whereas HDL-bound NEFA may be undetectable by the enzymatic method. If this analytical possibility is true, it suggests that HDL may be the major fatty acid binding protein in trout plasma (similar to carp; Krauskopf *et al.*, 1988), accounting for ~80% of the total NEFA carrying capacity of the plasma. This analytical possibility deserves further attention.

The present study provides evidence that during recovery, the majority of the ATP needed to synthesize CrP, ATP, and glycogen is provided for by lipid oxidation. NEFA, especially long chain NEFA, from both exogenous and endogenous sources are taken up by the mitochondria of white muscle *via* a carnitine dependent transport mechanism (CPT-1) and oxidized by β -oxidation yielding acetyl-CoA. Acetyl groups are accumulated in the muscle post-exercise and support ATP production through increased TCA cycle flux and oxidative phosphorylation. Increases in malonyl-CoA during recovery do not appear to limit fatty acid oxidation, but may represent elongation of fatty acids for mitochondrial oxidation. Lactate is saved from an oxidative fate during recovery by a rapid transformation of PDH into its inactive form, in addition to product

inhibition, thus providing further evidence that glycogen synthesis is likely the major fate of lactate during recovery.

Perspectives

The notion that lipid oxidation provides ATP to support recovery from exercise has been in the literature for about a decade (*e.g.* Moyes *et al.*, 1992), but this hypothesis has remained relatively untested in most organisms. Recently, Keins and Richer (1998) have demonstrated lipid utilization during recovery from high-intensity exercise in the human. This represents a major shift from the classical O₂ debt hypothesis where lactate oxidation was thought to fuel recovery metabolism. By measuring the activities of flux-generating enzymes (GS and PDH), their allosteric modulators, and changes in substrate concentrations, we are able to provide insight into the mechanisms that regulate lipid *versus* carbohydrate oxidation. Our data strongly suggest that lipid oxidation prevails during recovery. Comprehensive studies that examine the regulation of substrate selection such as presented herein will prove to be a powerful tool for elucidating how substrate selection occurs during high-intensity exercise and during graded exercise intensities. The elusive role of malonyl-CoA in regulating CPT-1 also deserves further attention.

CHAPTER 3

GLYCOGEN PHOSPHORYLASE AND PYRUVATE DEHYDROGENASE TRANSFORMATION IN WHITE MUSCLE OF TROUT DURING HIGH-INTENSITY EXERCISE

ABSTRACT

We examined the regulation of Phos and PDH in white muscle of rainbow trout during a continuous bout of high-intensity exercise that led to exhaustion in 52 s. The first 10 s of exercise were supported by CrP hydrolysis and glycolytic flux from an elevated glycogenolytic flux and yielded a total ATP turnover of $3.7 \mu\text{mol}\cdot\text{g}^{-1}$ wet tissue $\cdot\text{s}^{-1}$. The high glycolytic flux was achieved by a large transformation of Phos into Phos_a. Exercise performed from 10 s to exhaustion was at a lower ATP turnover rate (0.5 to $1.2 \mu\text{mol}\cdot\text{g}^{-1}$ wet tissue $\cdot\text{s}^{-1}$) and therefore at a lower power output. The lower ATP turnover was supported primarily by glycolysis and was reduced because of post-

transformational inhibition of Phos by the accumulation of glu 6-P. During exercise, there was a gradual activation of PDH, which was fully transformed into its active form by 30 s of exercise. Oxidative phosphorylation, from PDH activation, only contributed 2% to the total ATP turnover, and there was no significant activation of lipid oxidation. The time course of PDH activation was closely associated with an increase in estimated mitochondrial redox ($[NAD^+]/[NADH]$), suggesting that O_2 was not limiting during high-intensity exercise. Thus, anaerobiosis may not be responsible for lactate production in trout white muscle during high-intensity exercise.

INTRODUCTION

The classical scheme of substrate use during high-intensity exercise involves the temporally separate utilization of CrP followed by an activation of glycolysis and then oxidative phosphorylation (Meyer and Foley, 1996). In this scheme, the initial depletion of CrP at the onset of exercise results in the accumulation of P_i , ADP_f , AMP_f , IMP, and NH_3 , which are thought to activate glycolysis for further ATP production. Subsequently, the accumulation of pyruvate and lactate from glycolysis stimulates PDH for ATP production *via* oxidative phosphorylation. Trout white muscle is known to rely primarily on CrP and glycogen to support high-intensity exercise (Kieffer, 2000). However, the majority of research in trout muscle metabolism has focused on elucidating the pattern of metabolite recovery after high-intensity exercise (Black *et al.*, 1962; Wood, 1991; Milligan and Girard, 1993; Kieffer, 2000; Milligan *et al.*, 2000; Richards *et al.*, 2002b) and the factors that regulate recovery metabolism (Schulte *et al.*, 1992). As pointed out by numerous studies (*e.g.* Wood, 1991; Wang *et al.*, 1994a; Moyes and West, 1995), little information is available on the dynamics of substrate selection and the integrated mechanisms that regulate fuel use in trout white muscle *during* high-intensity exercise.

Dobson *et al.* (1987) examined the dynamics of “anaerobic” ATP production in trout swum at exercise intensities ranging from a 10 s sprint ($\sim 150\% U_{crit}$) to 30 min of burst swimming leading to exhaustion. Ten second sprints were solely supported by CrP hydrolysis. During longer (>10 min) and slower swimming ($\sim 120\% U_{crit}$), glycogen was the primary fuel utilized through the activation of glycolysis. Control points in glycolysis

were identified primarily at PFK-1. Evidence from mammalian studies further implicates the rate-limiting enzyme, Phos in regulating glycolytic flux by setting the upper limit for glycogen entry into glycolysis (Chasiotis *et al.*, 1982; Parolin *et al.*, 1999). Glycogen phosphorylase is regulated at the transformational level by reversible phosphorylation events and at the post-transformational level through substrate availability (P_i), product inhibition (glu 6-P), and changes in allosteric modulators (ADP_f and AMP_f ; Ren and Hultman, 1988; Heigenhauser and Parolin, 1999b). Phosphorylation of Phos by Phos kinase transforms Phos_b (low activity) into Phos_a (high activity), whereas the dephosphorylation by Phos phosphatase converts Phos_a to Phos_b (Krebs *et al.*, 1964). The transformation between Phos_b and Phos_a is regulated at the contractile level through Ca^{2+} release from the sarcoplasmic reticulum. Decreases in pH_i affect the transformation of Phos by inhibiting Phos kinase (Connett and Sahlin, 1996) and affect substrate availability by shifting the speciation of P_i (Katz *et al.*, 1990). To our knowledge, the transformation of Phos has not been examined in trout white muscle during high-intensity exercise.

Pyruvate dehydrogenase is the rate-limiting enzyme that sets the rate at which glycolytically derived pyruvate is decarboxylated for entry into the TCA cycle and oxidative phosphorylation. The catalytic rate of PDH is regulated by covalent modification (phosphorylation and dephosphorylation) and by product inhibition (Weiland, 1983). Dephosphorylation of PDH by PDH phosphatase transforms the inactive PDH_b into the active PDH_a while phosphorylation of PDH by PDH kinase transforms PDH_a into PDH_b. PDH kinase is allosterically stimulated by acetyl-CoA,

NADH, and ATP and is inhibited by ADP, CoA-SH, NAD^+ , and pyruvate. In mammalian muscle, PDH phosphatase is stimulated by Ca^{2+} release from the sarcoplasmic reticulum and hormonally by insulin (Randle, 1995). Elevations in mitochondrial NADH inhibit PDH phosphatase and reduce its transformation. In trout white muscle, Richards *et al.* (2002b) demonstrated that PDH was maximally activated at exhaustion. Richards *et al.* (2002b), suggested that this pathway could have contributed up to 14% of the total ATP production during a bout of high-intensity exercise. Furthermore, the relative transformation patterns and catalytic rates of Phos and PDH have recently been implicated in lactate production in human muscle (Parolin *et al.*, 1999; Spriet *et al.*, 2000).

The goal of the present study was to determine the role of the rate limiting enzymes, Phos and PDH, in regulating white muscle metabolism during a continuous bout of high-intensity exercise. To accomplish these objectives, we measured the transformation states of Phos and PDH, as well as their allosteric regulators (*e.g.* ADP_f , AMP_f , P_i , pH_i), in white muscle of trout during a typical high-intensity exercise regime. Insights into cellular O_2 limitations during high-intensity exercise were made through estimates of cytoplasmic and mitochondrial redox states ($[\text{NAD}^+]/[\text{NADH}]$).

METHODS

Animal Care

Adult rainbow trout (*Oncorhynchus mykiss*, Walbaum; 211 ± 8 g, $n=40$) were purchased from Humber Springs Trout Hatchery, Orangeville, Ontario, Canada. Trout were transported to our freshwater holding facility and held in 800 L tanks supplied with aerated, dechlorinated city of Hamilton tapwater at 15°C for two months before experimentation. Fish were fed daily to satiation with commercial trout pellets. One day before an experiment fish were placed individually into dark, aerated, 2.5 L acrylic boxes supplied with $100 \text{ mL} \cdot \text{min}^{-1}$ freshwater at 15°C . All experimental procedures fully comply to the Guiding Principles for Research Involving Animals and Human Beings and with Canadian Council of Animal Care guidelines.

Experimental Protocol

To exercise fish, we transferred individual fish without air exposure from their acrylic box to a 150 L circular tank filled with aerated fresh water at 15°C . Fish were chased with almost constant body contact to avoid burst-glide type swimming. White muscle samples were terminally sampled at rest (see below) and at 10.5 ± 0.3 s, 20.4 ± 0.3 s, 30.0 ± 0.2 s ($n=8$) during high-intensity exercise and at exhaustion (51.8 ± 3.2 s; $n=8$). Fish were considered exhausted when they did not attempt to escape from manual chase. To sample muscle as quickly as possible, fish were removed from the tank by hand (with no struggle by the fish) at a pre-specified time and killed by concussion. A 0.5 to 1 cm

thick cross section of the fish was taken posterior to the dorsal fin and immediately freeze clamped between two aluminium blocks cooled in liquid N₂. This sampling position is consistent with that used by other researchers (*e.g.* Dobson *et al.*, 1987; Schulte *et al.*, 1992; Milligan and Girard, 1993; Wang *et al.*, 1994a). The entire sampling procedure took < 5 s.

To obtain resting white muscle samples, trout were terminally anaesthetized in their boxes by adding 0.5 g·L⁻¹ 2-aminobenzoic acid ethyl ester (methanesulfonate salt; MS-222) to the surrounding water from a neutralised stock solution. At complete anaesthesia (~ 1 min), the fish was removed from the water and two muscle samples were taken. The first muscle sample was immediately freeze clamped as described above. The second muscle sample was freeze clamped after a 1 min delay to obtain resting Phos activities (Ren and Hultman, 1988). All muscle tissues were stored under liquid N₂ until analyzed.

Analytical Techniques

The frozen muscle was broken into pieces (50 to 100 mg) in an insulated mortar and pestle cooled in liquid N₂. As in other studies (*e.g.* Dobson *et al.*, 1987; Schulte *et al.*, 1992; Milligan and Girard, 1993; Wang *et al.*, 1994a), only white muscle dorsal of the lateral line was used. Several aliquots of muscle were stored separately in liquid N₂ for determination of PDH_a and total PDH activity as previously described by Richards *et al.* (2002b). A portion of the frozen muscle (~ 50 mg) was ground into a fine powder under liquid N₂ and used for pH_i measurements as described by Pörtner *et al.* (1991).

Total muscle NH_3 was determined on frozen muscle by the glutamate dehydrogenase methods as described by Wang *et al.* (1994b). The remaining muscle was lyophilized for 72 hr, dissected free of connective tissue, powdered, and stored dry at -80°C for subsequent analysis.

An aliquot of lyophilized muscle was used for determination of Phos activity. Briefly, a known weight (5 to 10 mg) of dry muscle was homogenized at -25°C in 200 μL of buffer containing 100 mM TRIS, 50 mM KCl, 10 mM EDTA, in 60% glycerol at pH 7.5. Homogenates were then diluted with 800 μL of the above buffer without glycerol and homogenized further at 0°C . Total Phos activity (Phos_{tot} ; in the presence of 3 mM AMP) and Phos_a (in the absence of AMP) were measured by following the production of glu 1-P spectrophotometrically at 15°C . Maximal velocity (V_{max}) and the mole fraction of Phos_a were calculated as described by Chasiotis *et al.* (1982).

For the determination of muscle glycogen, ~ 20 mg of lyophilized muscle were digested in 1 mL 30% KOH at 100°C . Glycogen was isolated as described by Hassid and Abraham (1957), and free glucose determined after digestion with amyloglucosidase (Bergmeyer, 1983).

For the extraction of metabolites from white muscle, aliquots of lyophilized muscle (~ 20 mg) were weighed into borosilicated tubes, with 1 mL of ice-cold 1 M HClO_4 added, and homogenized at the highest speed of a Virtis hand-held homogenizer for 20 s at 0°C . Homogenates were transferred to 1.5 mL bullet tubes, centrifuged for 5 min at 20,000 g at 4°C , and the supernatant was neutralised with 3 M K_2CO_3 . These extracts were assayed spectrophotometrically for ATP, CrP, creatine, lactate, pyruvate,

glucose, glu 6-P, fru 6-P, glu 1-P, gly 3-P, glycerol, L-glutamate, and α -ketoglutarate using methods described in Bergmeyer (1983). Muscle acetyl-CoA, CoA-SH, and acetyl-, free-, SCFA-, LCFA-, and total carnitine were determined on neutralised extracts by radiometric methods previously described in Richards *et al.* (2002b).

Calculations

The concentrations of ADP_f and AMP_f were calculated according to Dudley *et al.* (1987) using constants calculated from Schulte *et al.* (1992). The concentration of P_i was calculated as the difference between resting and exercise [PCr], less the accumulation of glu 6-P, fru 6-P, glu 1-P, and gly 3-P. Resting concentrations of P_i were estimated from Wang *et al.* (1994a) by subtracting [resting PCr], and [ATP] from measured total phosphate and were $\sim 0.75 \mu\text{mol/g}$ wet tissue, a value which agrees well with low resting P_i values reported by van den Thillart *et al.* (1989).

The rates of glycogenolysis and glycolysis (in $\mu\text{mol glycosyl units}\cdot\text{g}^{-1}$ wet weight $\cdot\text{s}^{-1}$) were estimated as described by Spriet *et al.* (1987) from the accumulation of glycolytic intermediates plus the flux of pyruvate through PDH_a during each time interval. The equations were as follows

$$\text{Glycogenolysis} = \Delta([\text{glu 6-P}] + [\text{fru 6-P}]) + (\Delta([\text{gly 3-P}] + [\text{lactate}] + [\text{pyruvate}])/2)/\text{time} + PDH/2$$

$$\text{Glycolysis} = (\Delta([\text{gly 3-P}] + [\text{lactate}] + [\text{pyruvate}])/2)/\text{time} + PDH_a/2$$

where Δ represents a change in tissue metabolite concentration over a specified time period and PDH represents the catalytic rate of PDH_a (in $\mu\text{mol glycosyl units}\cdot\text{g}^{-1}$ wet

weight·s⁻¹). The catalytic rate of PDH_a has been demonstrated to equal flux of pyruvate into the TCA cycle in mammalian muscle (Gibala *et al.*, 1998; Howlett *et al.*, 1998).

ATP turnover from CrP was calculated from the breakdown of CrP assuming 1 mole of ATP is produced per mole of CrP consumed. ATP turnover from glycolysis was calculated from the accumulation of lactate and the flux of pyruvate through PDH_a assuming 1.5 mole of ATP produced per lactate. The rate of ATP turnover from oxidative phosphorylation was calculated as the total acetyl-CoA production from the PDH_a catalytic rate for each time period, assuming 1 mole of acetyl-CoA from glycogenolysis would yield 15 mole of ATP.

The redox state (*i.e.* [NAD⁺]/[NADH]) of the cytoplasm was estimated from the apparent equilibrium of the lactate dehydrogenase reactions using measurements of pHi, lactate, and pyruvate and K_{eq} from Wang *et al.* (1994a). Mitochondrial redox was estimated from the glutamate dehydrogenase reaction using whole cell measurements of NH₃, glutamate, α-ketoglutarate, K_{eq} from Williamson *et al.* (1967), and estimates of mitochondrial pH from Moyes *et al.* (1988) for carp muscle.

Data Presentation and Statistical Analysis

All data are presented as means ± SE (n). All metabolite concentrations determined on lyophilized tissues were converted back to wet weights by taking into account a wet:dry ratio of 4:1 (Wang *et al.*, 1994a). Statistical analysis consisted of a one-way ANOVA followed by a Least-Significant Difference method of pairwise multiple comparisons. Results were considered significant at $P < 0.05$.

RESULTS

In response to manual chasing, trout swam vigorously for the first 20 s of exercise, then slowed, but maintained burst activity until exhaustion at 51.8 ± 3.2 s ($n=8$).

Adenylates, Creatine Phosphate, and Cellular Energy Status

White muscle [ATP] decreased by 28% during the first 10 s of exercise, then remained stable until 20 s and further decreased to 23% of resting [ATP] at exhaustion (Fig. 3-1A). During the initial 10 s of exercise there was a 63% decrease in [CrP] that remained lower than resting values and more or less stable for the duration of the exercise (Fig. 3-1B). These decreases in [CrP] were matched by stoichiometric increases in [Cr] and these relative differences account for the majority of the calculated increase in [P_i] (Table 3-1). The calculated [ADP_f] and [AMP_f] both increased rapidly within the first 10 s of exercise then gradually decreased until exhaustion at which point [ADP_f] and [AMP_f] were not significantly higher than resting values (Table 3-1). As a result, the ATP/ADP_f ratio decreased during the first 10 s of exercise and remained depressed until exhaustion. Intracellular pH decreased during the initial 10 s of exercise, stabilised, and then further decreased at exhaustion (Table 3-1). White muscle total NH₃ increased gradually over the entire bout of exercise (Table 3-1).

Glycogen Phosphorylase

The maximum Phos_{tot} activity (V_{\max}) was 0.85 ± 0.09 $\mu\text{mol} \cdot \text{g}^{-1}$ wet tissue $\cdot \text{s}^{-1}$ ($n=8$) at rest and did not change during exercise. In contrast, Phos_a activity increased from

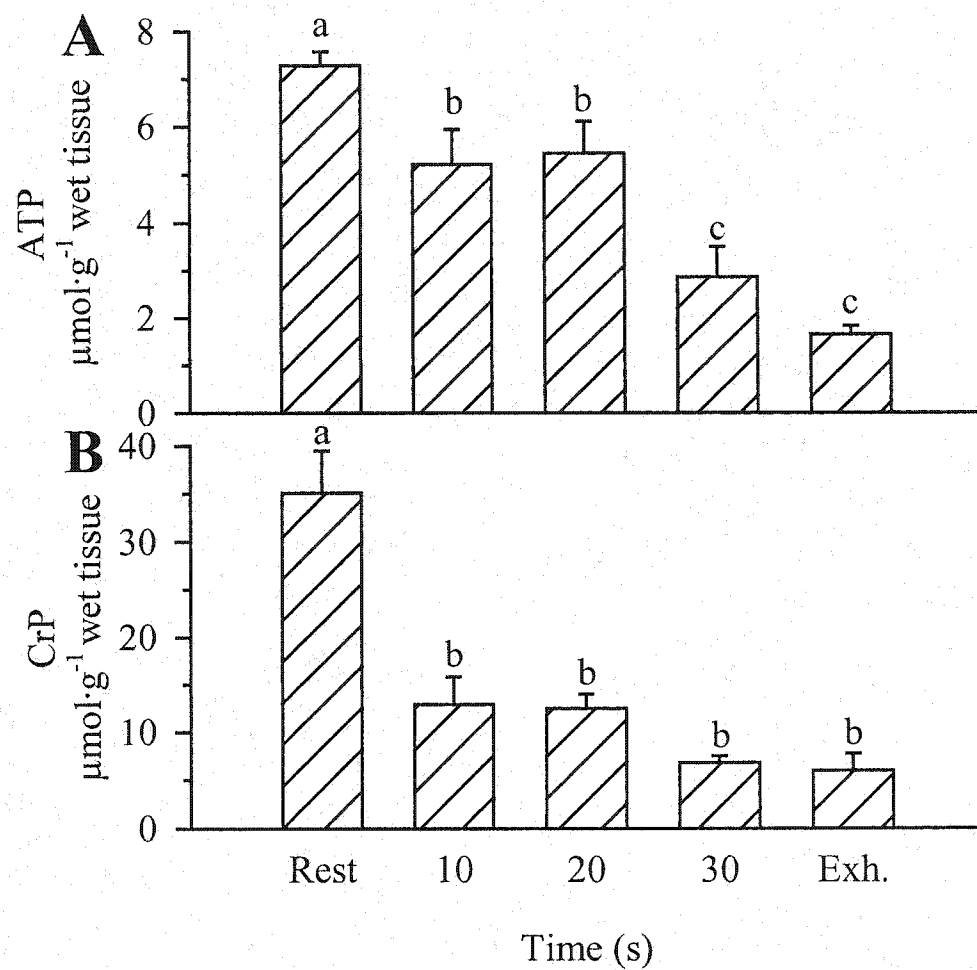


Figure 3-1:

White muscle ATP (A) and CrP (B) concentrations at rest and during high-intensity exercise. Exhaustion occurred at 51.8 s of manual chasing. Data are means \pm SE, $n = 8$ for each. Values with different letters are significantly different ($P < 0.05$).

Table 3-1: White muscle creatine, inorganic phosphate, adenylate, intracellular pH, and total NH₃ content in trout at rest and during high-intensity exercise.

Measure	Time				
	Rest	10 s	20 s	30 s	Exh.
Cr	23.2±2.2 ^a	45.4±5.2 ^b	45.8±1.5 ^b	53.3±1.8 ^b	52.2±1.8 ^b
P _i	1.0	22.3	21.2	27.5	28.1
ADP _f	15.6±1.9 ^a	56.0±9.6 ^b	40.9±4.3 ^{bd}	36.0±6.3 ^{cd}	22.4±4.8 ^{ac}
AMP _f	0.9±0.2 ^a	19.0±5.5 ^b	7.1±2.0 ^{ac}	13.1±3.7 ^{bc}	7.6±3.6 ^{ac}
ATP/ADP _f	516±118 ^a	190±49 ^b	173±25 ^b	87±13 ^b	102±29 ^b
pH _i	7.30±0.03 ^a	7.01±0.04 ^b	6.97±0.04 ^b	6.95±0.06 ^{bc}	6.85±0.04 ^c
NH ₃	0.91±0.28 ^a	2.12±0.40 ^a	3.63±0.72 ^b	4.47±0.65 ^b	6.21±0.51 ^c

Data are means ± SE (n=8). Cr, free creatine; P_i, free inorganic phosphate; ADP_f, free ADP; AMP_f, free AMP; pH_i, intracellular pH; NH₃, total ammonia. Cr, P_i, and total NH₃ are reported in μmol·g⁻¹ wet tissue, and ADP_f and AMP_f are reported in nmol·g⁻¹ wet tissue. Resting P_i was taken from Wang *et al.* (1994a) and changes in P_i due to exercise were estimated from changes in CrP (Fig. 3-1) glu 6-P, fru 6-P, glu 1-P, and gly 3-P (Table 3-2; see text for more details). Values with different letters are significantly different ($P<0.05$).

$0.16 \pm 0.02 \mu\text{mol} \cdot \text{g}^{-1} \text{ wet tissue} \cdot \text{s}^{-1}$ ($n=8$) at rest to $0.42 \pm 0.02 \mu\text{mol} \cdot \text{g}^{-1} \text{ wet tissue} \cdot \text{s}^{-1}$ ($n=8$; $P < 0.05$) at 10 s and then decreased significantly to $0.34 \pm 0.04 \mu\text{mol} \cdot \text{g}^{-1} \text{ wet tissue} \cdot \text{s}^{-1}$ at exhaustion ($n=8$; $P < 0.05$). As a result, the calculated transformation of Phos_b into Phos_a increased rapidly during the first 10 s of exercise, remained elevated until 30 s of exercise, and then decreased at exhaustion to levels that remained elevated compared with resting values (Fig. 3-2A).

Pyruvate Dehydrogenase

The total PDH (PDH_t) activity in white muscle of trout was $2.9 \pm 0.3 \text{ nmol} \cdot \text{g}^{-1} \text{ wet tissue} \cdot \text{s}^{-1}$ ($n=8$). In contrast, PDH_a activity increased gradually from resting values of $0.3 \pm 0.1 \text{ nmol} \cdot \text{g}^{-1} \text{ wet tissue} \cdot \text{s}^{-1}$ ($n=8$) to $3.0 \pm 0.3 \text{ nmol} \cdot \text{g}^{-1} \text{ wet tissue} \cdot \text{s}^{-1}$ ($n=8$) at 30 s and remained elevated until exhaustion. Consequently, the transformation of PDH_b into PDH_a increased gradually during the first 20 s of exercise, reaching 100% transformation at 30 s and exhaustion (Fig. 3-2B).

Muscle Metabolites

White muscle [glycogen] decreased by 35% during the first 10 s of exercise, remained more or less stable until 30 s, and then decreased at exhaustion to levels that were 16% of resting values (Fig. 3-3A). These changes in [glycogen] were matched by reciprocal increases in [lactate] and [pyruvate] (Fig. 3-3B and C). During the entire bout of exercise, there was a gradual accumulation of the glycolytic intermediates, glu 6-P, fru 6-P, and glu 1-P, all of which peaked in concentration at 20 s (Table 3-2). Muscle [gly 3-P] remained stable for the first 30 s of exercise and then increased at exhaustion

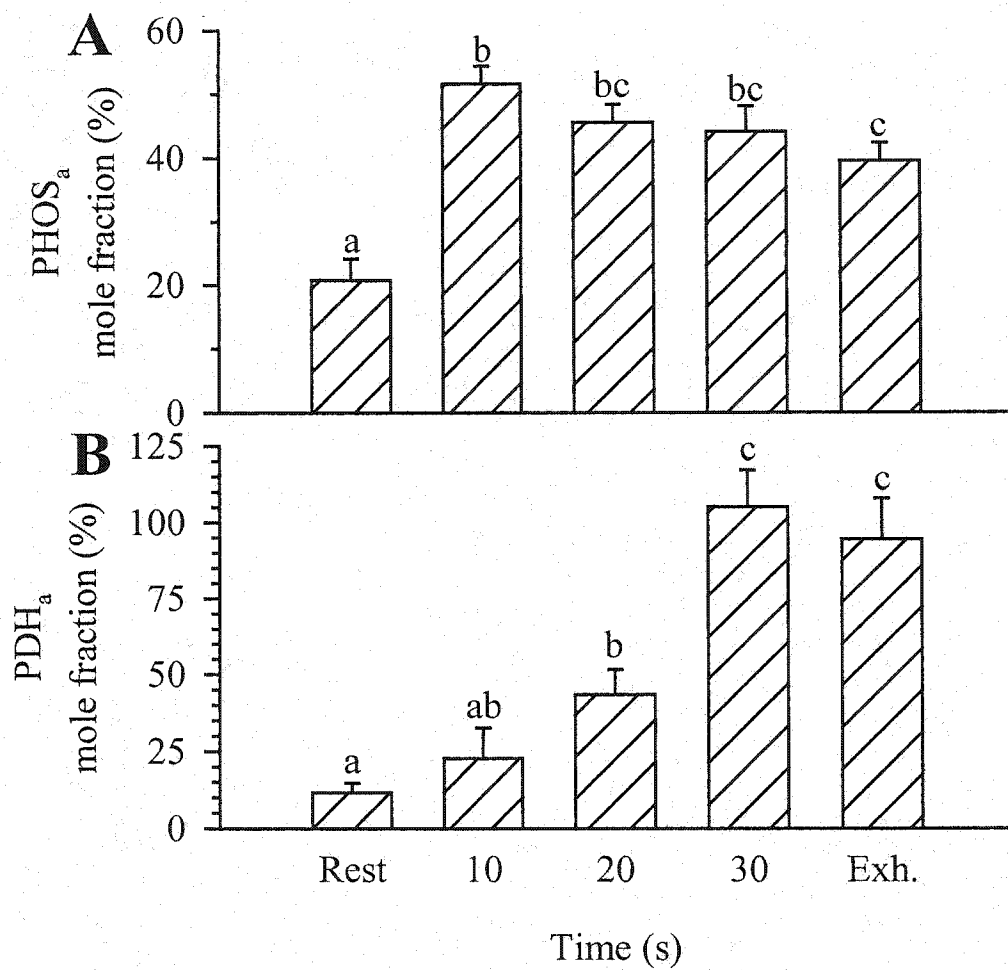


Figure 3-2:

White muscle glycogen phosphorylase (PHOS; A) and pyruvate dehydrogenase (PDH; B) mole fraction at rest and during high-intensity exercise. See figure 3-1 caption for other details.

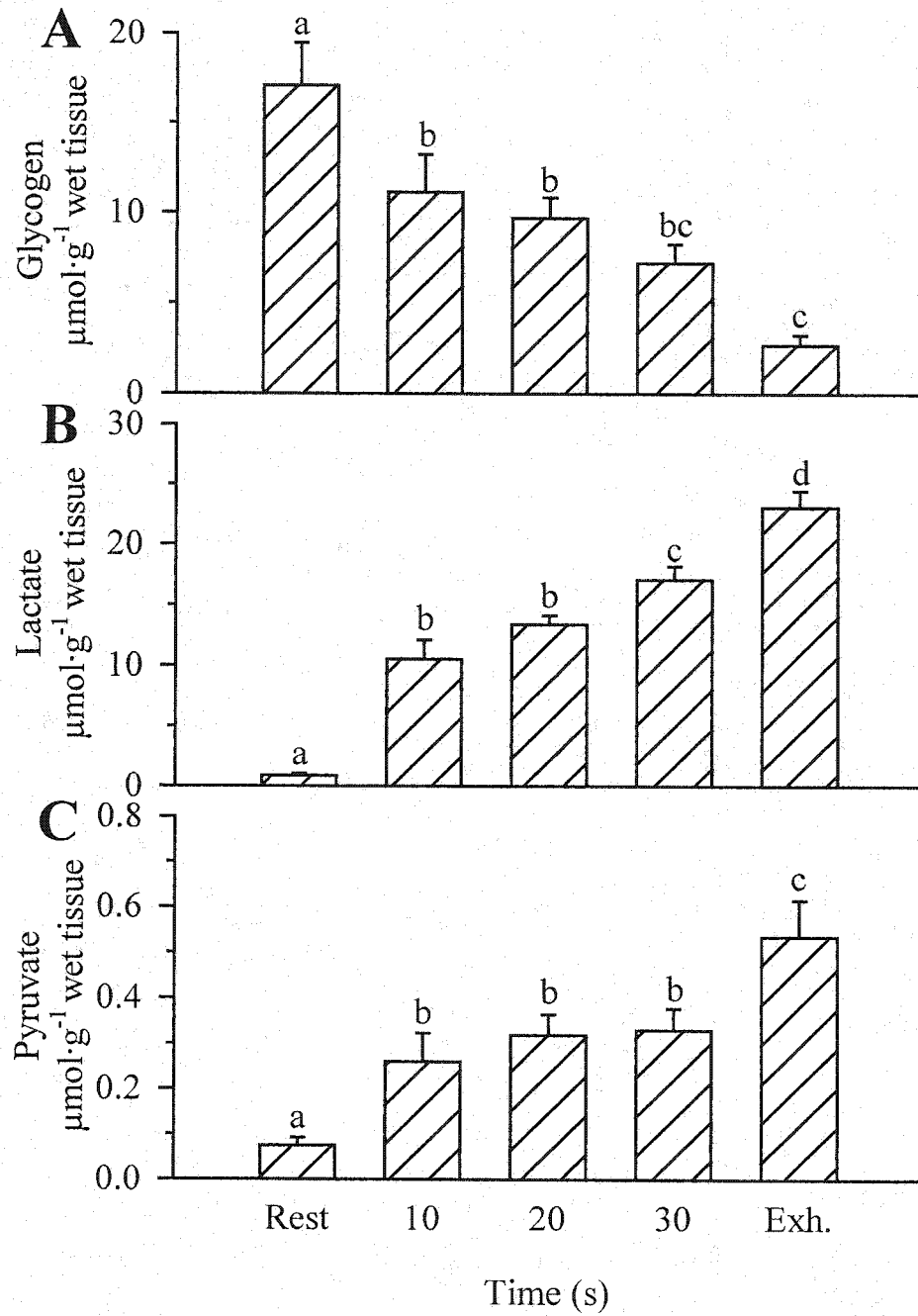


Figure 3-3:

White muscle glycogen (A), lactate (B), and pyruvate (C) concentrations at rest and during high-intensity exercise. Glycogen is expressed in glycosyl units. See figure 3-1 caption for other details.

Table 3-2: White muscle glycolytic intermediates, glycerol, glutamate, and α -ketoglutarate content at rest and during high-intensity exercise.

Measure	Time				
	Rest	10 s	20 s	30 s	Exh.
Glu 6-P	0.01±0.01 ^a	0.45±0.12 ^{ac}	1.33±0.39 ^b	0.91±0.29 ^{bc}	0.94±0.23 ^{bc}
Fru 6-P	0.01±0.01 ^a	0.16±0.09 ^{ac}	0.40±0.15 ^{bc}	0.13±0.05 ^{ac}	0.36±0.13 ^{bc}
Glu 1-P	0.23±0.05 ^a	0.40±0.13 ^{ac}	0.62±0.11 ^{bc}	0.37±0.08 ^{ac}	0.41±0.08 ^{ac}
Glucose	0.19±0.05 ^a	0.36±0.04 ^b	0.39±0.04 ^b	0.53±0.09 ^b	0.71±0.05 ^c
Gly 3-P	0.63±0.09 ^a	0.76±0.14 ^a	0.95±0.08 ^{ab}	0.85±0.14 ^{ab}	1.18±0.16 ^b
Glycerol	0.28±0.05 ^a	0.54±0.02 ^b	0.63±0.02 ^c	0.66±0.03 ^c	0.62±0.03 ^{bc}
Glutamate	2.66±0.40 ^a	2.51±0.35 ^a	2.12±0.13 ^a	2.85±0.23 ^a	2.68±0.39 ^a
α -ketoglu	0.05±0.01 ^a	0.06±0.01 ^a	0.08±0.02 ^a	0.14±0.07 ^a	0.07±0.01 ^a

Data are means \pm SE (n=8) in $\mu\text{mol}\cdot\text{g}^{-1}$ wet tissue. Glu 6-P, glucose 6-phosphate; Fru 6-P, fructose 6-phosphate; Glu 1-P, glucose 1-phosphate; Gly 3-P, glycerol 3-phosphate; α -ketoglu, α -ketoglutarate. Values with different letters are significantly different ($P<0.05$).

(Table 3-2). Muscle [glycerol] increased over the first 20 s of exercise then remained unchanged until exhaustion (Table 3-2). There were no changes in L-glutamate or α -ketoglutarate during exercise (Table 3-2). The calculated rates of glycogenolysis and glycolysis were both high during the first 10 s of exercise then decreased by ~60% during subsequent exercise (Fig. 3-4). There was higher glycogenolytic flux compared to glycolytic flux between 10 and 20 s, a trend that reversed between 20 and 30 s. Between 30 s and exhaustion calculated glycogenolytic and glycolytic fluxes were equal (Fig. 3-4).

White muscle [acetyl-CoA] did not change during high-intensity exercise except at 30 s where it decreased slightly (Table 3-3). However, muscle [CoA-SH] decreased by ~60% during the first 10 s of exercise, and remained lower than resting values until exhaustion (Table 3-3). There was no effect of exercise on muscle [acetyl-carnitine] (Table 3). Muscle [total carnitine] and [free carnitine] were variable, with a trend for a gradual decrease over the exercise period (Table 3-3). [SCFA-carnitine] and [LCFA-carnitine] did not change during exercise (Table 3-3).

Cytoplasmic redox did not change during exercise or at exhaustion (Fig. 3-5), but mitochondrial redox increased during high-intensity exercise (Fig. 3-5).

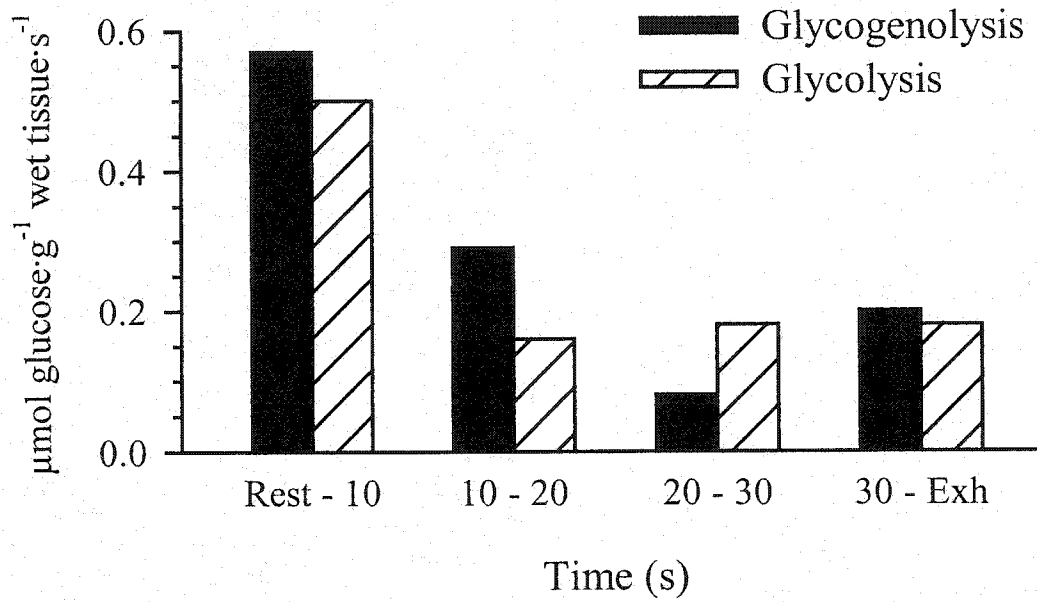


Figure 3-4:

Estimated white muscle glycogenolytic and glycolytic flux rates at rest and during high-intensity exercise.

Table 3-3: White muscle acetyl-CoA, CoA-SH, and carnitine derivative content at rest and during high-intensity exercise.

Measure	Time				
	Rest	10 s	20 s	30 s	Exh.
Acetyl-CoA	1.13±0.08 ^a	1.15±0.08 ^a	1.11±0.06 ^a	0.92±0.02 ^b	1.11±0.06 ^a
CoA-SH	3.93±0.66 ^a	1.59±0.26 ^b	2.26±0.31 ^b	1.71±0.39 ^b	1.34±0.19 ^b
Acetyl-Carn	19.0±2.1 ^a	36.8±10.2 ^a	20.4±2.7 ^a	25.1±6.0 ^a	33.3±9.4 ^a
Total-Carn	283.7±28.4 ^a	240.6±15.4 ^{ab}	233.8±13.1 ^{ab}	282.4±16.2 ^a	214.7±19.0 ^b
Free-Carn	190.1±19.4 ^a	140.3±13.3 ^{bc}	153.5±10.0 ^{abc}	150.1±17.2 ^{ac}	119.3±17.1 ^b
SCFA-Carn	93.7±11.4 ^a	100.3±10.8 ^a	80.3±8.0 ^a	110.1±10.8 ^a	92.4±13.9 ^a
LCFA-Carn	31.8±2.0 ^a	31.7±2.1 ^a	33.9±2.2 ^a	31.6±2.1 ^a	48.5±14 ^a

Data are means ± SE (n=8) in nmol·g⁻¹ wet tissue. Acetyl-CoA, acetyl-coenzyme A; CoA-SH, free coenzyme A; Acetyl-Carn, acetyl-carnitine; Total-Carn, total carnitine; Free-Carn, free carnitine; SCFA-Carn, short-chain fatty acyl carnitine; LCFA-Carn, long-chain fatty acyl carnitine. Values with different letters are significantly different ($P < 0.05$).

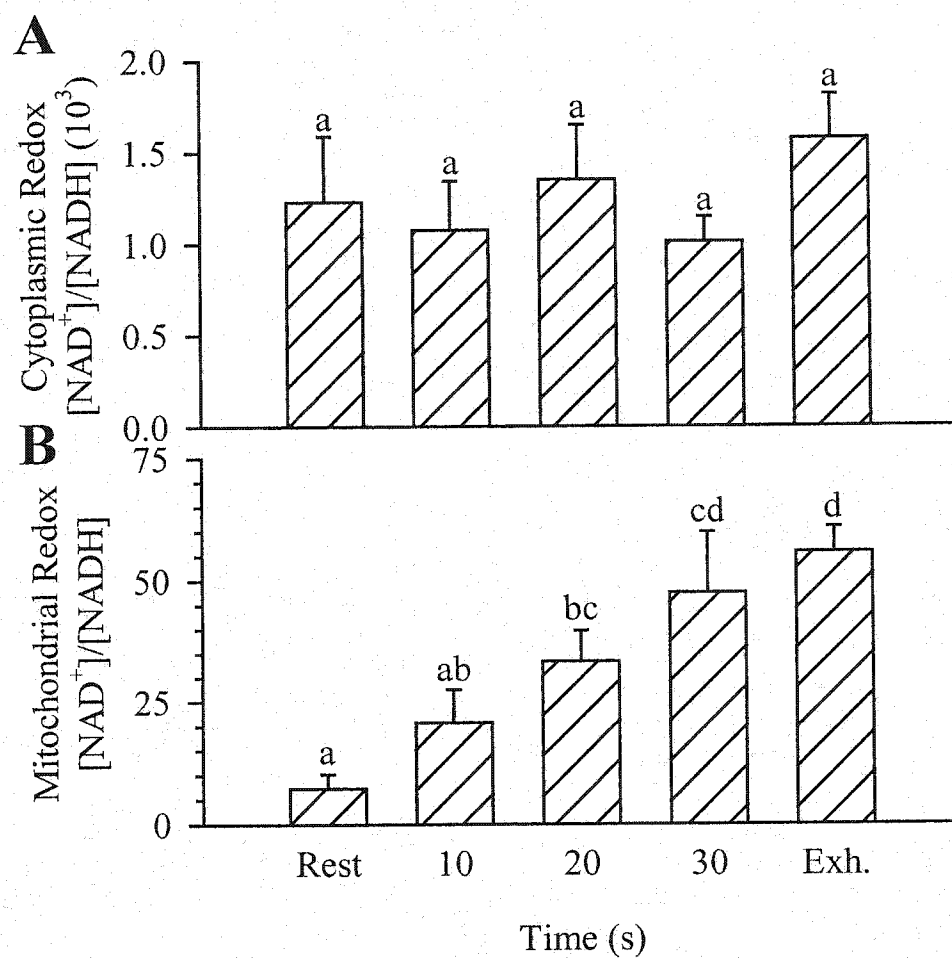


Figure 3-5:

Estimated white muscle cytoplasmic redox (A) and mitochondrial redox (B) states at rest and during exhaustive exercise. See figure 3-1 caption for other details.

DISCUSSION

The present study examined the regulation of substrate use in white muscle of rainbow trout during a continuous bout of high-intensity exercise leading to exhaustion. During the first 10 s of burst activity, high ATP turnover rates (Fig. 3-6) were supported primarily by CrP hydrolysis and glycolysis (Fig. 3-1B and 3-4) yielding dramatic decreases in muscle CrP (Fig. 3-1B) and glycogen with corresponding increases in lactate and pyruvate (Fig. 3-3). The entry of glycogen into glycolysis was the consequence of a very large and rapid transformation of Phos_b into Phos_a (Fig. 3-2A). Oxidative phosphorylation through PDH made only minor contributions to total ATP turnover during the first 10 s of exercise. Total ATP turnover rate during the first 10 s of exercise was $3.7 \mu\text{mol}\cdot\text{g}^{-1} \text{ wet tissue}\cdot\text{s}^{-1}$ which is in close agreement with the maximum ATP turnover rates estimated by Dobson *et al.* (1987) for trout white muscle ($3.1 \mu\text{mol}\cdot\text{g}^{-1} \text{ wet tissue}\cdot\text{s}^{-1}$).

During longer periods of exercise (>10 s), Phos remained significantly transformed compared to resting values (Fig. 3-2A), but glycogenolytic and glycolytic fluxes were reduced (Fig. 3-4) therefore leading to a lower ATP turnover from glycolysis (Fig. 3-6), and likely a correspondingly reduced power output. However, glycolysis still supported the majority of the total ATP turnover throughout the exercise period (Fig. 3-6). Creatine phosphate hydrolysis made only minor contributions to total ATP turnover after the initial 10 s of exercise. As exercise duration increased, there was a gradual transformation of PDH_b into PDH_a that was complete by 30 s of exercise; however,

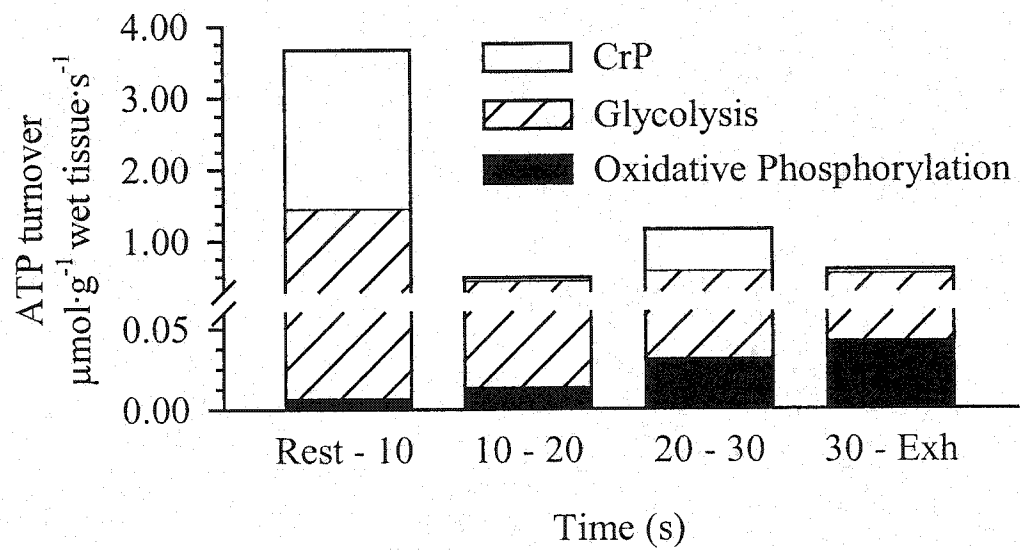


Figure 3-6:

White muscle ATP turnover rates during exhaustive exercise.

oxidative phosphorylation from carbohydrate only contributed ~ 2% to the total ATP turnover during the bout of exercise (Fig. 3-6).

Adenylates and CrP

Considerable debate surrounds the sequence of substrate utilization by muscle during maximal contraction. It is generally believed that CrP hydrolysis is tightly linked to myofibrillar ATPase activity through high affinity, high activity creatine kinase (Meyer and Foley, 1996). Once the ATP buffering capacity of CrP is exceeded, endogenous ATP is utilized, yielding increases in ADP_f, AMP_f, IMP, NH₃, and P_i, all of which act to stimulate glycogenolysis and ATP production *via* glycolysis. Indeed, during the first 10 s of exercise CrP was depleted by 63% whereas ATP only decreased by 28% (Fig. 3-1) indicating that a greater proportion of CrP was hydrolyzed before endogenous ATP. However, during the first 20 s of exercise, muscle [ATP] remained elevated compared with [ATP] at exhaustion, suggesting that complete depletion of ATP was not required to activate glycolysis. Decreasing [ATP] was associated with stoichiometric increases in NH₃ (Table 3-1), indicating the deamination of adenylates during high-intensity exercise and the formation of IMP (Schulte *et al.*, 1992).

Regulation of Glycogen Phosphorylase

It has long been recognised that glycogen is an important substrate for ATP production in fish white muscle during bouts of burst activity (Black *et al.*, 1962; Wang *et al.*, 1994a; Kieffer, 2000; Milligan *et al.*, 2000; Richards *et al.*, 2002b). During the initial 10 s of exercise, glycogenolytic flux and glycolytic flux supported ~40% of the

ATP turnover (Fig. 3-6). Subsequently, glycogenolytic and glycolytic flux both decreased by about two-thirds, but continued to contribute the largest portion to ATP turnover from 10 s of exercise to exhaustion.

The high glycogenolytic flux observed during the first 10 s of exercise (Fig. 3-4) was achieved primarily by a large transformation of Phos_b into Phos_a (Fig. 3-2A) probably caused by Ca²⁺ release from the sarcoplasmic reticulum activating Phos kinase (Krebs *et al.*, 1964). The calculated V_{\max} of Phos_a during the initial 10 s of exercise was $0.42 \mu\text{mol}\cdot\text{g}^{-1} \text{wet tissue}\cdot\text{s}^{-1}$, therefore the increase in activity due to Phos transformation was nearly sufficient to completely explain the observed glycogenolytic flux (Fig. 3-4). In addition to increased Phos activity due to the transformational modification, post-transformational modification of Phos_a also contributed to increased glycogenolysis. Accumulation of P_i from CrP hydrolysis (Table 3-1; Fig. 3-1B) increases substrate availability during the first 10 s of exercise, and the accumulation of AMP_f (Table 3-1) is thought to decrease the K_m of Phos_a for P_i and thus increase the catalytic rate (Ren and Hultman, 1988). In addition, the accumulation of the allosteric activators (ADP_f; Table 3-1), decreasing cellular energy status (ATP/ADP_f ratio; Table 3-1), and lack of product accumulation (glu 6-P; Table 3-2) would further allosterically increase Phos_a activity to allow for the observed glycogenolytic flux.

Glycogenolytic flux decreased linearly during the first 30 s of high-intensity exercise (Fig. 3-4). During this period of decreased glycogenolysis, Phos remained significantly transformed into Phos_a (Fig. 3-2A), and the calculated V_{\max} of Phos_a was 2 - 3 fold larger than the calculated glycogenolytic flux. As a result, after the initial 10 s of

exercise, Phos activity was primarily mediated *via* post-transformational modification. The decreased flux through Phos_a was likely mediated by an increase in product, glu 6-P (Table 3-2), acting to inhibit Phos_a in addition to the decrease in AMP_f observed at 20 s compared to 10 s (Table 3-1), which would reduce the activation of Phos_a. Glucose 6-phosphate remained elevated and AMP_f remained at lower than peak levels until exhaustion. Decreasing pH_i inhibits Phos kinase and would reduce the transformation of Phos (Connett and Sahlin, 1996). In addition, decreasing pH_i would shift the chemical form of P_i from the monoprotonated form, which is the active substrate for Phos_a to the diprotonated form, which does not act as a substrate for Phos_a (Katz *et al.*, 1990). Changes in muscle NH₃ suggest that at >10 s of exercise IMP was accumulating from the deamination of AMP, which is also thought to allosterically stimulate Phos_a (Chasiotis *et al.*, 1982). However, it appears that glu 6-P and AMP_f are the two important allosteric regulators of Phos activity in trout white muscle.

In general, the calculated fluxes through Phos and glycolysis were reasonably well matched throughout the exercise period (Fig. 3-4), although during the initial 20 s of exercise, there was greater glycogenolytic flux than glycolytic flux and this pattern was reversed between 20 and 30 s. This matching between Phos and glycolysis suggests that Phos transformation and catalytic rate directly set the upper limit for glycolytic flux and therefore pyruvate and lactate production. The slightly lower glycolytic rate observed during the first 20 s of exercise suggests that PFK-1 modifies the glycolytic rate set by Phos, a conclusion reinforced by the accumulation of fru 6-P and glu 6-P during this period (Table 3-2). Dobson *et al.* (1987) identified PFK-1 as a point of control in trout

white muscle during exercise but did not critically examine the role of Phos. Considering the very close matching between glycogenolytic flux and glycolytic flux observed in Fig. 3-4, it appears that the transformation state of Phos sets the upper limit for glycogenolytic and glycolytic flux in trout muscle, and flux is further modified by post-transformational modification of Phos and allosteric regulation of PFK-1 activity (Dobson and Hochachka, 1987; Peters and Spriet, 1995).

Regulation of Pyruvate Dehydrogenase

During exercise the transformation of PDH_b into PDH_a was slow to occur, taking 30 s of intense activity before PDH_b was fully transformed into PDH_a (Fig. 3-2B). The initial cue for the transformation of PDH is generally thought to be Ca²⁺ release from the sarcoplasmic reticulum (Randle, 1995). However, Ca²⁺ does not appear to be a potent stimulator of PDH phosphatase in trout white muscle because of the considerable delay in PDH transformation (Fig. 3-2B). Within 10 s of exercise, the energy status (ATP/ADP_f) of the cells was substantially reduced with a large and significant increase in ADP_f and pyruvate (Table 3-1; Fig. 3-3C), which should inhibit PDH kinase (Weiland, 1983) and further allow Ca²⁺ mediated activation of PDH phosphatase and thus PDH transformation. However, in trout white muscle there must be factors that override the stimulatory effects of Ca²⁺ on PDH and slow its transformation. Thus the reason for the delayed transformation of PDH appears, at first glance, to be paradoxical.

Two possible explanations exist to explain the delayed activation of PDH (Fig. 3-2B) in trout muscle. First, PDH is located within the mitochondrial matrix and is

therefore subject to regulation by changes in mitochondrial metabolites and energy status. During the transition from rest to 10 s of exercise there is a significant decrease in CoA-SH in the absence of any change in acetyl-CoA, thus the acetyl-CoA/CoA-SH ratio increases substantially during the first 10 s of exercise. This increase in acetyl-CoA/CoA-SH could stimulate PDH kinase (Heigenhauser and Parolin, 1999b) and slow the transformation of PDH at the onset of exercise. The reason for decreasing CoA-SH is also paradoxical, but likely CoA-SH is bound by TCA cycle intermediates or fatty acids (van der Vusse and Reneman, 1996). In human muscle, acetyl-CoA/CoA-SH is thought to contribute to PDH regulation only at rest, and its role in regulating PDH during exercise is thought to be minimal (Putman *et al.*, 1995). However, in fish muscle the importance of acetyl-CoA/CoA-SH may be greater.

The second plausible reason for the delayed PDH activation in trout muscle is related to the estimated redox state of the mitochondrial matrix. The pattern of PDH transformation (Fig. 3-2B) closely approximates the estimated changes in muscle mitochondrial redox (Fig. 3-5B). This suggests that increases in mitochondrial $[NAD^+]$ act to stimulate PDH phosphatase and inhibit PDH kinase in trout muscle. Therefore, it appears that PDH transformation may be more closely related to the redox state of the cellular compartment within which PDH is found than to the general energy status of the cell or Ca^{2+} release from the sarcoplasmic reticulum.

In all studies that have examined PDH activation to date, the activity of PDH is very closely matched to the total flux through the TCA cycle and thus oxidative phosphorylation (Gibala *et al.*, 1998; Howlett *et al.*, 1998). In previous work that

examined recovery metabolism in trout white muscle, Richards *et al.* (2002b) observed that immediately after high-intensity exercise PDH was fully transformed into PDH_a. In that study (Richards *et al.*, 2002b), we assumed an immediate transformation of PDH_b to PDH_a at the onset of exercise and calculated that oxidative phosphorylation from carbohydrate could contribute up to a maximum of 14% of the total ATP turnover during a 5 min bout of high-intensity exercise. However, in the present study, we have demonstrated that PDH transformation is slow to occur over a 52 s bout of exercise, therefore oxidative phosphorylation only contributes ~2% to total ATP turnover during high-intensity exercise in trout muscle. Despite the lack of ATP production *via* oxidative phosphorylation of carbohydrate in trout muscle, the pattern of PDH transformation has implications toward lactate metabolism.

Lactate Metabolism in Trout White Muscle

Classically, lactate production was thought to occur during high-intensity exercise because of a limitation of O₂ supply to the mitochondria preventing oxidative phosphorylation thus activating “anaerobic” glycolysis for ATP production. However, recent evidence suggests that O₂ may not be limiting in mammalian muscle during high-intensity exercise but rather that lactate production may be due to metabolic inertia where pyruvate production exceeds pyruvate oxidation (Heigenhauser and Parolin, 1999b; Spriet *et al.*, 2000). The present research contributes to the accumulating evidence that suggests that lactate accumulation is due to an imbalance between the transformation pattern and catalytic rates of Phos and PDH (e.g. Parolin *et al.*, 1999). The catalytic rate

of Phos_a in trout white muscle is about 140 times greater than the catalytic rate of PDH_a (Phos $\sim 0.42 \mu\text{mol}\cdot\text{g}^{-1}$ wet tissue $\cdot\text{s}^{-1}$ c.f. PDH $0.003 \mu\text{mol}\cdot\text{g}^{-1}$ wet tissue $\cdot\text{s}^{-1}$; see Results, chapter 3). The low mitochondrial content present within trout white muscle, and likely low copy number of PDH will accentuate the mismatch between glycolytic flux and pyruvate oxidation and will therefore yield lactate accumulation *via* lactate dehydrogenase.

Considerable controversy surrounds the issue whether an O₂ limitation in muscle cells plays a role in lactate formation during high-intensity exercise (Kasvinski and Meyer, 1977). Measurements of whole cell [NADH] and [NAD⁺] suggest that the cell becomes more reduced with increases in exercise intensity (Sahlin, 1985). However, as with the adenylates (ADP and AMP), changes in total [NADH] and [NAD⁺] do not provide information regarding the regulation of metabolism, only changes in free NADH and NAD⁺ provide insight into metabolic control. Based on the lactate dehydrogenase reaction, we have demonstrated that cytoplasmic redox remains constant during high-intensity exercise. This is in direct agreement with previous work from our laboratory (Wang *et al.*, 1994a), and further agrees with Dobson *et al.* (1987) in trout swum for 10 min at 120% U_{crit}.

Estimates of cytoplasmic redox do not yield information regarding the redox state of the mitochondrial matrix. Our estimates of mitochondrial NAD⁺/NADH in trout muscle during exercise suggests that the mitochondrial matrix becomes more oxidized as exercise progresses (Fig. 3-5B) and that this is associated with the progressive activation of PDH (Fig. 3-2B), supporting the idea that the electron transport chain is functioning.

However, it should be noted that the use of the glutamate dehydrogenase equilibrium to estimate mitochondrial redox state remains highly controversial (Kasvinski and Meyer, 1977), especially in white muscle with low mitochondrial number (Moyes *et al.*, 1992). Therefore, the conclusion that O₂ is not limiting at the mitochondrion during high-intensity exercise in trout muscle must be considered tentative and awaits confirmation by other methods.

If O₂ is not limiting in white muscle mitochondria during high-intensity exercise, it is possible that other substrates are also used for oxidative ATP production. Because lipid oxidation is very important during post-exercise recovery (Richards *et al.*, 2002b), we wished to determine whether lipid oxidation could contribute significantly to ATP production in trout white muscle during high-intensity exercise. There was a small, transient decrease in free carnitine within the first 10 s of exercise, but this decrease did not correspond to an increase in either SCFA or LCFA-carnitine and there were no increases in acetyl-CoA (Table 3-3). These data indicate that there was not an increase in fatty acid transport into the mitochondria by CPT-1.

In conclusion, we have demonstrated that during the first 10 s of high-intensity exercise CrP hydrolysis and glycolysis support ATP turnover. Subsequent exercise through to exhaustion is supported primarily by glycolytic ATP production. Entry of glycogen into glycolysis is regulated by the transformational and post-transformational modification of Phos. As exercise duration increased there was a near maximal activation of PDH, and the mismatch between Phos and PDH transformation can explain lactate production in fish muscle. Estimates of cytoplasmic and mitochondrial

NAD⁺/NADH, in addition, to the activation pattern of PDH suggest that O₂ is not limiting in white muscle cells during high-intensity exercise and is not the primary cause of lactate formation.

Perspectives

Many studies have examined the effects of exhaustive exercise on trout white muscle metabolism and looked at the pattern of metabolite recovery. However, the present study is the first to document changes in muscle metabolites during a continuous bout of exercise. Measurements of the transformation state of regulating enzymes (Phos, and PDH) and their allosteric modulators have added evidence to support the hypothesis that lactate production during exercise is not due to an O₂ limitation but rather to an imbalance between Phos and PDH transformation. The slow activation pattern of PDH in trout white muscle indicates that the major fate of lactate is not oxidation. Instead, lactate production in white muscle could be considered integral to white muscle design in fish, so that accumulated lactate is conserved as the substrate for glycogen synthesis during recovery (Schulte *et al.*, 1992; Milligan *et al.*, 2000; Richards *et al.*, 2002b). Further research should focus on isolating the role of PDH in white muscle lactate production through manipulation of PDH activity with dichloroacetate (a pharmacological analogue of pyruvate), hyperoxia, and hypoxia.

CHAPTER 4

SUBSTRATE UTILIZATION DURING GRADED AEROBIC EXERCISE IN RAINBOW TROUT

ABSTRACT

A biochemical approach was employed to examine the oxidative utilization of carbohydrate and lipid in red muscle of rainbow trout (*Oncorhynchus mykiss*) during sustained swimming at 30 and 60% U_{crit} (2, 15, and 240 min) and during non-sustainable swimming at 90% U_{crit} (2, 15, and 45 min). Measurements include PDH activity, glycogen, glycolytic intermediates, acetyl-CoA, acetyl-, total-, free-, SCFA-, and LCFA-carnitine, IMTG, malonyl-CoA, and whole body \dot{M}_{O_2} . During the first 2 min at 30 and 60% U_{crit} , there was enhanced oxidation of endogenous glycogen through 4 and 8 fold increases, respectively, in PDH activity, yielding 1.5 to 2.5 fold increases in acetyl-CoA and 2 to 6 fold increases in acetyl-carnitine concentrations. Within 15 min, PDH activity returned to control values (153.9 ± 30.1 nmol·g⁻¹ wet tissue·min⁻¹) and at 240 min there were small 1.7 to 2.6 fold increases in LCFA-carnitine and ~50% decreases in malonyl-CoA indicating an overall enhancement of lipid oxidation. Sustainable swimming at 30 and 60% U_{crit} was further characterized by 1.5 and 2.2 fold increases in \dot{M}_{O_2} ,

respectively. Non-sustainable swimming at 90% U_{crit} was characterized by a sustained ~10 fold elevation of red muscle PDH activity ($\sim 1600 \text{ nmol}\cdot\text{g}^{-1} \text{ wet tissue}\cdot\text{min}^{-1}$). Significant 67% decreases in white muscle CrP and 73% decreases in glycogen without matching increases in lactate point to significant recruitment of white muscle during high-speed swimming for power production, and the potential export of white muscle lactate to red muscle for oxidation. Overall, sustainable exercise at 30 and 60% U_{crit} is supported by approximately equal contributions of carbohydrate ($\sim 45\%$) and lipid ($\sim 35\%$) oxidation, whereas non-sustainable swimming is supported primarily by carbohydrate oxidation with only moderate contributions from lipid oxidation.

INTRODUCTION

Trunk musculature in trout is spatially divided into separate regions of red-oxidative and white-glycolytic fibres that are differentially recruited to propel the fish at different swimming speeds. Red muscle is almost solely recruited at swimming speeds up to 70 to 80% U_{crit} , beyond which white muscle is also recruited (Hudson, 1973; Johnson, 1981; Wilson and Egginton, 1994; Burgetz *et al.*, 1998). This spatial separation of red and white muscle in fish and their known recruitment patterns have long been recognized as advantages of fish in the study of muscle metabolism (Moyes and West, 1995). To this end, the rainbow trout has become the model organism for the study of white muscle metabolism during high-intensity exercise (Dobson *et al.*, 1987; Richards *et al.*, 2002a) and during post-exercise recovery (Milligan and Girard, 1993; Wang *et al.*, 1994a; Wood and Wang, 1999; Kieffer, 2000; Richards *et al.*, 2002b). However, high-intensity exercise, as imposed in most studies, is likely an event rarely experienced by fish in nature, except when human intervention is involved (*e.g.* angling). For the most part, fish probably swim naturally at speeds that can be sustained for prolonged periods (< 80% U_{crit} ; Krohn and Boisclair, 1994).

In addition to the spatial separation and different recruitment patterns of red and white muscle, the two muscle types have different substrate preferences. Red muscle contraction relies heavily on ATP generated from mitochondrial oxidative phosphorylation (maximum ATP production of 29 $\mu\text{mol ATP}\cdot\text{g}^{-1}\text{wet tissue}\cdot\text{min}^{-1}$; Moyes and West, 1995). Mitochondria isolated from red muscle of carp (*Cyprinus carpio*) have

a very high capacity to oxidize pyruvate, fatty acids and some amino acids (Moyes *et al.*, 1989). Conversely, white muscle mitochondrial ATP production is very low ($\sim 3.5 \mu\text{mol ATP}\cdot\text{g}^{-1}\text{ wet tissue}\cdot\text{min}^{-1}$; Moyes *et al.*, 1992), therefore, white muscle relies primarily on CrP hydrolysis and glycolysis (*i.e.* substrate level phosphorylation) for ATP production. However, during recovery from high-intensity exercise, white muscle mitochondria oxidize lipids to fuel CrP and glycogen synthesis (Moyes *et al.*, 1992; Richards *et al.*, 2002b). Thus, it is generally thought that at swimming speeds ≤ 70 to 80% U_{crit} red muscle contraction and swimming will be supported primarily by oxidative utilization of fuels and as swimming speed increases there will be greater reliance on ATP production by substrate level phosphorylation in white muscle.

Despite the variety of the substrates oxidized by red muscle mitochondria, considerable debate surrounds the pattern of substrate selection in fish swimming at speeds $\leq U_{\text{crit}}$ (Moyes and West, 1995). Classically, protein and lipids were considered to be the major fuels oxidized during sustained swimming while carbohydrate utilization was considered minimal (Dreidzic and Hochachka, 1987; Jobling, 1994). However, Lauff and Wood (1996), using respirometry, demonstrated that juvenile rainbow trout primarily oxidize lipid during swimming at 55% and 85% U_{crit} , that carbohydrate oxidation is the next most important, and that protein oxidation is kept minimal. White muscle glycolytic utilization of carbohydrate has been demonstrated to contribute to ATP turnover during the initial fast-start of swimming (Wokoma and Johnson, 1981) and at swimming speeds $\geq 70\%$ U_{crit} (Burgetz *et al.*, 1998). In addition, whole body oxidative

utilization of carbohydrate increases as swimming speed approaches U_{crit} (Lauff and Wood, 1996).

The objective of the present study was to determine the biochemical pathways involved in carbohydrate and lipid utilization by red and white muscle of rainbow trout during swimming at speeds which correspond to 30, 60, and 90% U_{crit} . Furthermore, insights into the regulation of lipid and carbohydrate oxidation were gained through measurements of PDH and malonyl-CoA. Previously, we have demonstrated the integral role for PDH transformation in regulating carbohydrate and lipid metabolism in white muscle during a bout of high-intensity exercise (Richards *et al.*, 2002a) and during recovery from exhaustive exercise (Richards *et al.*, 2002b). In the present study we measured the activity of PDH in both red and white muscle, and changes in oxidative metabolites (*e.g.* acetyl-CoA and carnitine) and glycolytic intermediates in red muscle during graded swimming in an attempt to isolate whether lipid or carbohydrate was oxidized during graded swimming.

METHODS

Animal Care

Rainbow trout (*Oncorhynchus mykiss*, Walbaum; 115.9 ± 7.9 g; 22.3 ± 0.4 cm; $n = 123$) were obtained from Humber Springs Trout Hatchery, Orangeville, Ontario, Canada. Fish were held under flow-through conditions in 800 L tanks supplied with aerated, dechlorinated city of Hamilton tapwater [composition as in Alsop and Wood (1997)] at 15°C for at least two months before experimentation. During holding, fish were fed daily with commercial trout pellets. On the day of an experiment, fish were removed from the tank before feeding. All experimental procedures comply with guidelines from the Canadian Council of Animal Care.

Critical Swimming Velocity (U_{crit})

In order to establish the swimming capacity of the population of fish under study, U_{crit} was determined on all fish at least two weeks before experimentation, according to the methodology outlined by Brett (1964). Briefly, groups of four to six fish were introduced into a Beamish-style swim tunnel (volume 156 L) and allowed to acclimatize for 1 h at a linear water velocity of $10 \text{ cm}\cdot\text{s}^{-1}$ at 15°C . This water velocity caused the fish to orient into the current, but all fish maintained station at the bottom of the tunnel with only periodic tail movements. After the acclimatization period, swimming speed was increased by $10 \text{ cm}\cdot\text{s}^{-1}$ every 30 minutes until each fish fatigued. Fatigue was established when a trout fell against the back screen of the swim tunnel three consecutive times after

being manually reintroduced into the current. Critical swimming speed was then calculated by

$$U_{\text{crit}} = V_{\text{ls}} + \left(\frac{t_{\text{s}}}{t_{\text{i}}} \cdot V_{\text{i}} \right)$$

where V_{ls} is the velocity ($\text{cm}\cdot\text{s}^{-1}$) of the last completed swimming period, t_{s} is the time (minutes) spent swimming at the final swimming speed, t_{i} is the time increment (minutes), and V_{i} is the velocity increment ($\text{cm}\cdot\text{s}^{-1}$). Mean U_{crit} for all trout was 68.5 ± 0.6 $\text{cm}\cdot\text{s}^{-1}$ or 3.2 ± 0.1 body lengths $\cdot\text{s}^{-1}$ ($n = 123$).

Experimental Protocol

On the day of an experiment, two to three fish were transferred into the swim tunnel and allowed to acclimatize for 1 h at a swimming speed of $10 \text{ cm}\cdot\text{s}^{-1}$. At the end of the acclimatization period, water velocity was increased over a 5 s period to one of three swimming speeds corresponding to 30% ($20 \text{ cm}\cdot\text{s}^{-1}$), 60% ($41 \text{ cm}\cdot\text{s}^{-1}$), and 90% ($62 \text{ cm}\cdot\text{s}^{-1}$) U_{crit} . Fish swimming at 30% and 60% U_{crit} were maintained for 2, 15, or 240 min (4 h) of swimming and fish swimming at 90% U_{crit} were maintained for 2, 15 and 45 min of swimming because the high speed fish fatigued between 50 and 80 min ($n = 9$). During swimming at 90% U_{crit} , periodic prodding was required to prevent fish from resting against the rear grid of the swim tunnel. Control fish were maintained at $10 \text{ cm}\cdot\text{s}^{-1}$ for either 2 min or 240 min after the initial acclimatization period. There were no differences between trout sampled at either control time, therefore in all Figures and Tables the controls are combined into a single point.

To sample fish while swimming, 25 mL clove oil (Sigma-Aldrich; Anderson *et al.*, 1997) was introduced into the 156 L swim tunnel, resulting in anaesthesia in 2 to 4 min at which time the fish fell against the back screen. Clove oil anaesthesia is an effective fish anaesthetic that has no detrimental effects on U_{crit} in juvenile or adult trout (Anderson *et al.*, 1997). Fish continued to swim during the onset of anaesthesia and only fell against the back screen within the final 10 to 25 s. Any fish that struggled was discarded. At complete anaesthesia, the fish were manually removed from the tunnel and a section of trunk extending posterior of the dorsal fin to the anterior side of the anal fin was excised from each fish and immediately freeze clamped between two metal plates pre-cooled in liquid N_2 . Muscle sampling took less than 15 s. All samples were stored at -86°C until analysis.

Analytical Techniques

Frozen muscle was broken into small pieces (50 to 100 mg) under liquid N_2 in an insulated mortar and pestle. Care was taken to obtain only white muscle from the area dorsal to the lateral line and red muscle along the lateral line. Several pieces of red and white muscle were stored separately in liquid N_2 for determination of PDH activity by radiometric assay as previously described by Richards *et al.* (2002b). The remaining broken muscle was lyophilized for 72 h, red and white muscle fibres were separated by careful dissection, cleaned of blood, bone and connective tissue, and each stored dry at -86°C for subsequent analysis.

For the extraction of metabolites from red and white muscle, aliquots of lyophilized muscle (~10 mg for red muscle, ~20 mg for white muscle) were weighed into borosilicated tubes, 1 mL of ice-cold 1 M HClO₄ added, and homogenized at the highest speed of a Virtis hand-held homogenizer for 20 s at 0°C. Red and white muscle homogenates were vortexed and 200 µL of homogenate slurry was removed and placed into a 1.5 mL centrifuge tube and frozen at -86°C for determination of glycogen. The remaining homogenate was centrifuged for 5 min at 4,800 g at 4°C and the supernatant was neutralized with 3 M K₂CO₃. The neutralized extract was centrifuged for 5 min at 20,000 g at 4°C and the supernatant was analyzed immediately for ATP, CrP, and lactate. Red muscle extracts were further assayed fluorometrically for glucose, glu 6-P, fru 6-P, glu 1-P, gly 3-P, and glycerol. All these assays followed methods described in Bergmeyer (1983) that were modified for fluorometry. Red muscle extracts were also analyzed for acetyl-CoA, CoA-SH, and acetyl-, free-, SCFA-, LCFA-, and total-carnitine by radiometric methods previously described in Richards *et al.* (2002b). Red muscle IMTG was determined on lyophilized tissue as described by Keins and Richter (1996). Malonyl-CoA was determined by high-performance liquid chromatography on a separate extraction as described in Richards *et al.* (2002b).

Respirometry

To determine \dot{M}_{O_2} in control fish and fish swimming at 30% and 60% U_{crit} , we used an identical acclimatization and swimming procedure as outlined above in the experimental protocol. Oxygen consumption was not determined on fish swimming at

90% U_{crit} because our metabolic data indicated that the fish were not in steady state at this swimming speed. In view of the high volume of the swim tunnel it was necessary to swim fish in groups to achieve measurable changes in water P_{O_2} . Briefly, four fish were introduced into the swim tunnel and allowed to acclimatize for 1 h at a speed of 10 $cm \cdot s^{-1}$. At the end of the acclimatization period, water speed was either increased to 30% or 60% U_{crit} or left at the control speed. After two hours of swimming at the predetermined speed, the swim tunnel was sealed from the atmosphere and an initial water sample was taken. Swimming was maintained for an additional 1.5 to 2 h at which point another water sample was taken. Water samples (4 mL) were taken into gas-tight syringes and P_{O_2} was measured using a Radiometer E5046 oxygen electrode thermostatted to 15°C and attached to a Cameron Instruments OM-200 meter. Oxygen consumption, in $\mu mol \cdot kg^{-1} \cdot wet \ weight \cdot min^{-1}$, was calculated by the Fick Principle:

$$\dot{M}_{O_2} = \frac{[\Delta P_{O_2} \cdot \alpha O_2 \cdot volume]}{[fish \ weight \cdot time]}$$

where ΔP_{O_2} is the difference in P_{O_2} over the duration of the respirometry period, αO_2 is the solubility coefficient of O_2 in water at 15°C (2.0111 $\mu mol / (torr \cdot L)$; Boutilier *et al.*, 1984), volume is the volume of water in the swim tunnel (156 L), fish weight is the total weight of all four fish (kg), and time is the duration of the respirometry period (min). ATP turnover was calculated from \dot{M}_{O_2} assuming a standard P: O_2 ratio of 6 (Hultman, 1967).

Data Presentation and Statistical Analysis

All data are presented as means \pm SE (n), where n = number of fish for all metabolite data and n = number of groups for \dot{M}_{O_2} measurements. All muscle metabolite concentrations determined on lyophilized tissue were converted back to wet weights by taking into account a wet:dry ratio of 4:1 (Wang *et al.*, 1994a). Statistical analysis consisted of a one-way ANOVA followed by a Least-Significant Difference method of pairwise multiple comparisons. Results were considered significant at $P < 0.05$.

RESULTS

Respirometry

Compared to control trout, \dot{M}_{O_2} increased by 1.5 and 2.2 fold in trout swimming at 30% and 60% U_{crit} , respectively (Fig. 4-1).

Red Muscle

CrP and Adenylates

Red muscle [CrP] did not change in trout swam at 30% U_{crit} for up to 240 min (Fig. 4-2A). In trout swimming at 60% U_{crit} , there was a 26% drop in [CrP] at 2 min, but by 15 min [CrP] had returned to control values and remained unchanged for up to 240 min. At 90% U_{crit} , there was a 54% decrease in [CrP] at 2 min which remained depressed compared to control values for up to 45 min. Swimming speed or duration had no effect on red muscle [ATP] (Fig. 4-2B).

Glycogen, Glycolytic Intermediates, and IMTG

There were no effects of swimming at 30% U_{crit} for 240 min or 60% U_{crit} for up to 15 min on red muscle [glycogen] (Fig. 4-3A). However, by 240 min of swimming at 60% U_{crit} there was a significant 34% decrease in [glycogen] compared to control trout. At 90% U_{crit} , there was a significant 46% decrease in [glycogen] by 15 min of swimming which remained depressed by 57% compared to control trout at 45 min. Red muscle [lactate] did not change in trout swam at 30 or 60% U_{crit} for up to 240 min (Fig. 4-3B).

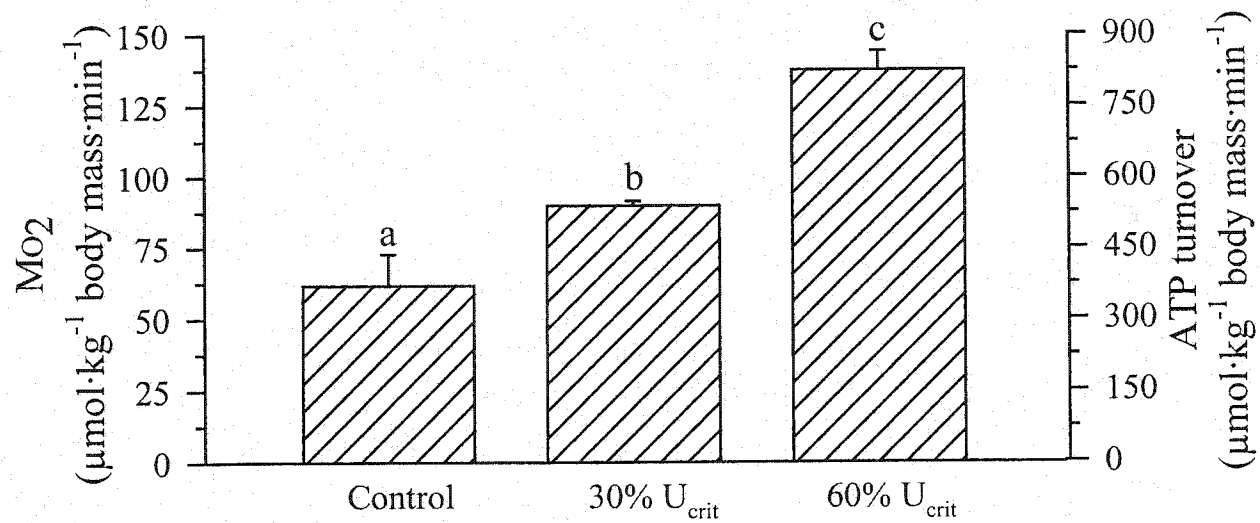


Figure 4-1:

Oxygen consumption (left axis) and theoretical ATP turnover (right axis) in control fish and in fish swam at 30% and 60% U_{crit}. See text for details. Data are means ± SE, from left to right n = 5, 5, and 10. Bars with different letters are significantly different ($P < 0.05$).

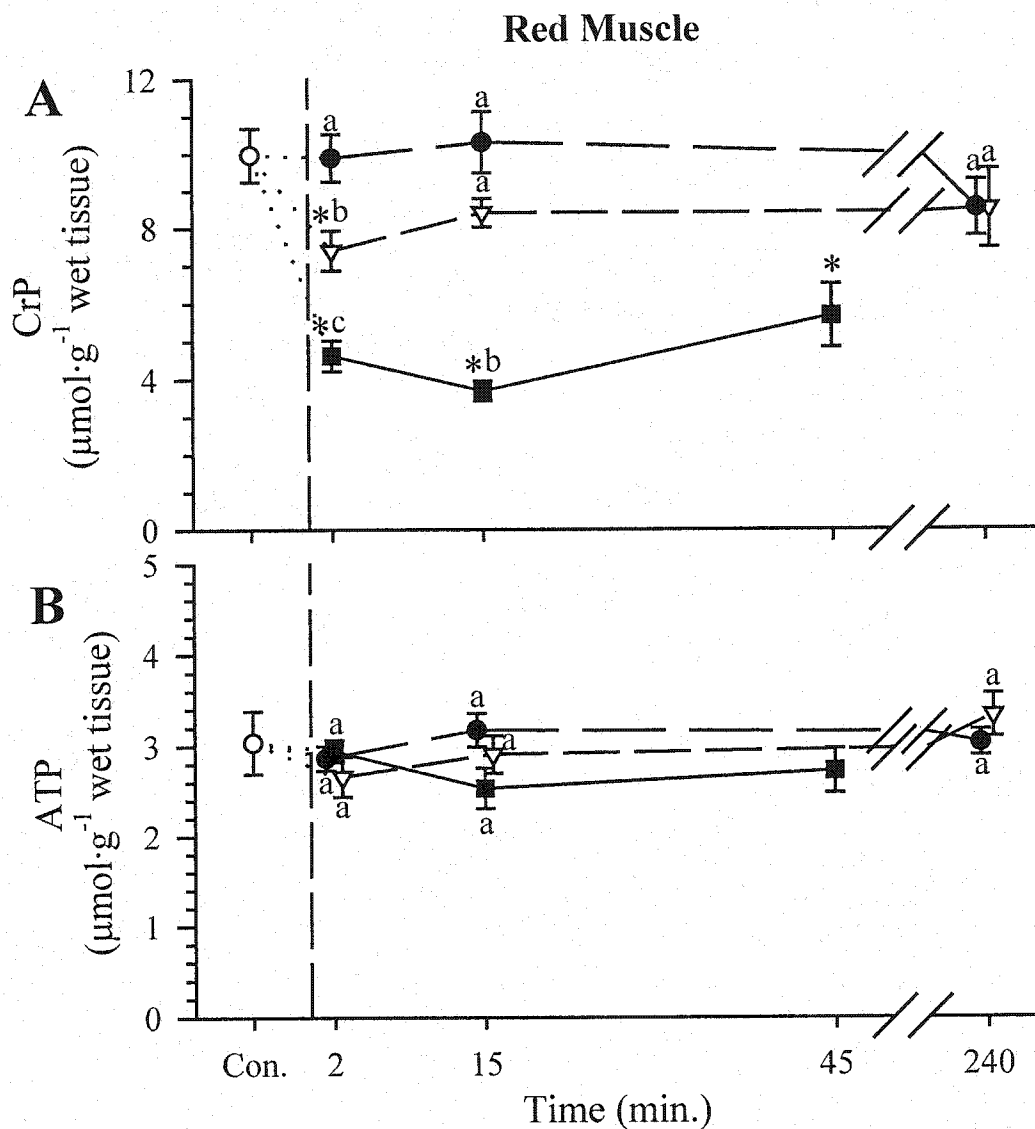


Figure 4-2:

Red muscle CrP (A) and ATP (B) concentrations in control fish (○) and in fish swam at 30% U_{crit} (●) and 60% U_{crit} (▽) for up to 240 min and at 90% U_{crit} (■) for up to 45 min. Vertical dashed line represents the start of exercise. Overlapping data points are offset for clarity. Data are means \pm SE, $n = 8$ for each point. Note the break in the horizontal axis. Asterisks represent significant ($P < 0.05$) differences from control fish and points with different letters are significantly ($P < 0.05$) different within a time period.

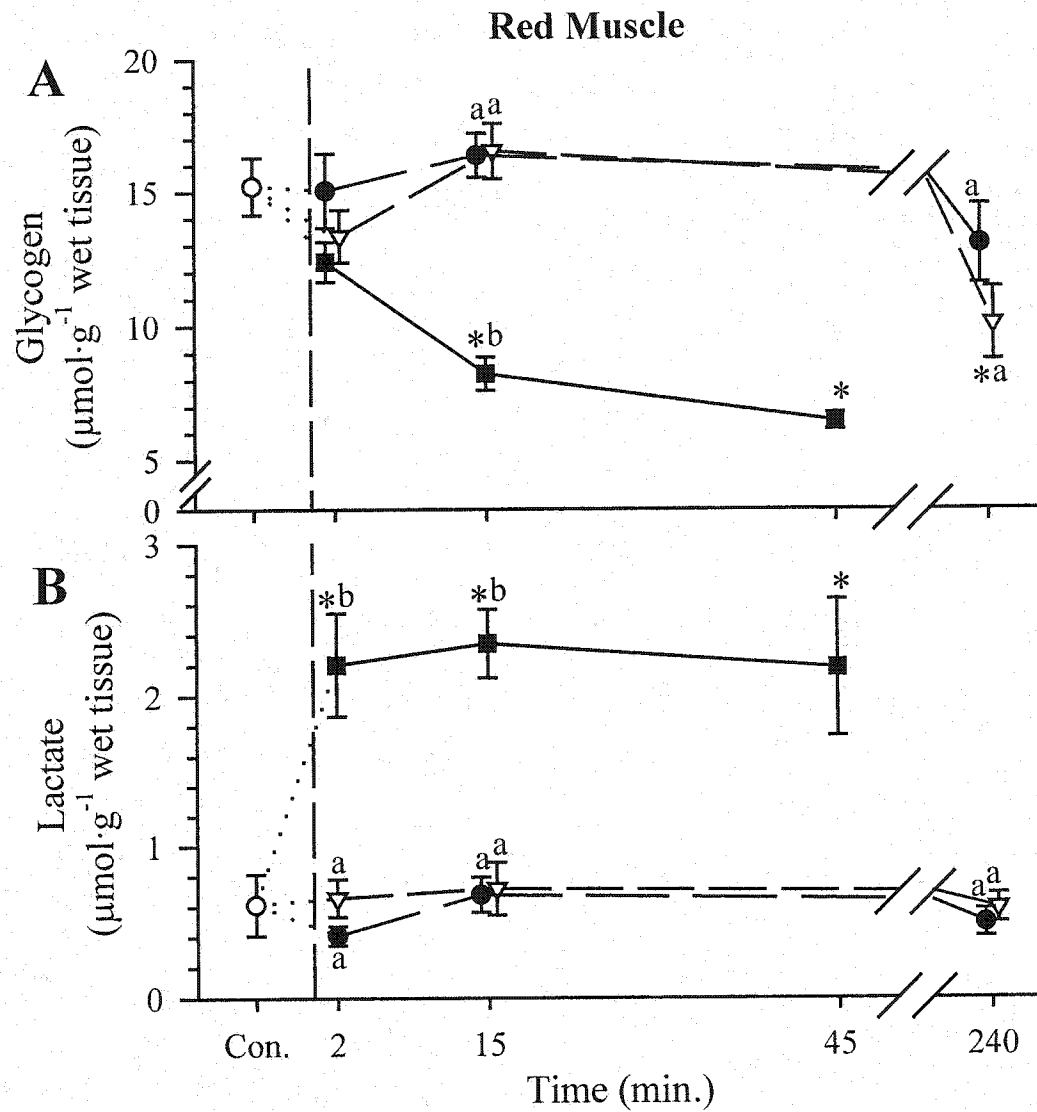


Figure 4-3:

Red muscle glycogen (A) and lactate (B) concentrations in control fish (○) and in fish swam at 30% U_{crit} (●) and 60% U_{crit} (▽) for up to 240 min and at 90% U_{crit} (■) for up to 45 min. See figure 4-2 caption for other details.

However, after swimming for 2 min at 90% U_{crit} , there was a 3.6 fold increase in red muscle [lactate] which remained elevated and at a stable level compared to control trout for up to 45 min. The rise in red muscle [lactate] ($\sim 1.6 \mu\text{mol}\cdot\text{g}^{-1}$ wet tissues) was slight relative to the fall in [glycogen] ($\sim 9 \mu\text{mol}$ glucosyl units $\cdot\text{g}^{-1}$ wet tissue = $18 \mu\text{mol}$ lactate units $\cdot\text{g}^{-1}$ wet tissue).

Red muscle [glucose] remained constant compared to controls in trout swimming at 30 and 60% U_{crit} for up to 240 min (Fig. 4-4A). At 90% U_{crit} , muscle [glucose] remained at control values for the first 2 min, then increased by 1.9 fold by 15 min and remained elevated for up to 45 min. There was no effect of swimming at 30% U_{crit} on red muscle [glu 6-P] (Fig. 4-4B). At 60% U_{crit} , muscle [glu 6-P] increased by 2.2 fold within 2 min, but returned to control values by 15 min and remained low until 240 min. In trout swimming at 90% U_{crit} , there was a 6.2 fold increase in [glu 6-P] which gradually returned to control values by 45 min. Swimming at 30 and 60% U_{crit} for up to 240 min did not affect red muscle [fru 6-P] except a small, but significant decrease in [fru 6-P] at 240 min of swimming at 30% U_{crit} (Table 4-1). Swimming at 90% U_{crit} caused an initial 2.3 fold increase in [fru 6-P] which returned to control values by 15 min and remained low at 45 min. Red muscle [glu 1-P] was below detection in all fish independent of swimming speed or duration (data not shown). Red muscle [gly 3-P] did not change in trout swimming at 30% U_{crit} for up to 240 min (Table 4-1). However, at 60% U_{crit} , there was a significant 27 fold increase in [gly 3-P] at 15 min of swimming which decreased to control values by 240 min. In trout swimming at 90% U_{crit} , there was a 29 fold increase in [gly 3-P] which further increased to 55 fold at 45 min. There was no effect of

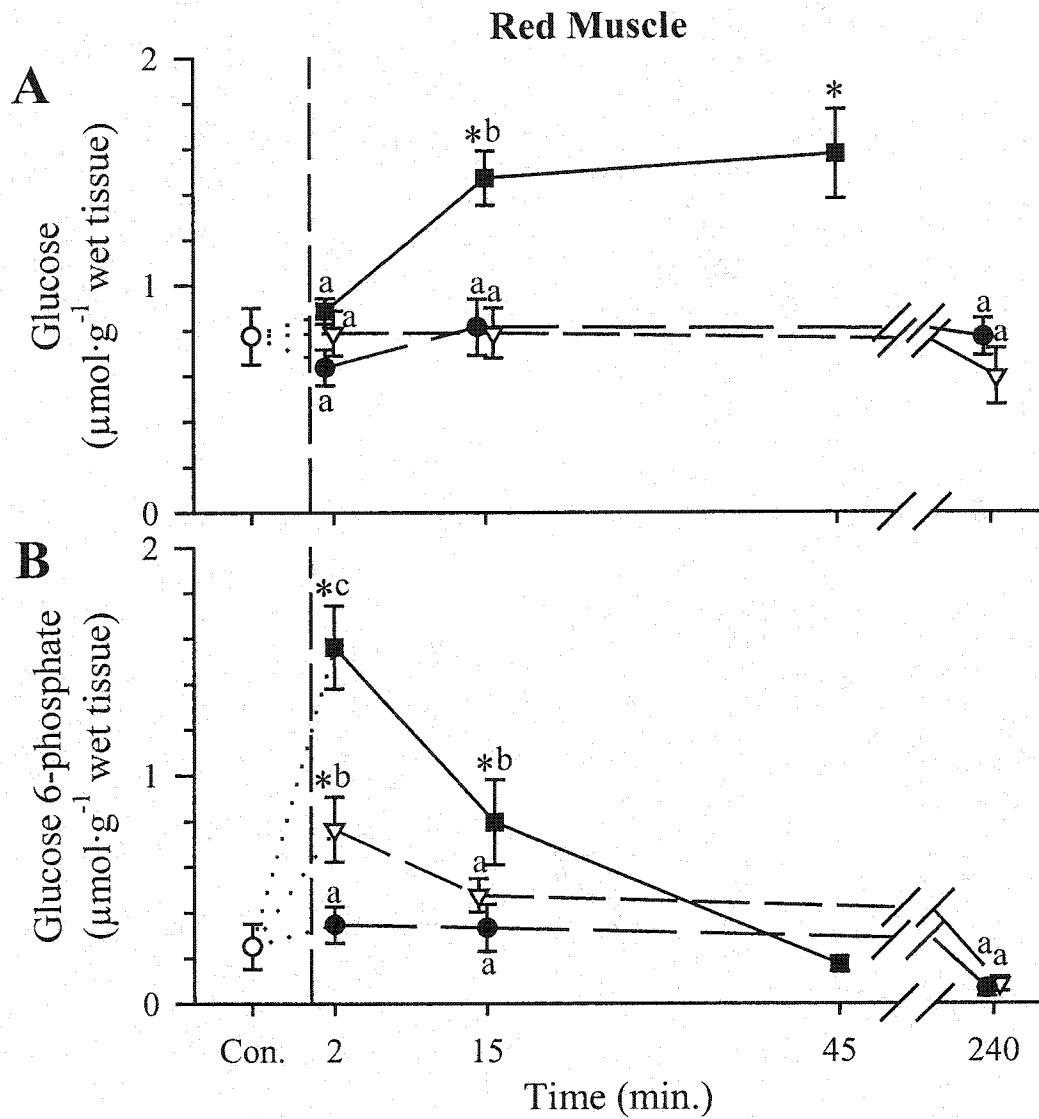


Figure 4-4:

Red muscle glucose (A) and glucose 6-phosphate (B) concentrations in control fish (○) and in fish swam at 30% U_{crit} (●) and 60% U_{crit} (▽) for up to 240 min and at 90% U_{crit} (■) for up to 45 min. See figure 4-2 caption for other details.

Table 4-1: Red muscle glycolytic intermediates, glycerol, IMTG, CoA-SH, and SCFA-carnitine concentrations in control fish and fish swam at 30, 60, and 90% U_{crit} for up to 240 min.

Measure	Control	30 % U_{crit}			60% U_{crit}			90% U_{crit}		
		2 min	15 min	240 min	2 min	15 min	240 min	2 min	15 min	45 min
Fru 6-P	0.11±0.02	0.06±0.02 ^a	0.12±0.03 ^a	0.01±0.0 ^{*a}	0.12±0.05 ^a	0.09±0.02 ^a	0.09±0.05 ^a	0.25±0.04 ^{*b}	0.15±0.03 ^a	0.08±0.01
Gly 3-P	0.04±0.03	0.03±0.03 ^a	0.12±0.07 ^a	0.09±0.09 ^a	0.32±0.09 ^{ab}	1.07±0.39 ^b	0.13±0.07 ^a	1.17±0.12 ^{*c}	2.82±0.29 ^{*c}	2.19±0.58 [*]
Glycerol	2.03±0.37	0.93±0.19 ^a	1.58±0.25 ^a	1.94±0.41 ^a	1.27±0.45 ^a	1.71±0.37 ^a	2.22±0.50 ^a	1.19±0.55 ^a	2.57±0.53 ^a	5.20±0.96 [*]
IMTG	207±10	198±12 ^a	200±11 ^a	181±12 ^a	212±16 ^a	197±22 ^a	203±24 ^a	201±8 ^a	197±8 ^a	216±24
CoA-SH	53.6±5.4	48.5±7.3 ^a	56.8±6.0 ^a	50.0±3.8 ^a	57.6±6.2 ^a	37.4±4.6 ^b	49.1±4.9 ^a	46.7±3.8 ^a	38.4±5.1 ^{*b}	46.8±4.4
SCFA-Car	13±25	49±12 ^a	61±9 ^a	33±10 ^a	49±4 ^a	10±8 ^a	31±19 ^a	1±1 ^a	1±1 ^b	30±19

Data are means ± SE in $\mu\text{mol}\cdot\text{g}^{-1}$ wet tissue except for CoA-SH and SCFA-Car which are in $\text{nmol}\cdot\text{g}^{-1}$ wet tissue, n = 8 for each except IMTG where, from left to right, n = 8, 8, 6, 8, 4, 5, 7, 7, 8, and 7. Fru 6-P, fructose 6-phosphate; Gly 3-P, glycerol 3-phosphate; IMTG, intramuscular triacylglycerol; CoA-SH, free CoA; SCFA-carnitine, short chain fatty acid-carnitine. IMTG is expressed in terms of glycerol units (1 glycerol = 3 fatty acids from IMTG). Asterisks represent a significant difference from control. Within a time period, values with different letters are significantly different.

swimming speed or duration on red muscle [glycerol] except in fish swimming at 90% U_{crit} for 45 min where [glycerol] was 2.6 fold higher than in control fish. Swimming speed or duration had no effect on red muscle [IMTG] (Table 4-1).

Pyruvate Dehydrogenase Activity

During the first 2 min of swimming, red muscle PDH activity increased by 4, 8, and 12 fold in trout swimming at 30, 60, and 90% U_{crit} , respectively (Fig. 4-5). In trout that continued swimming at 30 and 60% U_{crit} , PDH activity returned to values that were not statistically higher than control fish, and remained low for the full 240 min of swimming. However, in trout that continued swimming at 90% U_{crit} , PDH activity remained approximately 10 to 12 fold higher than in control trout for the duration of swimming.

Acetyl Group Accumulation and Carnitine

In general, swimming speed and duration had no effect on red muscle [CoA-SH] except at 15 min where trout swimming at 60 and 90% U_{crit} exhibited significantly lower [CoA-SH] (by ~ 30%) compared to control trout and trout swimming at 30% U_{crit} (Table 4-1). During the first 2 min of swimming, red muscle [acetyl-CoA] increased by 1.5, 2.6, and 3.6 fold in trout swimming at 30, 60, and 90% U_{crit} , respectively, and remained stable at these elevated concentrations for the duration of the swimming (Fig. 4-6A). Similarly, during the first 2 min of swimming, there were 2.1, 6.0, and 7.5 fold increases in [acetyl-carnitine] in trout swimming at 30, 60, and 90% U_{crit} , respectively (Fig. 4-6B). These elevations in [acetyl-carnitine] remained constant for the duration of the swimming.

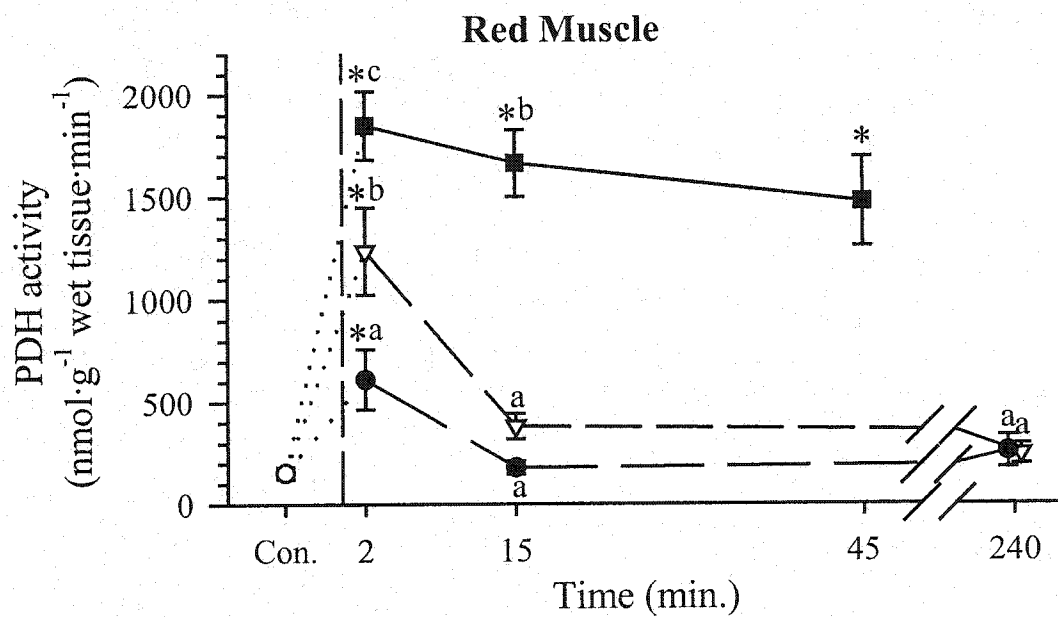


Figure 4-5:

Red muscle pyruvate dehydrogenase activity in control fish (○) and in fish swam at 30% U_{crit} (●) and 60% U_{crit} (∇) for up to 240 min and at 90% U_{crit} (■) for up to 45 min. See figure 4-2 caption for other details.

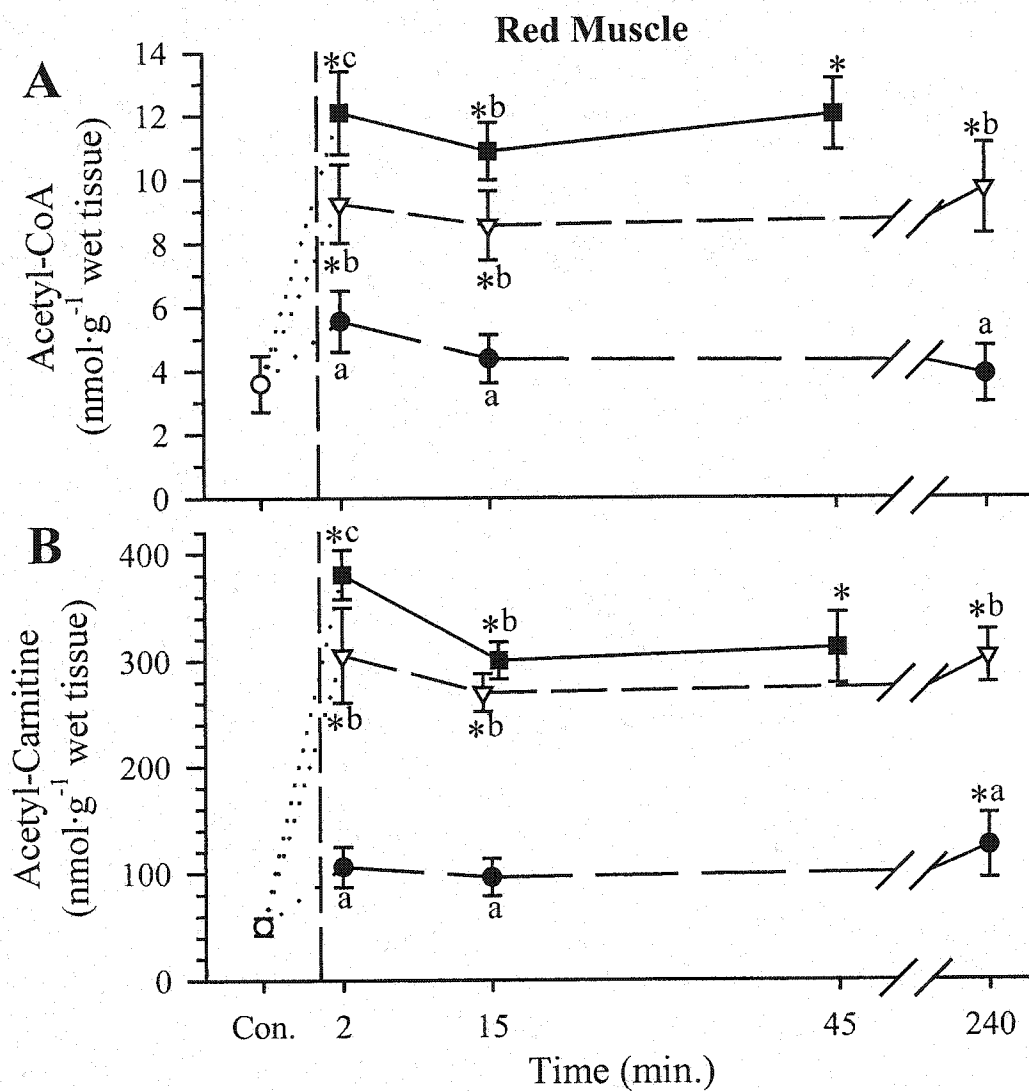


Figure 4-6:

Red muscle acetyl-CoA (A) and acetyl-carnitine (B) concentrations in control fish (○) and in fish swam at 30% U_{crit} (●) and 60% U_{crit} (▽) for up to 240 min and at 90% U_{crit} (■) for up to 45 min. See figure 4-2 caption for other details.

Swimming speed and duration had no effect on red muscle [total-carnitine], except for a minor, but significant elevation at 2 min of swimming at 60% U_{crit} (Fig. 4-7A). Red muscle [free-carnitine] decreased by 18%, 56%, and 59% in trout swimming for 2 min at 30, 60, and 90% U_{crit} , respectively and remained close to these values for the duration of swimming (Fig. 4-7B). Swimming speed or duration had no effect on red muscle [SCFA-carnitine] except trout swimming at 90% U_{crit} for 15 min had significantly lower [SCFA-carnitine] than in trout swimming at 30% U_{crit} (Table 4-1). Long chain fatty acyl carnitine remained constant compared to control trout for the first 15 min of swimming at 30% U_{crit} then increased by 1.7 fold by 240 min (Fig. 4-7C). At 60% U_{crit} , there was an initial 1.6 fold increase in [LCFA-carnitine] at 2 min, and a 2.5 fold increase by 240 min. Swimming at 90% U_{crit} for 45 min did not affect [LCFA-carnitine].

Malonyl-CoA

Red muscle [malonyl-CoA] did not change within the first 2 min of swimming at any of the three swimming speeds (Fig. 4-8). However, by 15 min, there were significant 55% decreases in [malonyl-CoA] in fish swimming at 30 and 60% U_{crit} . [Malonyl-CoA] remained at these levels for up to 240 min of swimming at these two speeds. In contrast, in trout swimming at 90% U_{crit} , there was a gradual decrease in muscle malonyl-CoA to values that were 36% lower than control fish at 45 min.

White Muscle

White muscle [CrP] did not change in trout swimming at 30% U_{crit} for up to 240 min or in trout swimming at 60% U_{crit} for up to 15 min (Fig. 4-9A). At 240 min of

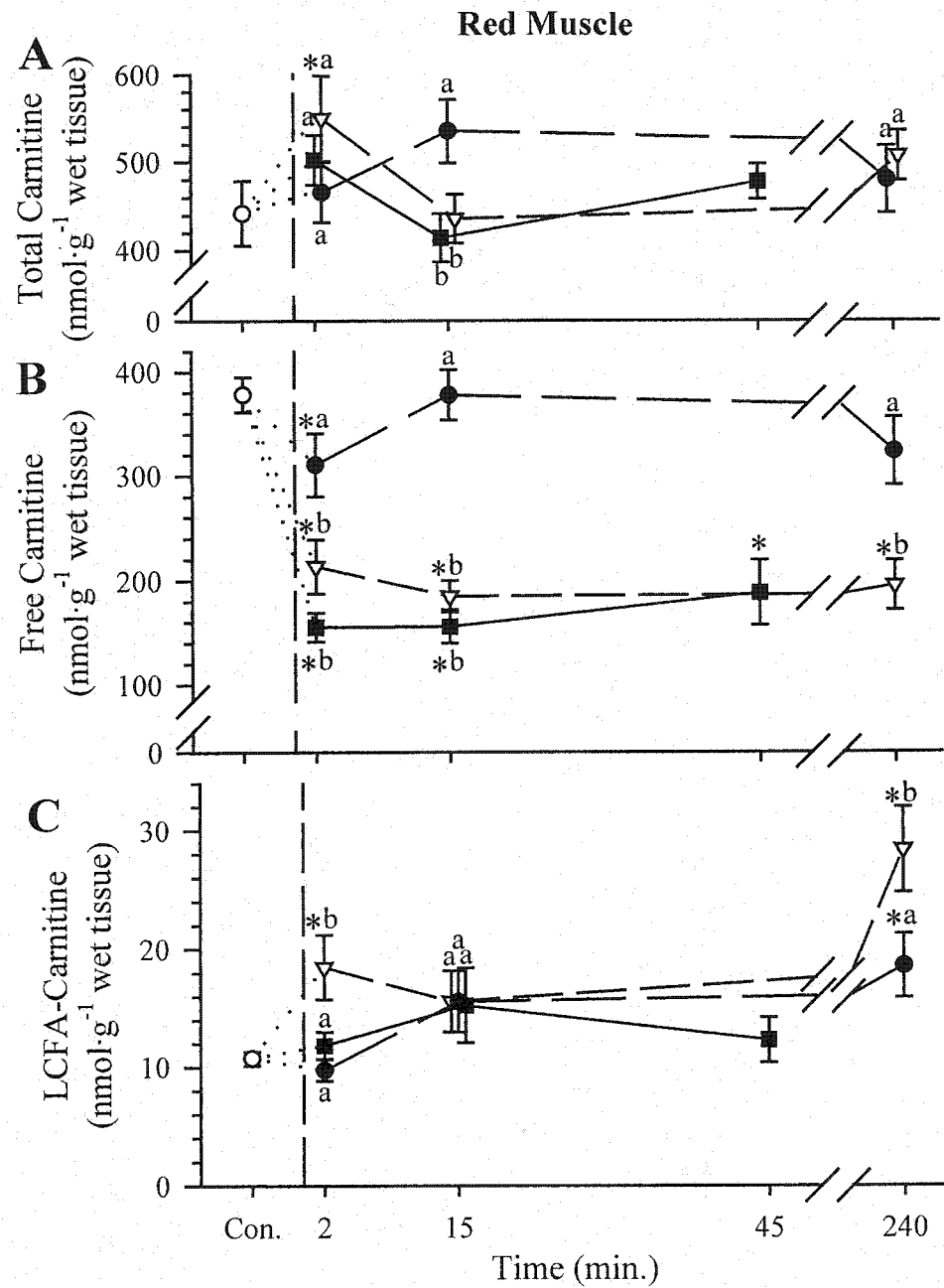


Figure 4-7:

Red muscle total carnitine (A), free carnitine (B), and long chain fatty acyl-carnitine (C) concentrations in control fish (○) and in fish swam at 30% U_{crit} (●) and 60% U_{crit} (▽) for up to 240 min and at 90% U_{crit} (■) for up to 45 min. LCFA-Carnitine, long chain fatty acid-carnitine. See figure 4-2 caption for other details.

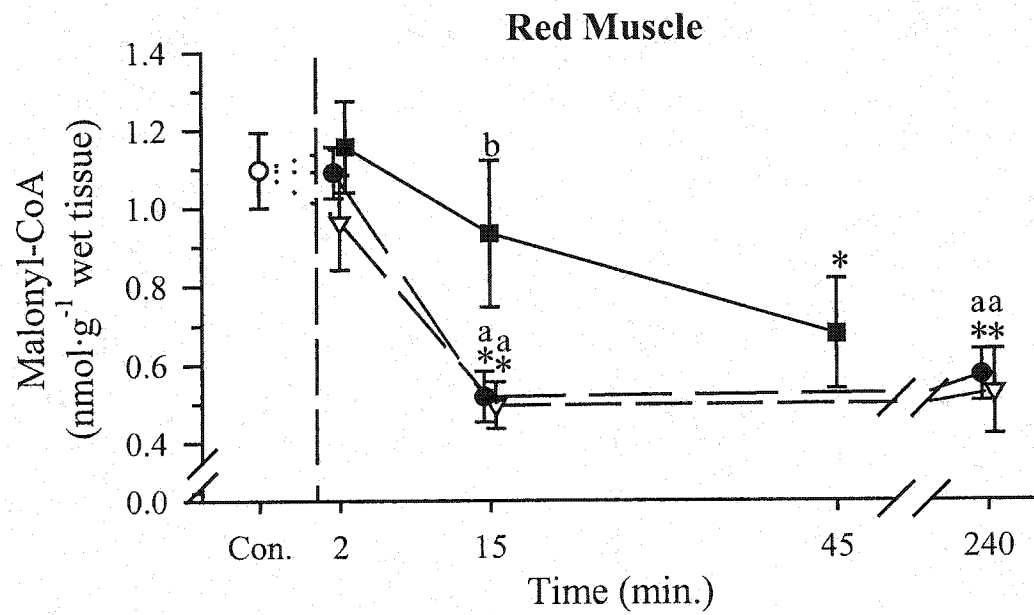


Figure 4-8:

Red muscle malonyl-CoA concentrations in control fish (○) and in fish swam at 30% U_{crit} (●) and 60% U_{crit} (∇) for up to 240 min and at 90% U_{crit} (■) for up to 45 min. See figure 4-2 caption for other details.

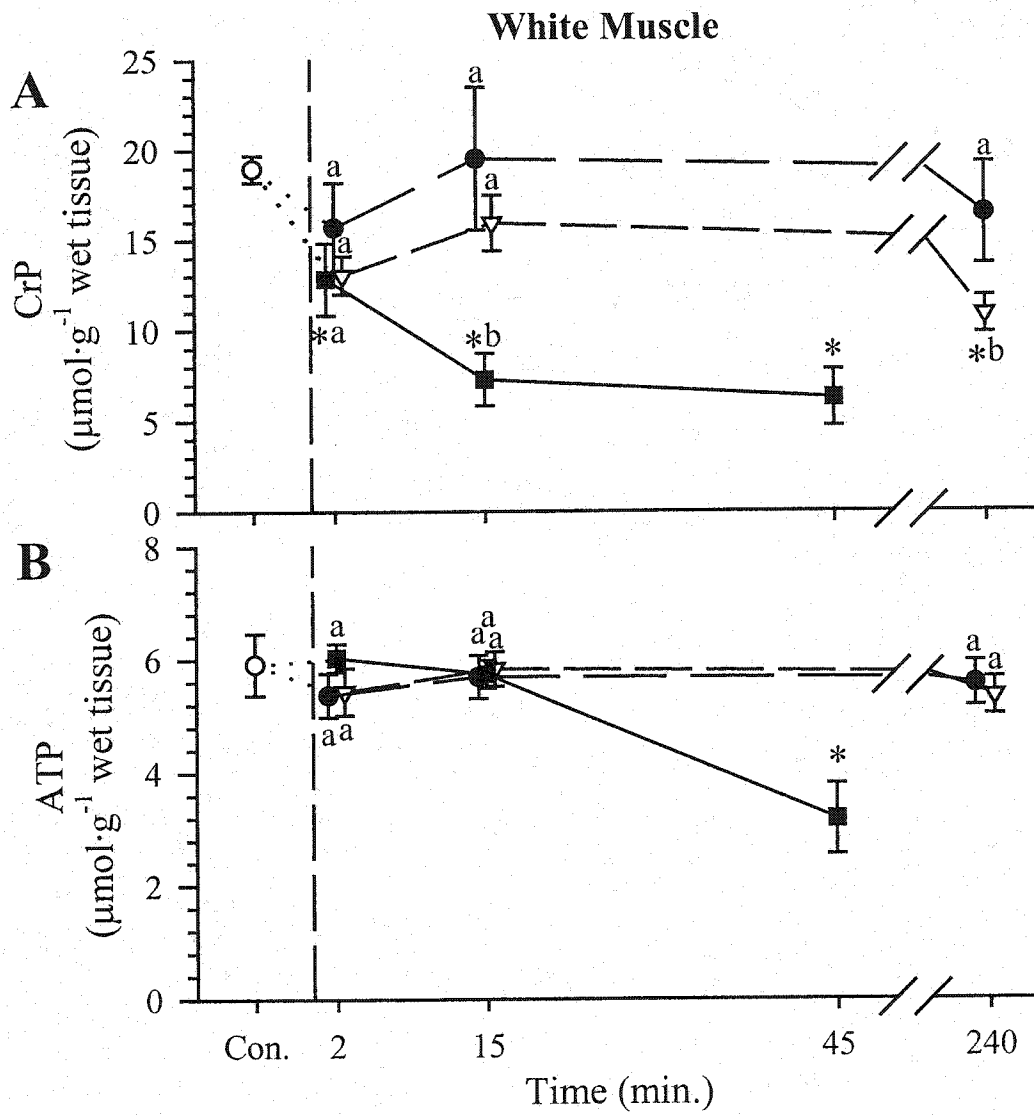


Figure 4-9:

White muscle CrP (A) and ATP (B) concentrations in control fish (\circ) and in fish swam at 30% U_{crit} (\bullet) and 60% U_{crit} (∇) for up to 240 min and at 90% U_{crit} (\blacksquare) for up to 45 min.

See figure 4-2 caption for other details.

swimming at 60% U_{crit} , there was a 43% decrease in [CrP]. At 90% U_{crit} , muscle [CrP] decreased by 33% at 2 min and 67% at 45 min. White muscle [ATP] did not change in trout swimming at 30 or 60% U_{crit} for up to 240 min (Fig. 4-9B). At 90% U_{crit} , white muscle [ATP] remained at control values for the first 15 min of swimming, but decreased by 46% at 45 min.

White muscle glycogen remained relatively stable in white muscle of trout swimming at 30% U_{crit} , except for a small, but significant decrease at 15 min which recovered by 240 min (Fig. 4-10A). There was an initial 18% decrease in white muscle glycogen at 2 min of swimming at 60% U_{crit} , which further decreased (34%) by 240 min. At 90% U_{crit} , there was a 33% decrease in [glycogen] at 2 min which continued to decrease (73%) at 45 min. There were no changes in white muscle lactate in trout swimming at 30 and 60% U_{crit} for 240 min (Fig. 4-10B). Trout swum at 90% U_{crit} for 2 min showed a 3.8 fold increase in white muscle lactate which further increased at 15 min to 7.3 times control values, then remained constant until 45 min. This rise in white muscle lactate at 90% U_{crit} ($\sim 8 \mu\text{mol}\cdot\text{g}^{-1}$ wet tissue) only accounts for $\sim 30\%$ of the relative fall in white muscle glycogen ($\sim 13 \mu\text{mol glycosyl units}\cdot\text{g}^{-1}$ wet tissue = $\sim 26 \mu\text{mol lactate units}\cdot\text{g}^{-1}$ wet tissue).

There was no effect of swimming speed or duration on white muscle PDH activity: mean PDH activity was $39.8 \pm 4.4 \text{ nmol}\cdot\text{g}^{-1}$ wet tissue $\cdot\text{min}^{-1}$ ($n = 35$; data not shown).

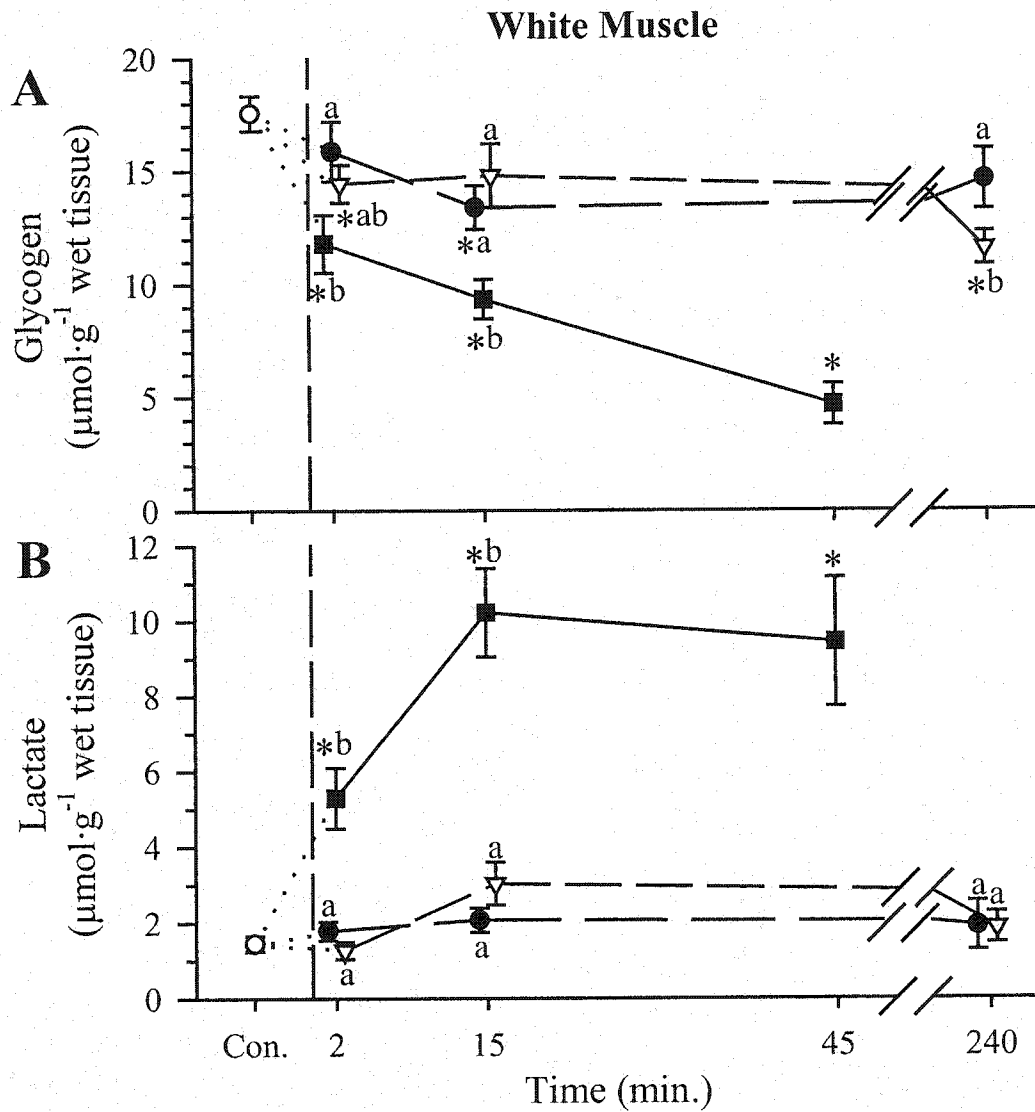


Figure 4-10:

White muscle glycogen (A) and lactate (B) concentrations in control fish (○) and in fish swam at 30% U_{crit} (●) and 60% U_{crit} (▽) for up to 240 min and at 90% U_{crit} (■) for up to 45 min. See figure 4-2 caption for other details.

DISCUSSION

Overview

The present study is the first to examine the temporal dynamics of substrate utilization by red and white muscle in trout during sustainable (30% and 60% U_{crit} ; 240 min) and non-sustainable (90% U_{crit} ; 45 min) swimming. Sustainable swimming is powered predominantly by red muscle with only minor changes in cellular energy status and small depletions of intramuscular substrates. Furthermore, there is a shift from the initial oxidation of carbohydrate observed during the first 2 min of swimming to oxidation of lipid during sustained swimming (>15 min). Non-sustainable swimming at 90% U_{crit} is characterized by large depletions of intramuscular fuels, with oxidative phosphorylation of carbohydrate predominating in red muscle, and substrate level phosphorylation predominating in white muscle.

Our data generally agree with previous data suggesting that mainly red muscle is used to power swimming at speeds up to 70 or 80% U_{crit} beyond which both red and white muscle contribute (Hudson, 1973; Johnson, 1981; Wilson and Egginton, 1994; Burgetz *et al.*, 1998). At all swimming speeds, there were some changes in red muscle metabolites (*e.g.* Fig. 4-2A and Fig. 4-6A and B) and PDH activity (Fig. 4-5) indicating that red muscle contributes to power production during swimming. During sustained swimming, there were moderate decreases in white muscle CrP after 240 min at 60% U_{crit} (Fig. 4-9A), and small decreases in white muscle [glycogen] at 2 min and 240 min at 60%

U_{crit} , and at 15 min at 30% U_{crit} (Fig. 4-10A). These minor decreases in [CrP] and [glycogen] suggest that there is an early but minor recruitment of white muscle during sustainable swimming, possibly to power bursts. The larger depletions of white muscle [CrP] observed over 45 min at 90% U_{crit} (Fig. 4-9A) along with decreases in white muscle [ATP] (Fig. 4-9B) and [glycogen] (Fig. 4-10A), point to a substantial recruitment of white muscle during non-sustainable swimming. These results are in general agreement with those of Burgetz *et al.* (1998) who demonstrated a significant contribution of white muscle “anaerobic” metabolism in trout at $\geq 70\%$ U_{crit} . The recruitment of white muscle probably acts to enhance swimming performance of the fish by providing greater power output, but may also play a role in supplying lactate to the red muscle for oxidation, particularly at high swimming speeds (Moyes and West, 1995). Note that lactate buildup was less than glycogen depletion in white muscle at 60 and 90% U_{crit} (Fig. 4-10A and B), suggesting that lactate was exported for oxidation elsewhere.

Sustainable Swimming

During sustainable swimming at 30 and 60% U_{crit} there were increases in \dot{M}_{O_2} and thus the oxidative generation of ATP (Fig. 4-1). Our \dot{M}_{O_2} data agree well with those previously reported for trout at rest ($90 \mu\text{mol}\cdot\text{kg}^{-1}$ body mass $\cdot\text{min}^{-1}$) and during swimming at 45% and 75% U_{crit} (107 and $158 \mu\text{mol}\cdot\text{kg}^{-1}$ body mass $\cdot\text{min}^{-1}$, respectively; Kieffer *et al.*, 1998). Similarly, Lauff and Wood (1996) found resting rainbow trout had an \dot{M}_{O_2} of $92 \mu\text{mol}\cdot\text{kg}^{-1}$ body mass $\cdot\text{min}^{-1}$ which increased to $150 \mu\text{mol}\cdot\text{kg}^{-1}$ body

mass·min⁻¹ at 55% U_{crit}. These increases in \dot{M}_{O_2} with increasing swimming speeds are probably reflective of increases in red muscle ATP turnover from oxidative phosphorylation which occur in the absence of changes in red muscle [ATP] (Fig. 4-2B) and with only very minor changes in red muscle [CrP] (Fig. 4-1A) and [glycogen] (Fig. 4-3A).

Substrate utilization at 30 and 60% U_{crit} can be divided into two portions: an initial acclimatization period that relies on carbohydrate oxidation followed by a prolonged period which is characterized by enhanced lipid oxidation. During the first 2 min at 60% U_{crit}, there were increases in glycolytic flux as indicated by the accumulation of glu 6-P (Fig. 4-4B), and small, non-significant decreases in glycogen (Fig. 4-3A). By analogy to the data of Richards *et al.* (2002a), this increase in glycolysis was likely mediated through the transformation of the rate limiting enzyme glycogen phosphorylase (Phos) into its active form. Enhanced glycolysis, without an increase in lactate production (Fig. 4-2B), yields sufficient substrate for the speed-dependent increases in PDH activity (Fig. 4-5). These increases in PDH activity observed at 30 and 60% U_{crit} indicate an increase in the catalytic rate of PDH and an increase in TCA cycle flux and oxidative phosphorylation.

Pyruvate dehydrogenase is the rate-limiting enzyme that sets the rate of entry of glycolytically derived pyruvate into the TCA cycle for ATP production through oxidative phosphorylation. In mammals, the activity of PDH is regulated by reversible covalent modification and by product inhibition (Weiland, 1983). At the onset of exercise, Ca²⁺ release from the sarcoplasmic reticulum probably acted as the initial cue to activate red

muscle PDH through a stimulation of PDH phosphatase that dephosphorylates and activates PDH. The increase in PDH activity at 2 min of swimming (Fig. 4-5) resulted in product accumulation as acetyl-CoA (Fig. 4-6A). However, acetyl-CoA was kept relatively low within the mitochondria through the formation of acetyl-carnitine (Fig. 4-6B) which can be transported from the mitochondrial matrix to the cytoplasm. Total acetyl group accumulation accounted for only 7 and 19% of the total PDH activity during the first 2 min at 30 and 60% U_{crit} , respectively indicating a large increase in acetyl-CoA oxidation.

During sustained swimming (15 - 240 min) at 30 and 60% U_{crit} , PDH activity (Fig. 4-5) returned to control values indicating a relative reduction in carbohydrate oxidation and a shift toward lipid oxidation. Inactivation of PDH at 15 min was probably due to a mechanism similar to that proposed by the glucose-fatty acid cycle in mammals (van der Vusse and Reneman, 1996; Randle, 1998). We have previously demonstrated that the regulation of PDH transformation in fish white muscle during high-intensity exercise (Richards *et al.*, 2002a) and recovery (Richards *et al.*, 2002b) is similar to that described in mammals. During submaximal exercise, increases in β -oxidation cause a sustained elevation in acetyl-CoA/CoA-SH ratio (*c.f.* Fig. 4-6A and Table 4-1) and an increase in NADH/NAD⁺. These increases in acetyl-CoA and NADH may override the stimulatory effects of Ca²⁺ on PDH phosphatase. They may also reduce the transformation of PDH through a stimulation of PDH kinase that results in the phosphorylation and deactivation of PDH. Increases in PDH kinase activity have been proposed to explain reduced carbohydrate oxidation in rat red quadriceps muscle during

sustained exercise (Denyer *et al.*, 1991). Sustained elevations of muscle acetyl-CoA would also inhibit PDH activity *in vivo* through product inhibition.

During sustained exercise at 60% U_{crit} (240 min) there was a small, but significant decrease in red muscle glycogen (Fig. 4-3A) suggesting that some carbohydrate utilization persisted. Based on measured red muscle PDH activity observed during sustained swimming (15 to 240 min) and taking into account that red muscle constitutes 7% of the body mass (Moyes and West, 1995), carbohydrate oxidation at 30 and 60% U_{crit} could account for 47 and 44% of the \dot{M}_{O_2} , respectively. If we assume in the present study that 20% of the \dot{M}_{O_2} is due to protein oxidation (from Lauff and Wood, 1996), then the remaining increase in \dot{M}_{O_2} observed at 30 and 60% U_{crit} (33 and 36% of \dot{M}_{O_2} , respectively) must be due to increased lipid oxidation. Thus, sustained swimming is supported by the oxidation of ~45% carbohydrate, ~35% lipid, and ~20% protein. These relative increases in \dot{M}_{O_2} due to lipid oxidation at 30 and 60% U_{crit} can be supported by the oxidation of only 4.4 and 7.3 $\mu\text{mol}\cdot\text{g}^{-1}$ wet weight palmitate over the period swimming (15 to 240 min). The oxidation of these low concentrations of palmitate would yield changes in red muscle IMTG of only 1.5 to 2.4 μmol glycerol units $\cdot\text{g}^{-1}$ red muscle from 15 to 240 min, concentrations that are well within the error of IMTG measurement (Table 4-1). Therefore, in the present study, no changes in IMTG were expected or observed.

However, during sustained swimming there were small, but significant increases in LCFA-carnitine at 240 min of swimming at 30 and 60% U_{crit} (Fig. 7C). In muscle,

carnitine plays two major roles (Brass, 2000). First, carnitine acts to transport long chain fatty acids into the mitochondria for β -oxidation. Second, excess acetyl groups from acetyl-CoA are bound to carnitine, forming acetyl-carnitine, which keeps acetyl-CoA relatively low within the mitochondria in order to sustain flux through β -oxidation. In trout red muscle, there is a clear reliance on carnitine to bind acetyl groups from acetyl-CoA production (Fig. 4-6 and 4-7). However, the significant increase in LCFA-carnitine observed during sustained swimming (Fig. 4-7C) implicates long chain fatty acid oxidation as the source of acetyl-groups. Short chain fatty acyl carnitines are also oxidized at high rates in mitochondria isolated from carp red muscle (Moyes *et al.*, 1989), but we show no increase in SCFA-carnitine during exercise (Table 4-1). However, this does not preclude the oxidation of short chain fatty acids because their movement across the inner mitochondrial membrane is not necessarily dependent on carnitine (van der Vusse and Reneman, 1996).

The binding of long chain fatty acids to carnitine is catalyzed by CPT-1 which is thought to be the rate-limiting step in fatty acid oxidation (van der Vusse and Reneman, 1996). Based on mammalian research, CPT-1 is thought to be regulated *in vivo* by malonyl-CoA production (Ruderman *et al.*, 1999). Malonyl-CoA is the first committed step in fatty acid synthesis and is formed by the carboxylation of acetyl-CoA by acetyl-CoA carboxylase. During sustained exercise at 30 and 60% U_{crit} (≥ 15 min), malonyl-CoA concentrations decreased in trout red muscle (Fig. 4-8) despite a sustained elevation in acetyl-CoA (Fig. 4-6A). These decreases in malonyl-CoA would relieve the resting inhibition of CPT-1 during sustained exercise and enhance fatty acid oxidation through

an increase in carnitine dependent transport of fatty acids into the mitochondria. In rat muscle, submaximal exercise reduces the concentration of malonyl-CoA, as seen in trout red muscle (Fig. 4-8) and relieves inhibition of CPT-1. However, the response of CPT-1 to malonyl-CoA is not consistent between species. In the human, submaximal exercise at 60% $V_{O_{2Max}}$ does not cause a decrease in muscle malonyl-CoA despite the fact that this exercise intensity is characterized by enhanced lipid oxidation (Romijn *et al.*, 1993). In trout, malonyl-CoA may play different roles in red *versus* white muscle. We have previously demonstrated that during recovery from high-intensity exercise, a period characterized by enhanced white muscle lipid oxidation (low PDH activity, elevated acetyl-CoA, decreased IMTG, and increased LCFA-carnitine), there are increases rather than decreases in white muscle malonyl-CoA (Richards *et al.*, 2002b). These increases in malonyl-CoA observed in white muscle during recovery from high-intensity exercise may act to elongate fatty acids for oxidation as proposed by Richards *et al.* (2002b). These apparent differences in the regulation of CPT-1 by malonyl-CoA in red and white muscle of trout deserve further experimental attention.

In the present study, we provide indirect biochemical evidence for lipid oxidation during sustained exercise. Our results agree well with those of Lauff and Wood (1996) who assessed substrate use in juvenile trout by respirometry. However, direct evidence for enhanced lipid oxidation during sustained exercise remains to be demonstrated. Bernard *et al.* (1999) demonstrated that TAG:NEFA cycling is not enhanced during endurance exercise in trout. However, basal rates of TAG:NEFA cycle are high in trout and could not limit fatty acid utilization during exercise. Quantification of the absolute

rates and relative contributions of endogenous and exogenous lipids in fuelling sustained swimming needs to be addressed in isolated muscles using pulse-chase techniques (Dyke, 1997).

Non-sustainable Swimming

During swimming at 90% U_{crit} , there was a sustained elevation of red muscle PDH activity (Fig. 4-5) and a large depletion of red muscle glycogen and accumulation of lactate (Fig. 4-3A and B), indicating that trout rely heavily on carbohydrate as the substrate for high speed swimming. In particular, the high PDH activity indicates that oxidative phosphorylation of carbohydrate is a major source of ATP at 90% U_{crit} . In addition, there was a significant contribution of white muscle CrP hydrolysis (Fig. 4-9A) and glycolysis (Fig. 4-10A and B) to total power output and a potential role of the white muscle supplying substrate, as lactate, to the red muscle.

The contributions of lipid oxidation during swimming at 90% U_{crit} are likely smaller than at 30 and 60% U_{crit} . In the present study, we did not observe changes in LCFA-carnitine in red muscle of trout swimming at 90% U_{crit} for 45 min and only demonstrated decreases in red muscle malonyl-CoA concentrations at 45 min of swimming. However, given the large ATP turnover generated by a small amount of lipid oxidation, we cannot rule out the contributions of lipid oxidation to non-sustainable exercise. These data agree with those of Lauff and Wood (1996) indicating a greater reliance on carbohydrate as the carbon source during high-speed swimming at 80% U_{crit} and a lower, but sustained utilization of lipid fuels.

In trout swimming at 90% U_{crit} , ~25% of the substrate required to support the measured red muscle PDH activity could have been supplied by endogenous glycogen. The remaining substrate for red muscle PDH could have been supplied by hepatic glucose release or by white muscle lactate production (Moyes and West, 1995). However, Shanghavi and Weber (1999) have recently demonstrated the hepatic glucose release does not contribute significantly to red muscle ATP turnover in trout swimming at ~70% U_{crit} . Numerous studies have proposed that the large store of white muscle glycogen could contribute substrate to red muscle during steady state exercise (e.g. Wokoma and Johnson, 1981). In the present study, there were decreases in white muscle glycogen at 60 and 90% U_{crit} that could not be accounted for by white muscle lactate accumulation. Taking into account the fact that white muscle PDH activity did not change with swimming speed or duration (PDH activity was ~40 $\text{nmol}\cdot\text{g}^{-1}\cdot\text{wet weight}\cdot\text{min}^{-1}$), the difference between glycogen depletion, and combined lactate accumulation and oxidation reveals a discrepancy of 2.0 and 15.6 $\mu\text{mol}\cdot\text{g}^{-1}$ white muscle lactate at 60 and 90% U_{crit} , respectively. Based on whole body calculations, if this lactate were transported from the white muscle to the red muscle for oxidation, the discrepancy could provide an additional 37% ($0.06\ \mu\text{mol}\cdot\text{g}^{-1}\cdot\text{red muscle}\cdot\text{min}^{-1}$) and 211.5% ($2.96\ \mu\text{mol}\cdot\text{g}^{-1}\cdot\text{red muscle}\cdot\text{min}^{-1}$) of the total substrate needed for the measured red muscle PDH activity at 60 and 90% U_{crit} , respectively. However, based on estimates of lactate turnover in trout plasma during swimming at 85% U_{crit} ($9.7\ \mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$; Weber, 1991), the primary circulation has the capacity to shuttle 10% of the substrate required. Clearly, more work is needed to resolve this apparent discrepancy.

In conclusion, the present biochemical evidence indicates that sustained swimming in trout (30 and 60% U_{crit}) is primarily supported by red muscle contraction where carbohydrate serves as the initial substrate for ATP production. During extended periods of sustained swimming there is a partial shift in substrate utilization away from carbohydrate to an activation of long chain fatty acid oxidation. Non-sustainable swimming at 90% U_{crit} relies extensively on carbohydrate as the substrate for ATP production throughout swimming through both red muscle oxidative phosphorylation and white muscle glycolysis. The likely contributions of lipid oxidation to ATP production during swimming at 90% U_{crit} are lower than at 30 and 60% U_{crit} , but may still be important.

CHAPTER 5

PALMITATE MOVEMENT ACROSS RED AND WHITE MUSCLE MEMBRANES OF RAINBOW TROUT

ABSTRACT

We examined the movement of palmitate, as [³H]-palmitate, across giant sarcolemmal vesicles prepared from red and white muscle of rainbow trout (*Oncorhynchus mykiss*). Palmitate uptake by trout muscle is partially through a low-affinity, high-capacity carrier. Red and white muscle carriers have similar affinities for palmitate (apparent $K_m = 26 \pm 6$ and 33 ± 8 nM, respectively, reported in terms of free fatty acid (FFA) concentration); however, red muscle has significantly higher maximal uptake compared to white muscle ($V_{max} = 476 \pm 41$ and 229 ± 23 pmol·mg⁻¹ protein·s⁻¹, respectively; $P < 0.05$). Maximal palmitate uptake into red and white muscle of trout was 600 to 750 times higher than previously reported in rat red and white muscle (Bonen *et al.*, 1998, *Am. J. Physiol.* **275**: E471-E478). Palmitate uptake was dependent on temperature in both red and white muscle with maximum Q_{10} values of 3.3 and 2.2, respectively. Phloretin (250 μM) inhibited palmitate influx into red and white muscle vesicles by ~40% and the sulphhydryl group modifier HgCl₂ (2.5 mM) inhibited palmitate

uptake by 20 to 30%. The anion-exchange inhibitor, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS; 250 μM) inhibited palmitate influx into white muscle vesicles by 30%, but had no effect on palmitate influx into red muscle vesicles. Aqueous-heptane partitioning was used to assess the effects of phloretin, DIDS, and HgCl_2 on fatty acid binding characteristics to albumin. The addition of phloretin, DIDS, or HgCl_2 to a fatty acid-albumin mixture increased free palmitate by ~ 2 fold. At low concentrations of free palmitate (2.5 nM), adding high concentrations (111 μM total) of oleate (18:0) caused a 50% reduction in palmitate uptake by red and white muscle vesicles, but high concentrations (100 μM) of octanoate (8:0) caused no inhibition of uptake. Five days of swimming at $\sim 60 U_{\text{crit}}$ and 9 days of chronic cortisol elevation *in vivo*, both of which are known to stimulate lipid metabolism, had no effect on the rate of palmitate movement into red or white muscle vesicles. It appears that palmitate uptake by red and white muscle of trout is facilitated by a saturable, phloretin-, DIDS-, and mercury-inhibitable transport mechanism, but the transport process may not contribute to the regulation of lipid oxidation in red or white muscle.

INTRODUCTION

Rainbow trout are known to rely heavily on fatty acid oxidation in red muscle during periods of sustained “aerobic” swimming (Lauff and Wood, 1996; Richards *et al.*, 2002c) and in white muscle during recovery from high-intensity exercise (Moyes *et al.*, 1992; Wang *et al.*, 1994a; Richards *et al.*, 2002b). Lipid, usually as TAG, is stored in, and mobilized from, visceral adipose tissue, liver, and muscle; however, the relative contributions of each of these lipid pools in supplying substrate for muscle during exercise and recovery are not known (Moyes and West, 1995). Rates of TAG:NEFA cycling in trout are high (Bernard *et al.*, 1999) suggesting that there is a high rate of fatty acid exchange between tissues of trout, either for re-esterification or oxidation. Fatty acids are moved between tissues in plasma bound to albumin and high-density lipoproteins (Davidson *et al.*, 1988). Intracellular fatty acid binding proteins have been identified in teleost fish (Stewart and Driedzic, 1988) and act to transport fatty acids from the point of cellular entry to the mitochondria for oxidation or to sites of intracellular esterification and storage. However, no information is available on the mechanism of fatty acid movement across muscle cell membranes in fish.

Considerable controversy surrounds the mechanism of cellular fatty acid uptake in mammals (Luiken *et al.*, 1999a). Investigations using artificial, protein free, phospholipid bilayers, suggest that simple “flip-flop” diffusion of fatty acids across cell membranes is rapid and can account for rates of fatty acid uptake by cells observed *in vivo* (Hamilton and Kamp, 1999). However, at physiological pH, LCFA exist as an

ionized, anionic molecule. Thus, it has been argued that in true biological membranes, with membrane bound, anionic proteins, fatty acids cannot interact with the phospholipid membrane and thus diffusion of fatty acids into cells is limited. Furthermore, the rate of dissociation of LCFA from plasma carrier proteins (albumin) is thought to be insufficient for diffusion to explain the observed rates of fatty acid uptake into cells (Bassingthwaite *et al.*, 1989). In light of these kinetic constraints on diffusion as a means for fatty acid uptake into cells, considerable effort has focused on the potential involvement of specific proteins in facilitating both fatty acid sequestration from the plasma and fatty acid movement across cell membranes (Bonen *et al.*, 1998a).

There is a growing body of literature suggesting that fatty acid movement across mammalian cells membranes is, in part, facilitated by specific membrane-bound proteins (Luiken *et al.*, 1999a). Abumrad *et al.* (1981) first demonstrated LCFA permeation into isolated adipocytes was *via* a mechanism which displayed saturation kinetics, and which could be inhibited by protein-modifying drugs (phloretin, DIDS; Abumrad *et al.*, 1984) and competing LCFA. Recently, similar biochemical indices of protein-mediated uptake have been demonstrated in mammalian muscle using isolated giant sarcolemmal vesicles (Bonen *et al.*, 1998b). Furthermore, many proteins implicated in fatty acid movement across cell membranes have been cloned in mammals (Abumrad *et al.*, 1993; Luiken *et al.*, 2000). Specifically, proteins involved in sequestering fatty acids from the plasma (FABPpm) and proteins involved in the translocation of fatty acids across the membrane have been identified (Bonen *et al.*, 1998a). Antibodies to these proteins are effective at blocking LCFA uptake by isolated muscle vesicles (Bonen *et al.*, 1998b; Luiken *et al.*,

1999b). Cellular redistribution of fatty acid translocase (FAT; rat homolog to human CD36) from intracellular stores to the sarcolemma has been implicated as a contributing mechanism for the acute regulation of fatty acid uptake by rat muscle (Bonen *et al.*, 2000).

The primary objective of the present study was to determine if LCFA uptake by red and white muscle of trout occurs *via* a carrier-mediated process. Furthermore, insights into the importance of membrane transport in regulating overall lipid oxidation in muscle were gained by examining the effects of long-term exercise and chronic cortisol elevations, both known to stimulate lipid metabolism, on LCFA uptake by muscle. To accomplish these goals, we examined palmitate movement across giant sarcolemmal vesicles isolated from red and white muscle of trout. Giant sarcolemmal vesicles have previously been used in trout to examine the kinetics of lactate and glucose exchange across white muscle membranes (Labree and Milligan, 1999; Legate *et al.*, 2001). Giant sarcolemmal vesicles are ideal for investigating membrane transport processes because they are entirely right-side out (Pilegaard *et al.*, 1993) and are devoid of metabolic machinery (*i.e.* mitochondria; Bonen *et al.*, 1998b). Therefore, metabolite transport can be examined in the absence of metabolism.

MATERIALS AND METHODS

Animal Care

Adult rainbow trout (*Oncorhynchus mykiss*, Walbaum; 300 - 450 g) were purchased from Humber Springs Trout Hatchery, Orangeville, Ontario, Canada. Trout were transported to our freshwater holding facility and held in 800 L tanks supplied with aerated, dechlorinated city of Hamilton tapwater [composition as in Alsop and Wood (1997)] at 12°C for at least one month before experimentation. Fish were fed to satiation every other day with commercial trout pellets. Before tissue sampling, trout were carefully netted from their tank with minimal disturbance and killed by concussion. All experimental procedures fully comply with Canadian Council of Animal Care guidelines.

Giant Sarcolemmal Vesicles

Vesicles were isolated from red and white muscle using a modified protocol from Labree and Milligan (1999). Briefly, freshly killed trout were skinned along the lateral surface, and red muscle (~10 g) was dissected from both sides. White muscle (50 to 60 g) was then taken from the dorsal epaxial muscle mass. Both the red and white muscle were placed into separate petri dishes containing 30 mL of filtered vesicle preparation buffer (140 mM KCl, 5 mM MOPS, 3.5 mM phenylmethyl sulphonyl fluoride (PMSF) with 0.25% DMSO, pH 7.4). Muscle was extensively scored perpendicular to the myosepta with a scalpel blade and the muscle pieces were transferred to a flask containing ~1:1 wt:vol of vesicle preparation buffer containing 8 000 units of Type IV

collagenase (Worthington Biochemical), 1 mM CaCl₂, and 0.3 mg·ml⁻¹ of aprotinin. The flask was shaken gently in a water bath at 37°C for 1 hr. Following the incubation, the muscle pieces were allowed to settle and the supernatant was filtered through 4 layers of cheesecloth into a graduated cylinder. The remaining muscle pieces were washed with one third of the volume of the supernatant with 10 mM EDTA in vesicle preparation buffer. The muscle pieces were allowed to settle and the supernatant was filtered and combined with the previous supernatant. For the red muscle, the supernatant was placed into a 15 mL conical vial and centrifuged for 10 min at 50 g. This centrifugation step caused lipid to accumulate at the top of the muscle supernatant, which was gently removed by aspiration. For the white muscle preparation, centrifugation was not necessary. The supernatants were then added to Percoll (Sigma) and 1.4 M KCl in a ratio of 1:0.33:0.0575 and the resulting solution was bottom loaded into a round bottom, 14 mL centrifuge tube containing 3 mL of 5.75% 5(*N*-2,3-dihydroxypropylacetoamido)-2,4,6-triiodo-*N,N'*-bis(2,3-dihydroxypropyl)-isophthalamide (Nycodenz) in vesicle preparation buffer. The tubes were topped with 2 mL of vesicle preparation buffer and centrifuged at 200 g for 45 min at 20°C with a slow acceleration and deceleration (Allegra™ 21R centrifuge; Beckmann Coulter, Palo Alto, CA). The vesicles floated through the Percoll and Nycodenz layers and accumulated at the interface between the Nycodenz layer and vesicle preparation buffer. Vesicles were gently removed with a transfer pipette, combined into 15 mL conical vials, and centrifuged at 2 000 g for 30 min. The supernatant was removed by careful aspiration and the pellet was suspended in a known volume of buffer containing 140 mM KCl, 5 mM MOPS, pH 7.4 to yield a

protein concentration of $\sim 1 \mu\text{g}\cdot\mu\text{L}^{-1}$. Vesicle protein was determined using the method of Bradford (1976) with BSA standards (Sigma). Each vesicle preparation was visually examined using a microscope to ensure the formation of vesicles. Vesicles were sized with a haemocytometer.

Fatty Acid Transport

Palmitate uptake was measured using a reaction mixture containing $14.1 \mu\text{M}$ unlabelled palmitate and $0.3 \mu\text{Ci}$ of 9, 10- $[\text{}^3\text{H}](\text{N})$ palmitate (American Radiolabelled Chemicals, St. Louis, MO), and $0.05 \mu\text{Ci}$ of 1,2- $[\text{}^{14}\text{C}]$ polyethylene glycol (PEG-4000; Mol. Wt. $4000 \text{ g}\cdot\text{mol}^{-1}$; American Radiolabelled Chemicals, St. Louis, MO) in a solution containing fatty acid free BSA (ICN Biochemicals, Aurora, OH), 140 mM KCl, 5 mM MOPS, pH 7.4. $[\text{}^{14}\text{C}]$ -PEG-4000 served as an extra-vesicular space marker. The concentration of BSA was adjusted to give molar ratios of fatty acid to BSA from 0.2 to 5 which resulted in a free palmitate concentrations ranging from 1.1 to 95.9 nM (Richieri *et al.*, 1993). Transport studies were carried out in 1.7 mL polyethylene microcentrifuge tubes containing $50 \mu\text{L}$ of vesicle suspension ($\sim 50 \mu\text{g}$ of protein). Palmitate uptake was initiated by gently titrating $50 \mu\text{L}$ of fatty acid reaction mixture with the vesicle suspension. The assay temperature was 20°C . The uptake was stopped by the addition of 1.4 mL of ice-cold stop solution containing 2.5 mM HgCl_2 , 0.1% BSA, 140 mM KCl, 5 mM MOPS, pH 7.4. The centrifuge tube was then centrifuged at $12\,000 \text{ g}$ for 1 min at 20°C and the supernatant gently aspirated away from the vesicle pellet. The sides of the centrifuge tube were cleared of any droplets, and the tip of the centrifuge tube containing

the vesicle pellet was cleaved into a 20 mL glass scintillation vial using a pair of pet-clippers. A 10 μ L sample of the reaction mixture was also taken and added to a scintillation vial. Scintillation cocktail (5 mL; ACS, Amersham) was added to each vial, the vials shaken, and allowed to stand in the dark for 1 h before counting. The vials were counted on a LKB scintillation counter (Rackbeta 1217) using a double label counting program. The amount of [3 H] palmitate in the extra-vesicular space was calculated as described in Labree and Milligan (1999). Net [3 H] palmitate uptake was calculated from the radioactivity in the vesicle pellet minus the extra-vesicular radioactivity. Net [3 H] palmitate uptake was converted to pmol of palmitate uptake using the specific activity (μ Ci/mmol) and is expressed per milligram of protein.

A time course for palmitate uptake into red and white muscle vesicles at 20°C was determined by incubating vesicles for up to 30 s with either 4.8 nM or 33.9 nM free palmitate (14.1 μ M total palmitate uptake, FA:BSA ratio (v)= 0.93 or 3.78, respectively; Richieri *et al.*, 1993). Unless otherwise stated, a transport period of 10 s was used in all subsequent transport studies. To examine the concentration-dependence of palmitate uptake by red and white muscle vesicles, v in the reaction mixtures was adjusted to 0.2, 0.4, 0.9, 1.9, 3.0, 3.7, 4.2, and 5.0, which yielded free palmitate concentrations of 1.1, 2.0, 4.8, 10.2, 20.5, 33.9, 50.2, and 95.9 nM, respectively (Richieri *et al.*, 1993). In these solutions, total unlabelled palmitate and [3 H]-palmitate remained constant, thus specific activity (μ Ci/mmol) remained constant. The effects of temperature on palmitate uptake were examined in red and white muscle vesicles equilibrated to 20, 10, and 0°C using free palmitate concentrations of 4.8 nM.

The effects of known pharmacological blockers on fatty acid transport on palmitate uptake by red and white muscle vesicles was determined in presence of 14.1 μM total palmitate. Free palmitate concentrations were determined using heptane-aqueous partitioning (described below). Vesicles were incubated in the presence of 250 μM phloretin in 0.3% DMSO, 250 μM DIDS in 0.3% DMSO, and 2.5 mM HgCl_2 in the dark for 0.5 h before transport studies were carried out. Transport controls (*i.e.* vesicle preparation minus inhibitor) were run concurrently with experimental treatments and contained an equivalent amount of any vehicle (*e.g.* DMSO). The effects of phloretin, DIDS, and HgCl_2 on palmitate binding to BSA were assessed using heptane-aqueous partitioning of bound and unbound palmitate in our solutions. Similar procedures have been used to characterize the basic kinetics of fatty acid interactions with albumin (*e.g.* Spector *et al.*, 1969). Briefly, 50 μL of reaction mixture containing experimental concentrations of the pharmacological agent and appropriate vehicle was added to 450 μL of 140 mM KCl, 5 mM MOPS, pH 7.4 in a siliconized borosilicate vial. To the top of the aqueous solution, 500 μL of n-heptane was added, the tubes were tightly seals with teflon-lined caps and allowed to incubate stirred overnight at 25°C. Fifty μL of the aqueous phase and heptane phase were then counted for [^3H] palmitate. The reaction media used to construct a concentration-dependence relationship were also run in a similar manner to the above reaction media. These provided a standard curve to which our unknown solutions were compared in order to determine the effects of pharmacological agents on free palmitate concentrations.

To examine the effects of other competing fatty acids on palmitate uptake by vesicles, transport studies were conducted in the presence of excess oleate (18:0; 111 μM) and octanoate (8:0; 100 μM). Reaction mixtures contained 7.4 μM total palmitate in 0.1% fatty acid free BSA ($\nu = \sim 0.5$) which resulted in a low free palmitate concentration (2.5 nM; Richieri *et al.*, 1993). To this reaction medium, high concentrations of competing fatty acid were added. To determine the interactions between palmitate and the competing fatty acid for binding to albumin we used the calculations outlined by Abumrad *et al.* (1984). Expected changes in palmitate uptake by the muscle vesicles were assessed by comparing the predicted change in free palmitate with the known responses of the vesicles to changes in free palmitate determined above (see Fig. 5-2). Octanoate does not compete with LCFA for binding to albumin, therefore no correction was required for changes in free palmitate in the presence of excess octanoate (Bonen *et al.*, 1998b).

To determine if palmitate movement into muscle was affected by long-term exercise, trout (389 ± 44 ; 32.2 ± 0.4 cm, $n = 12$) were swum at $60 \text{ cm} \cdot \text{s}^{-1}$ (1.9 ± 0.1 body lengths $\cdot \text{s}^{-1}$) for 5 days in a darkened, 156 L, Beamish-style swim tunnel served with a flow-through ($500 \text{ mL} \cdot \text{min}^{-1}$) of fresh water. Non-exercised fish were held in darkened Plexiglas boxes of approximately the same volume as the swimming chamber of the swim tunnel and were served with a similar flow-through ($500 \text{ mL} \cdot \text{min}^{-1}$) of fresh water. Fish were checked eight times daily, and any fish that was not swimming for the entire period was discarded. At the end of the 5 days, fish were removed from fish box or tunnel, killed by concussion, vesicles were prepared from their red and white muscle as

described above, and transport studies were conducted at a free palmitate concentration of 4.8 nM.

Chronic elevations in plasma cortisol in trout have been reported to stimulate lipid metabolism (De Boeck *et al.*, 2001). To determine if chronic cortisol treatment affected palmitate movement into red and white muscle, trout were fitted with coconut oil implants containing cortisol (Gregory and Wood, 1999). Briefly, adult trout (364 ± 21 g, $n = 8$) were anaesthetized with MS-222 anesthetic and injected peritoneally with $10 \mu\text{L} \cdot \text{g}^{-1}$ body weight of liquid coconut oil at 25°C containing cortisol to yield a dose of $250 \text{ mg cortisol} \cdot \text{kg}^{-1}$ of fish (cortisol = $11\beta, 17\alpha, 21$ -trihydroxypregn-4-ene-3,20-dione; Sigma; Gregory and Wood, 1999). A second group of fish (sham; 348 ± 30 g, $n = 8$) were treated identically to the cortisol implanted fish, but were injected with coconut oil that did not contain cortisol. The coconut oil quickly solidifies at 12°C , forming a solid implant that slowly releases cortisol to the circulation. Fish were revived and the cortisol and sham fish were placed into separate 200 L tanks for 9 days. At the end of 9 days, fish were quickly killed by concussion, blood samples were taken for cortisol determination, vesicles were prepared from their red and white muscle, and transport studies were conducted at 4.8 nM free palmitate. Blood cells were immediately separated from the plasma by centrifugation and frozen in liquid N_2 . Cortisol concentrations were determined on thawed plasma using a ^{125}I radioimmunoassay (ICN Biochemicals, Aurora, CA).

Data Presentation and Statistical Analysis

All data are presented as means \pm SE (n). A non-linear regression model was used to describe the effects of changes in free palmitate concentration on palmitate uptake by the red and white muscle vesicles, and to determine the K_m and V_{max} constants of the transport. Significant differences between treatments and paired controls were determined using a two-tailed paired Student's *t*-test. One-way ANOVA followed by a Least-Significant Difference method of pairwise multiple comparisons was used to evaluate the effects of temperature on palmitate uptake. Results were considered significant at $P < 0.05$.

RESULTS

Vesicles

Sarcolemmal vesicles isolated from trout red and white muscle were spherical in shape with a diameter of 14.0 ± 0.8 (n = 84; size range 5 to 25 μm) and 13.8 ± 0.9 (n = 112; size range 5 to 55 μm), respectively.

Palmitate Transport

Palmitate uptake by red and white muscle vesicles at 20°C was linear for at least 30 and 15 s, respectively, when incubated with 4.8 nM free palmitate (Fig. 5-1A). In vesicles incubated with 33.9 nM free palmitate, palmitate uptake by red and white muscle vesicles was linear over 20 s (Fig. 5-1B). At all time points, red muscle vesicles accumulated between 2.3 to 3.6 times more palmitate than white muscle vesicles.

Palmitate uptake by red and white muscle vesicles at 20°C rapidly increased and saturated as external free palmitate was increased up to 95 nM (Fig. 5-2). Uptake of palmitate saturated at approximately twice the rate in red muscle vesicles compared to white muscle vesicles. Red and white muscle vesicles exhibited similar affinities (K_m) for palmitate uptake, but the maximum transport capacity (V_{max}) was 2.1 fold higher in red muscle vesicles than in white muscle vesicles (Table 5-1).

The addition of 250 μM phloretin and 250 μM DIDS in 0.3% DMSO to the transport medium caused 1.9 and 2.0 fold increases in free palmitate concentrations, respectively compared to the addition of 0.3% DMSO alone (Table 5-2). The addition of

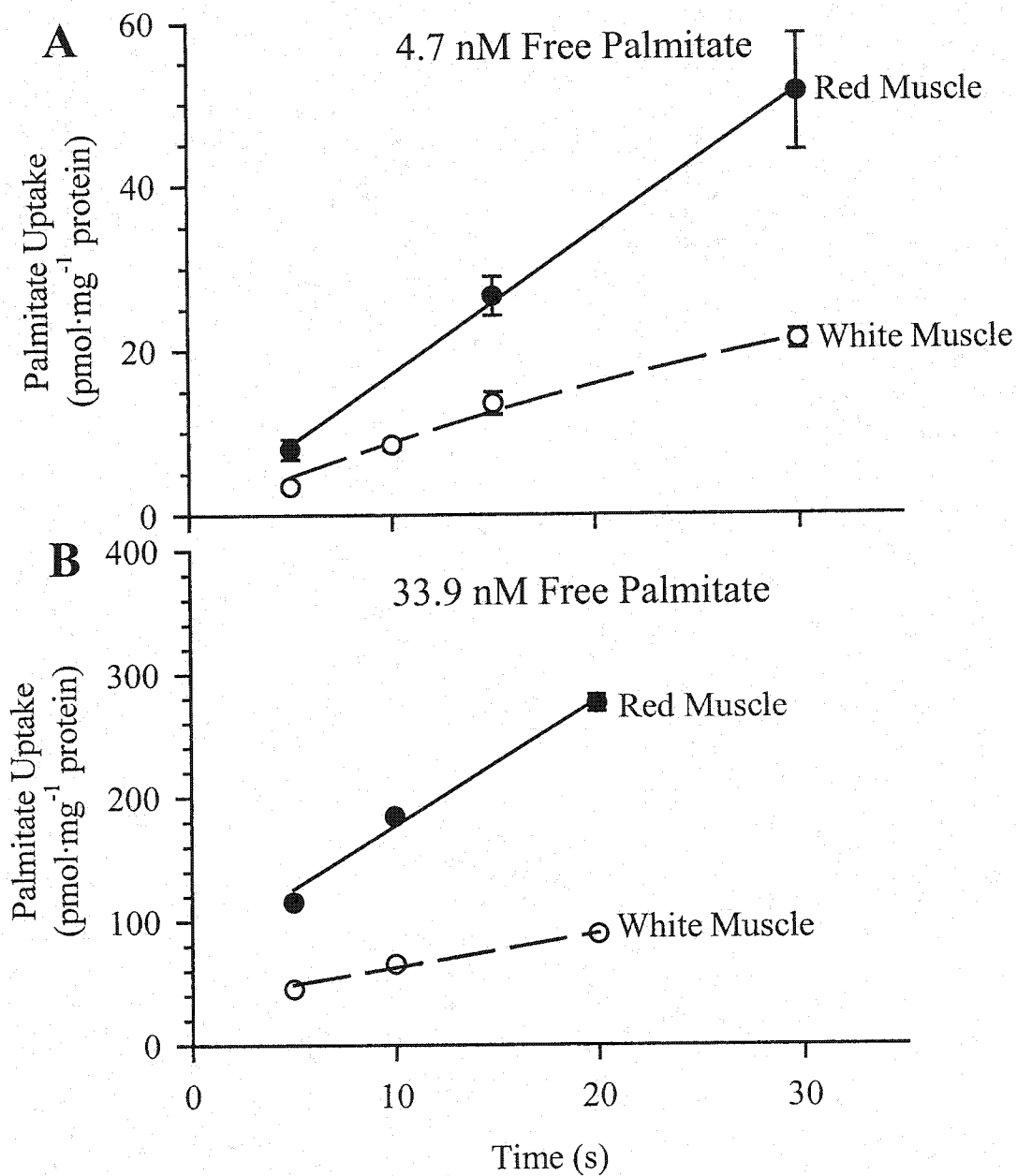


Figure 5-1:

Time course at 20°C of palmitate uptake by sarcolemmal vesicles isolated from trout red (●) and white (○) muscle during exposure to 4.8 (A) or 33.9 (B) nM free palmitate. Data are means ± SE. In A, n = 4 for each and in B, n = 2 for each.

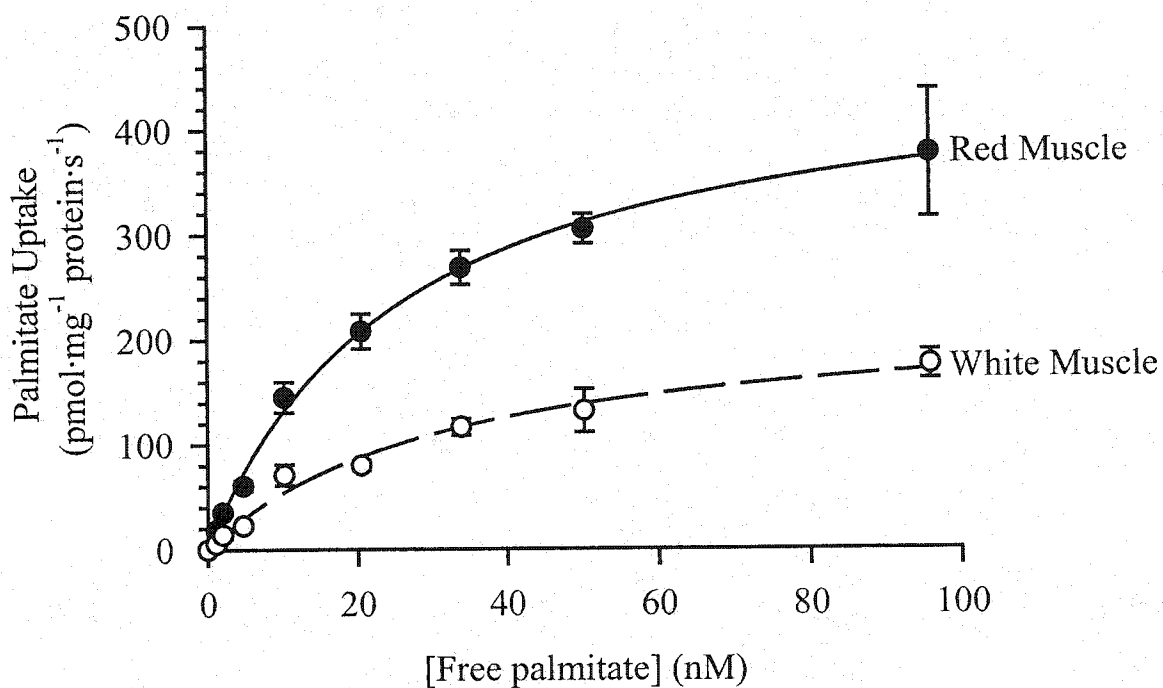


Figure 5-2:

Concentration-dependence of palmitate uptake at 20°C by sarcolemmal vesicles isolated from trout red (●) and white (○) muscle using increasing concentrations of free palmitate. The transport period was 10 s. Free palmitate concentrations were calculated according to Richieri *et al.* (1993). Data are means ± SE. From left to right, n = 6, 6, 6, 5, 6, 5, 6, and 5 for red muscle vesicles, and n = 5, 5, 5, 5, 4, 5, 5, and 5 for white muscle vesicles.

Table 5-1: K_m and V_{max} for palmitate uptake into vesicles isolated from red and white muscle. Means \pm SEM (n=9).

	K_m (nM free palmitate)	V_{max} (pmol \cdot mg $^{-1}$ protein \cdot s $^{-1}$)
Red Muscle	26.4 \pm 5.7	476.4 \pm 41.5
White Muscle	32.8 \pm 7.7	229.4 \pm 23.0
Significance	NS	$P < 0.05$

Table 5-2: Effects of phloretin, DIDS, and HgCl₂ on free palmitate concentration during transport.

	Free palmitate (nM)
Control	4.65±0.53
0.3% DMSO	4.74±0.04
250 μM Phloretin	9.03±0.53
250 μM DIDS	9.39±0.09
2.5 mM HgCl ₂	8.55±0.68

Control represents measurements done in the absence of a pharmacological agent and without carrier. Phloretin and DIDS were dissolved in 0.3% DMSO and therefore their carrier control is 0.3% DMSO.

2.5 mM HgCl₂ to the transport medium caused a 1.8 fold increase in free palmitate compared to controls. Taking these changes in free palmitate into account, palmitate uptake into both red and white muscle vesicles was inhibited by ~ 40% with the addition of 250 μM phloretin (Fig. 5-3A and B). There was no significant effect of 250 μM DIDS on palmitate uptake by red muscle vesicles (Fig. 5-3A), but DIDS caused a ~20% decrease in palmitate uptake by white muscle vesicles (Fig. 5-3B). The addition of 2.5 mM HgCl₂ reduced palmitate uptake by 21 and 29% in red and white muscle vesicles, respectively (Fig. 5-3A and B).

Palmitate uptake by red and white muscle vesicles was inhibited by 49 and 48%, respectively in the presence of 111 μM oleate (18:0; Fig. 5-4A and B). However, 100 μM octanoate (8:0) did not significantly affect palmitate uptake by either red or white muscle vesicles. Decreases in temperature from 20°C, to 10 and to 0°C decreased palmitate uptake by red muscle vesicles by 60 and 85%, respectively (Fig. 5-5A) and gave Q₁₀ values between 2.5 and 3.3. The same step-wise decreases in temperature decreased palmitate uptake by white muscle vesicles by 49 and 77% (Fig. 5-5B) and gave Q₁₀ values between 1.9 and 2.2.

Five days of exercise at 1.9 BL·s⁻¹ did not affect palmitate uptake by red or white muscle vesicles (Fig. 5-6). Furthermore, fish implanted with coconut oil implants containing cortisol (yielding plasma cortisol concentrations of 238 ± 110 ng/mL, n=6) did not exhibit different palmitate uptake rates in either red or white muscle vesicles compared to sham implanted fish (coconut oil with no cortisol; plasma cortisol = 14.8 ± 7.2 ng/mL; n=6; data not shown).

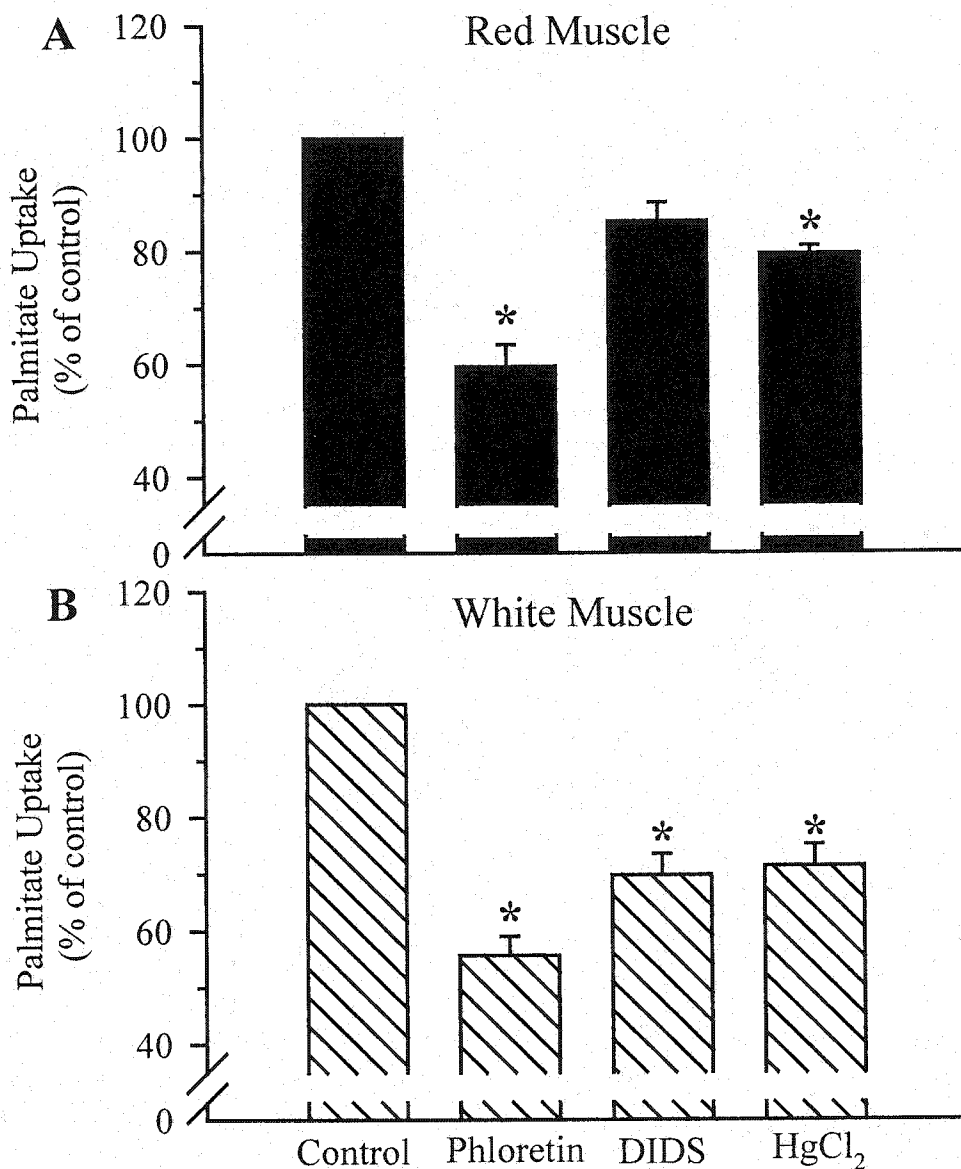


Figure 5.3:

Inhibition of palmitate uptake by 250 μ M phloretin, 250 μ M DIDS, and 2.5 mM HgCl₂ in sarcolemmal vesicles isolated from trout red (A; filled bars) and white (B; striped bars) muscle. Data are means \pm SE and are expressed relative to untreated control (100%), n = 5 for each bar. Asterisks represent a significant difference compared to controls ($P < 0.05$).

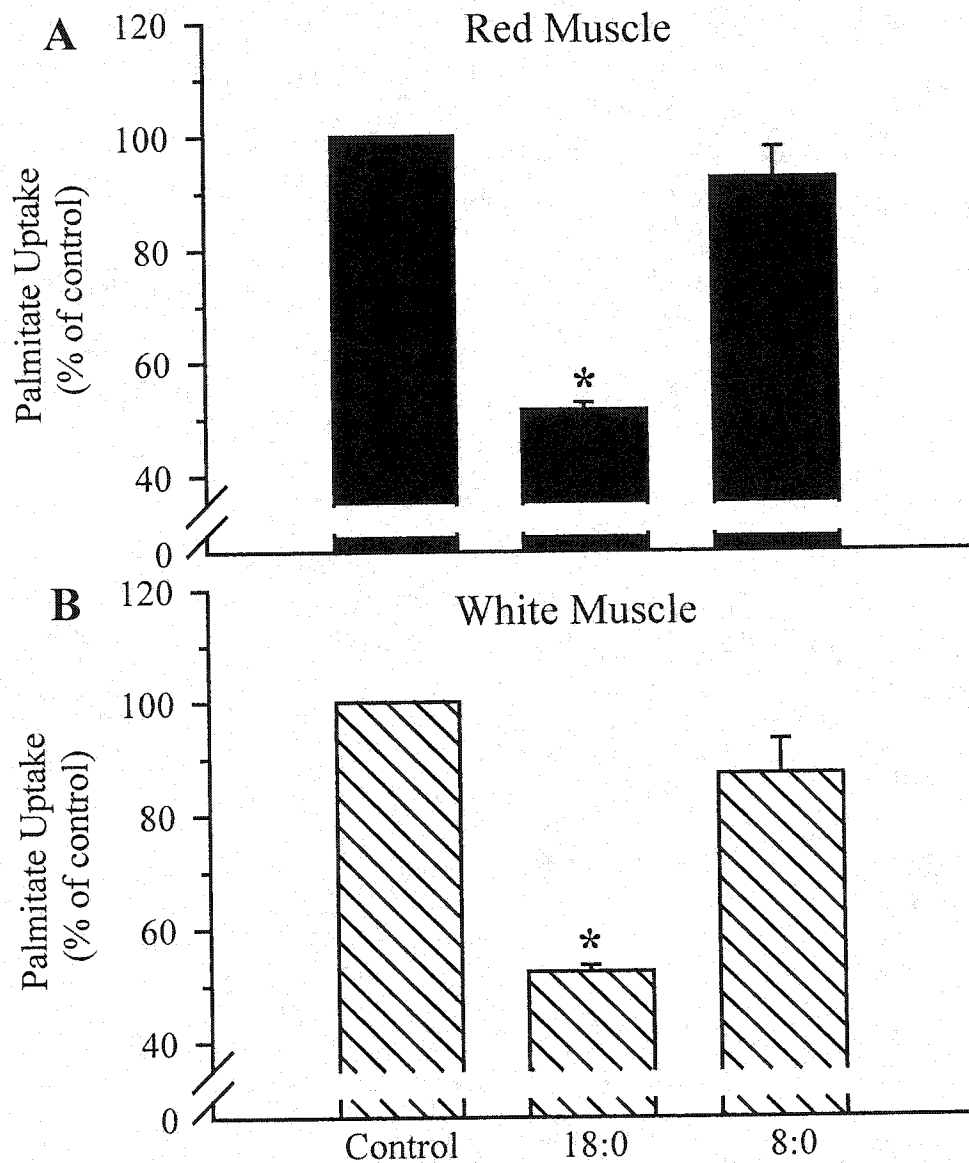


Figure 5-4:

Competition for palmitate uptake by sarcolemmal vesicles isolated from trout red (A; filled bars) and white (B; striped bars) muscle with 100 μ M oleate (18:0) and 100 μ M octanoate (8:0). Vesicles were incubated with 2.5 nM free palmitate. Data are means \pm SE and are expressed relative to untreated control (100%), $n = 5$ for each bar. Asterisks represent a significant difference compared to controls ($P < 0.05$).

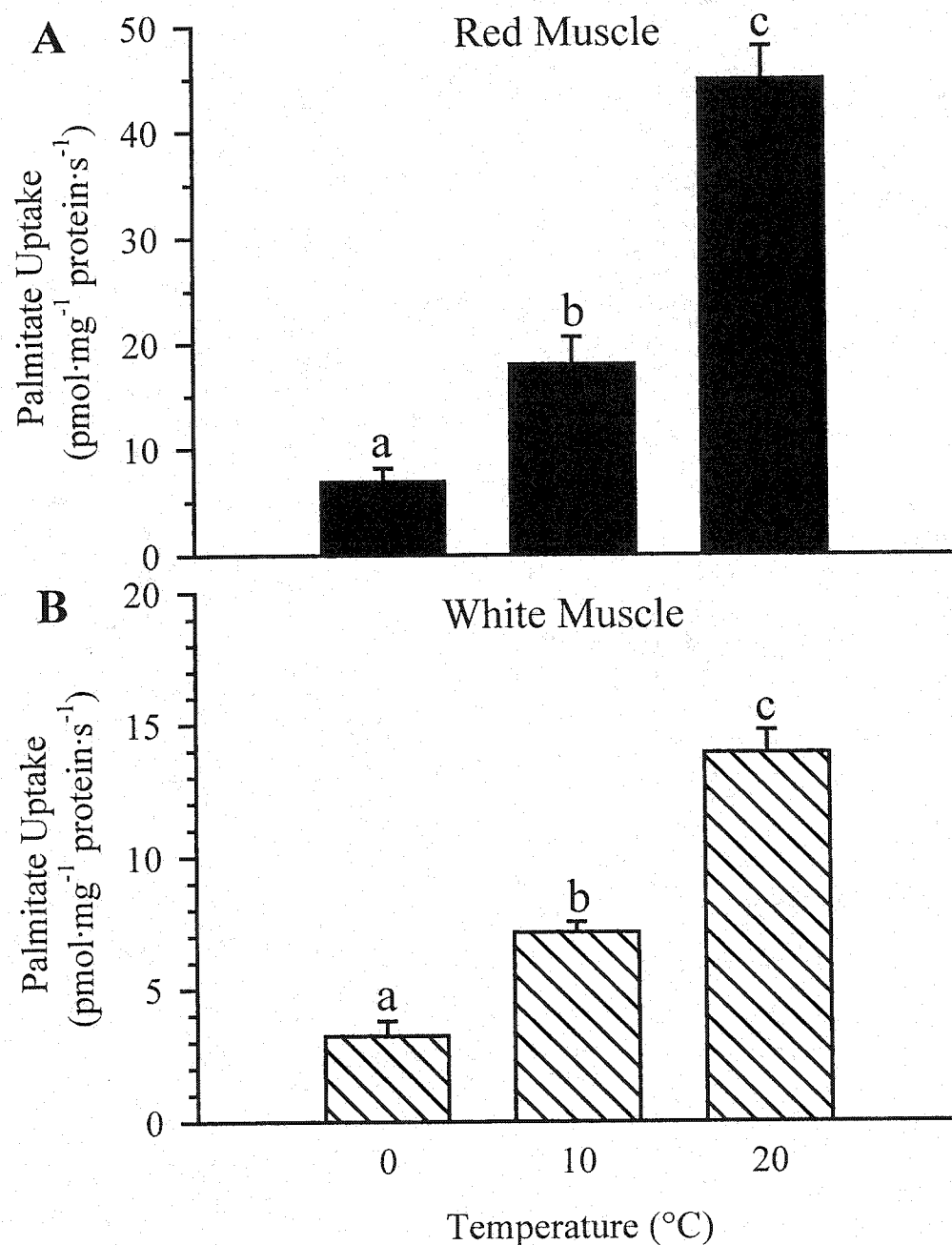


Figure 5-5:

Effects of temperature on palmitate uptake by sarcolemmal vesicles isolated from trout red (A; filled bars) and white (B; striped bars) muscle. Vesicles were incubated with 4.8 nM free palmitate. Data are means \pm SE, $n = 5$ for each bar. Bars with different letters are significantly different ($P < 0.05$).

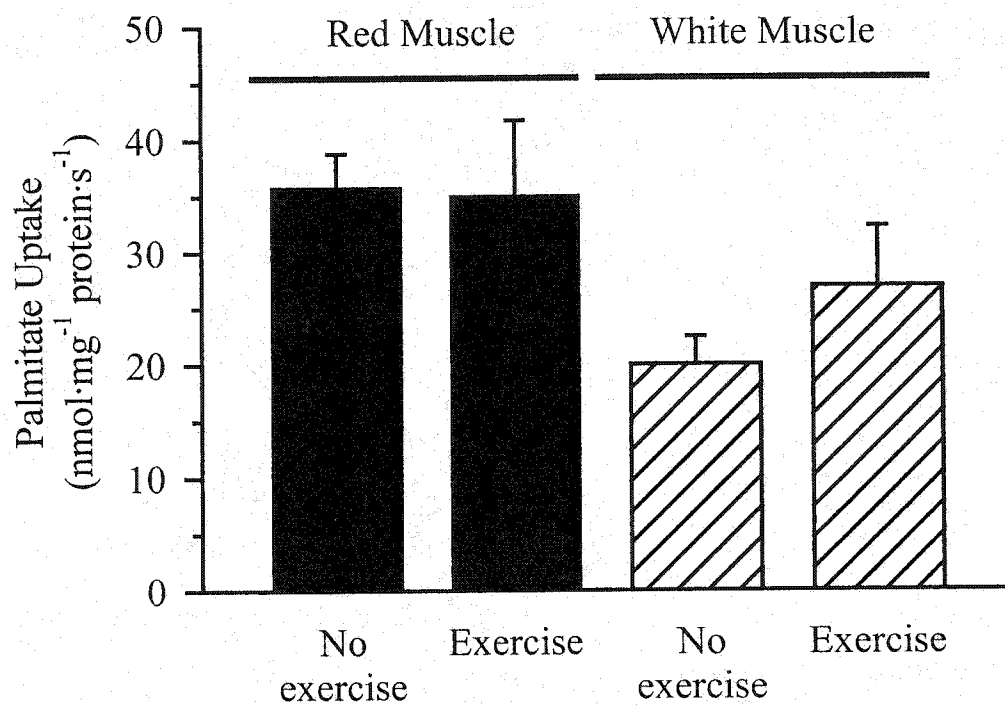


Figure 5-6:

Effects of 4 days of exercise at 1.9 body lengths·s⁻¹ on palmitate uptake by sarcolemmal vesicles isolated from trout red (filled bars) and white (striped bars) muscle. Vesicles were incubated with 4.8 nM free palmitate. Data are means \pm SE, n = 6 for each bar. There were no significant ($P > 0.05$) effects of exercise on palmitate uptake.

DISCUSSION

Sarcolemmal Vesicles

Giant sarcolemmal vesicles have become a powerful tool in elucidating the biochemical and molecular mechanisms of lactate (Pilegaard *et al.*, 1993; Juel *et al.*, 1994), glucose (Ploug *et al.*, 1992), and fatty acids (Bonen *et al.*, 1998b; Turcotte *et al.*, 2000; Glatz *et al.*, 2001) movement across muscle cell membranes in mammals. Giant sarcolemmal vesicles have also been used to examine the kinetics and pharmacology of lactate retention in rainbow trout white muscle (Labree and Milligan, 1999) and to characterize the kinetics of glucose movement across the white muscle of trout, American eel (*Anguilla rostrata*) and black bullhead catfish (*Ameiurus melas*; Legate *et al.*, 2001).

Compared to other preparations used to examine metabolite transport across muscle cell membranes (*e.g.* perfused muscle; Bonen *et al.*, 1994), giant sarcolemmal vesicles are advantageous because the vesicles are oriented right-side out, they contain intra-cellular fluid, and the transport process is divorced from subsequent metabolism (Bonen *et al.*, 1994). In particular, the presence of intracellular fluid within these vesicles makes them ideal for examining fatty acid movement across membranes because of the presence of cytoplasmic fatty acid binding protein (cytFABP) within the vesicle. This intracellular protein acts as a sink for fatty acid binding and provides the gradient for fatty acid uptake into the myocytes (Bonen *et al.*, 1998a). In the present study, vesicles prepared from red and white muscle of trout were spherical in shape and were roughly

the same size (~14 μm). Red and white muscle vesicles accumulated palmitate in a linear fashion over time (Fig. 5-1A and B) indicating that during the short assay period used in the present study (10 s), the concentration of cytFABP did not limit the rate of palmitate uptake.

Evidence for Carrier Mediated Uptake of Fatty Acids by Trout Muscle

The present study is the first to examine the movement of palmitate across giant sarcolemmal vesicles from red and white muscle of rainbow trout. Although considerable controversy surrounds the mechanisms involved in fatty acid movement across cell membranes (Hamilton *et al.*, 2001), we provide biochemical and pharmacological evidence to suggest that palmitate uptake by trout muscle is carrier-mediated. Palmitate uptake by red and white muscle vesicles occurs *via* a saturable mechanism displaying the characteristics of a low-affinity, high-capacity carrier (Fig. 5-2 and Table 5-1). These saturation kinetics were only observed when fatty acid concentrations were expressed as free palmitate (Richieri *et al.*, 1993), indicating that it is the unbound fatty acid that is transported.

Inhibition of palmitate uptake in the presence of protein modifying agents (phloretin, DIDS, and HgCl_2 ; Fig. 5-3) further suggests that membrane-bound proteins are involved in palmitate uptake. Phloretin is known to modify surface proteins and has proven effective in partially inhibiting fatty acid movement across trout red and white muscle cell membranes (Fig. 5-3A and B) and in muscle of other model systems (Abumrad *et al.*, 1981; Bonen *et al.*, 1998b; Luiken *et al.*, 1999b). HgCl_2 , a non-specific,

membrane permeable modifier of sulphhydryl groups on proteins caused minor decreases in palmitate uptake by red and white muscle vesicles of trout (Fig. 5-3A and B). The addition of DIDS, a non-permeable anion exchange inhibitor, to the vesicle suspension caused a minor decrease in palmitate uptake by white muscle vesicles, but did not significantly affect palmitate uptake by red muscle vesicles. Treatment of isolated rat adipocytes with 200 μ M DIDS has been shown to cause strong, irreversible blockade of oleate uptake (Abumrad *et al.*, 1984). In all cases, phloretin, DIDS, and HgCl_2 were shown to interact with albumin and cause an increase in free palmitate during the transport studies. Abumrad *et al.* (1981) have also demonstrated reduced binding of LCFA to albumin in the presence of phloretin. We further demonstrate that DIDS and HgCl_2 interact with albumin and cause a ~ 2 fold increase in free palmitate.

Competition between oleate and palmitate for uptake by red and white muscle vesicles further support the involvement of specific sites for LCFA binding to, or transport across, red and white muscle vesicles (Fig. 5-4). Short chain fatty acids (*e.g.* octanoate) do not compete with palmitate for uptake, indicating that the transport mechanism shows specificity for LCFA (Fig. 5-4). The permeation of SCFA into vesicles is probably *via* another transport mechanism, although simple diffusion of SCFA has also been proposed (Bonen *et al.*, 1998b). Decreases in incubation temperature from 20°C to 0°C dramatically decreased palmitate uptake by red and white muscle vesicles and demonstrate maximum Q_{10} values of 3.3 and 2.2, respectively (Fig. 5-5). These high Q_{10} values suggest that temperature has an impact upon palmitate transport rather than diffusion.

Taken together our biochemical and pharmacological results provide strong evidence suggesting that palmitate uptake into red and white muscle vesicles is *via* a carrier-mediated process. These results are in agreement with previous findings in rat muscle vesicles suggesting that palmitate uptake occurs *via* a low-affinity, high-capacity carrier protein (Bonen *et al.*, 1998b; Luiken *et al.*, 1999b). However, controversy still remains in the literature regarding the basic mechanism of fatty acid movement across muscle membranes. Hamilton and Kamp (1999) argue strongly in favour of diffusion of fatty acid through the lipid bilayer as the mechanism for fatty acid uptake into cells. However, most of their conclusions are based on the model phospholipid vesicle that is devoid of membrane bound proteins which would be ionized at physiological pH. Without assistance, fatty acids would be inhibited from interacting with the lipid bilayer for permeation.

Recent evidence points to the involvement of several proteins in facilitating LCFA uptake into various cell types (Abumrad *et al.*, 1981, 1984; Bonen *et al.*, 1998a,b; Luiken *et al.*, 2000). Putative fatty acid transport proteins have been identified in mammals including FABPpm, a fatty acid translocase (FAT/CD36), and a fatty acid transport protein (FATP; reviewed by Bonen *et al.*, 1998a). The precise role of each protein and their potential interactions to ultimately facilitate fatty acid movement across membranes is not known. However, expression studies have demonstrated a clear correlative relationship between the expression pattern of each protein and the rate of LCFA uptake by isolated vesicles (Bonen *et al.*, 1998a, 1999; Ibrahimi *et al.*, 1999). Theoretically, coordination of these proteins to facilitate LCFA transport into cells

involves the sequestering of fatty acids from the blood, either bound to albumin or as the free fatty acid, by FABPpm. Fatty acids bound to FABPpm are probably then donated to one or both of FAT or FATP which may act to facilitate the translocation of fatty acids across the plasma membranes (Bonnen *et al.*, 1998a). Based on the biochemical and pharmacological similarities between the present study and those studies demonstrating the involvement of the above proteins, it seems likely that a similar group of proteins are probably involved in fatty acid transport in trout red and white muscle. Expression studies are required to confirm the identity of the proteins involved in fatty acid movement across trout muscle cell membranes.

Differences in Transport between Red Muscle and White Muscle

Vesicles prepared from red and white muscle of trout transport palmitate with the same affinity; however, red muscle vesicles transport palmitate at approximately twice the maximum rate as white muscle vesicles (Fig. 5-2 and Table 5-1). These differences in transport capacity (V_{\max}) are probably due to a greater number of transport proteins in the red muscle membranes compared to the white muscle membrane. The red and white muscle vesicles made from trout were roughly the same size, thus the differences in palmitate uptake by red and white muscle vesicles is not due to differences in surface-to-volume ratios. The greater fatty acid transport capacity observed in red muscle compared to white muscle is probably related to the relative importance of lipid oxidation in each tissue. Red muscle of fish is known to have higher mitochondrial density compared to white muscle (citrate synthase activity 49 and 3.5 $\mu\text{mol}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}$, respectively; Moyes

et al., 1989) and red muscle has a higher capacity for LCFA oxidation. Furthermore, red muscle of trout is known to utilize lipid as a major substrate during prolonged periods of sustainable exercise ($<U_{crit}$; Lauff and Wood, 1996; Richards *et al.*, 2002c). In contrast, white muscle of fish relies primarily on substrate level phosphorylation to produce ATP to power swimming, and only relies on oxidative phosphorylation of lipid fuels during periods of post-exercise recovery (Richards *et al.*, 2002b).

Despite the differential transport of palmitate into trout red and white muscle vesicles, the maximal transport capacity of trout red and white muscle vesicles are 600 and 750 fold higher, respectively than that observed in similarly sized red and white muscle vesicles from rats (Bonen *et al.*, 1998b). These higher transport capacities attest to the importance of lipid as a substrate for metabolic energy production in trout.

Influence of Experimental Treatments on Fatty Acid Transport

Long periods of sustained swimming have been shown to increase the oxidative capacity of white and red muscle of trout (Johnson and Moon, 1980). Furthermore, sustained swimming in trout has been characterized to rely heavily on fatty acid oxidation during swimming at speeds up to 60% U_{crit} (Lauff and Wood, 1996; Richards *et al.*, 2002c). Indeed, in general, fatty acids have now been accepted as the major fuel for sustained exercise in teleost fish (Moyes and West, 1995). However, debate exists as to the source of lipid for oxidation (exogenous NEFA or endogenous TAG), and also how the rate of lipid oxidation is regulated in fish muscle (Moyes and West, 1995). In mammals, the rate of fatty acid oxidation is, in part, regulated by the rate of fatty acid

movement into the muscle cell. Chronic electrical stimulation of muscle (7 days at 10 Hz) in rats has been shown to increase the expression of FAT/CD36 and to result in an increase in palmitate uptake compared to nonstimulated muscle (Bonen *et al.*, 1999). Furthermore, LCFA uptake by rat muscle can be enhanced on an acute time scale by the cellular redistribution of FAT/CD36 from intracellular stores to the sarcolemma (Bonen *et al.*, 2000). In the present study, prolonged exercise (5 days at $1.9 \text{ bl}\cdot\text{s}^{-1}$; $\sim 60\% U_{\text{crit}}$) did not enhance palmitate movement into either red or white muscle vesicles. In addition, cortisol implants, which are known to enhance whole body lipid oxidation (De Boeck *et al.*, 2001), did not increase the rate of palmitate uptake by red and white muscle vesicles. However, the fact that palmitate uptake into trout red and white muscle vesicles is 600 to 750 fold higher than observed in rat muscle vesicles (Bonen *et al.*, 1998b) suggests that the rate of LCFA transport into trout muscle would never limit oxidation, thus precluding the transport step as an important point for the regulation of lipid oxidation in muscle. Furthermore, Bernard *et al.* (1999) demonstrated that prolonged swimming in trout did not cause a change in TAG:NEFA cycling in trout, suggesting that the rate of fatty acid delivery to muscles during exercise is also not a limiting step in the pathway of lipid oxidation by muscle.

Richards *et al.* (2002b,c) postulated that the rate of fatty acid oxidation in trout muscle is probably regulated at CPT-1, the enzyme responsible for catalyzing the binding of fatty acids to carnitine for transport into the mitochondria for oxidation (van der Vusse and Reneman, 1996). Carnitine palmitoyltransferase-1 is regulated *in vivo* by changes in malonyl-CoA. Richards *et al.* (2002c) have previously demonstrated that decreases in

red muscle malonyl-CoA concentrations occur during sustained exercise. Decreases in malonyl-CoA have been implicated in reducing the resting inhibition of CPT-1 and enhancing lipid oxidation in mammals. However, detailed studies into the role of malonyl-CoA in regulating CPT-1 in fish muscle need to be done.

Conclusions

In conclusion, we have demonstrated that LCFA uptake by trout red and white muscle membrane vesicles occurs *via* a saturable mechanism that is sensitive to inhibition by phloretin, DIDS, and HgCl₂, is competitively inhibited by other LCFA, and has a Q₁₀ for uptake of 2 to 3. Taken together, these data provide strong evidence that fatty acid uptake into the red and white muscle of trout is *via* a carrier-mediated process.

Furthermore, palmitate uptake into red muscle has a maximal rate that is twice that of white muscle, confirming the importance of fatty acid oxidation in fuelling red muscle contraction compared to white muscle contraction. Rates of fatty acid oxidation do not appear to be regulated at the point of uptake because long-term “aerobic” swimming and cortisol implantation, both of which have been demonstrated to enhance fatty acid oxidation, do not stimulate uptake. However, further investigations into the regulation of fatty acid oxidation are needed.

CHAPTER 6

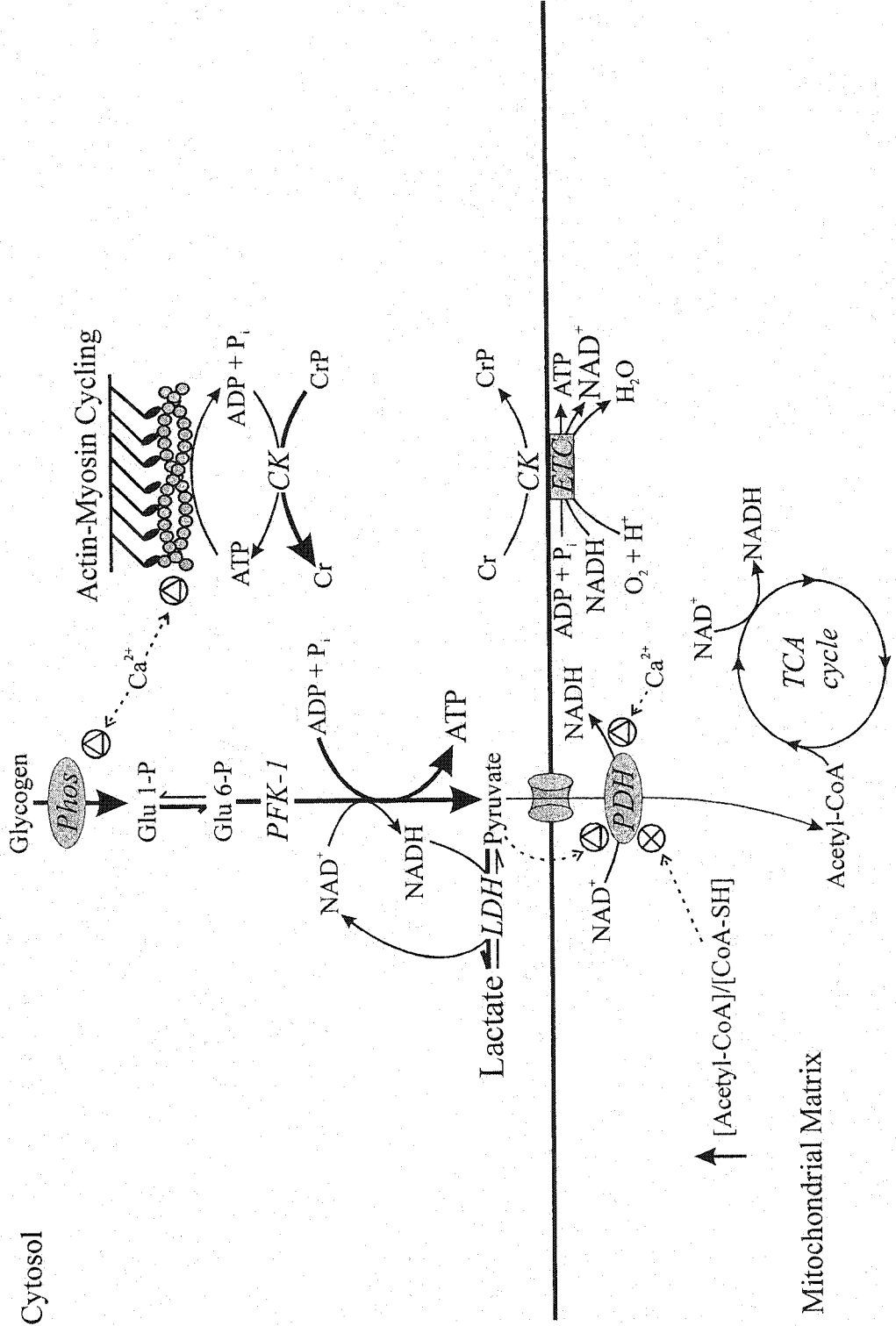
SUMMARY OF FINDINGS AND CONCLUSIONS

SUMMARY OF FINDINGS

ATP Production during High-Intensity Exercise

During high-intensity exercise, white muscle contraction is supported primarily by CrP hydrolysis and glycolysis leading to lactate accumulation (Fig. 6-1). At the onset of exercise, Ca^{2+} release from the sarcoplasmic reticulum acts to simultaneously initiate actin-myosin cycling, while allosterically stimulating Phos kinase to transform Phos_b into Phos_a and rapidly increases the rate of glycogen entry into glycolysis. Furthermore, there is a high degree of coordination between the initiation of CrP hydrolysis and glycolysis suggesting that the initiation of pathways for ATP production are not temporally separated, but are well integrated. The transformation of PDH during high-intensity exercise is slow to occur probably due to an increase in [acetyl-CoA]/[CoA-SH] (Fig. 6-1). Mismatches between the transformation patterns of, and the maximal catalytic rates of Phos and PDH explain lactate production in trout white muscle and challenge the dogma that lactate production during intense exercise is related to “anaerobiosis”.

Extracellular Space



Cytosol

Mitochondrial Matrix

↑ [Acetyl-CoA]/[CoA-SH]

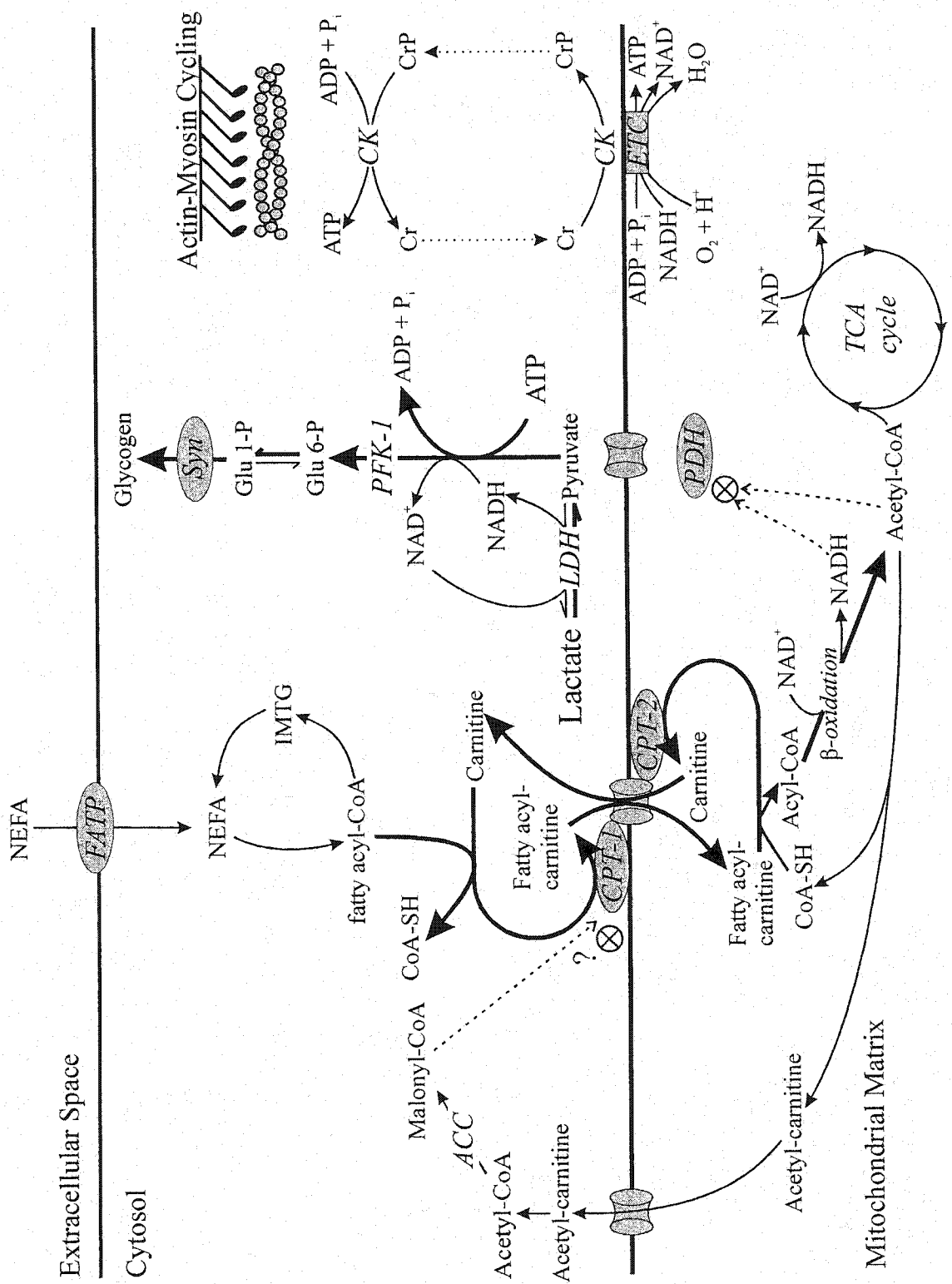
Indeed, estimates of the mitochondrial redox state ($[NAD^+]/[NADH]$) indicate that the mitochondrial matrix becomes oxidized during exercise suggesting that the electron transport chain is functioning during high-intensity exercise and that O_2 is not limiting during high-intensity exercise.

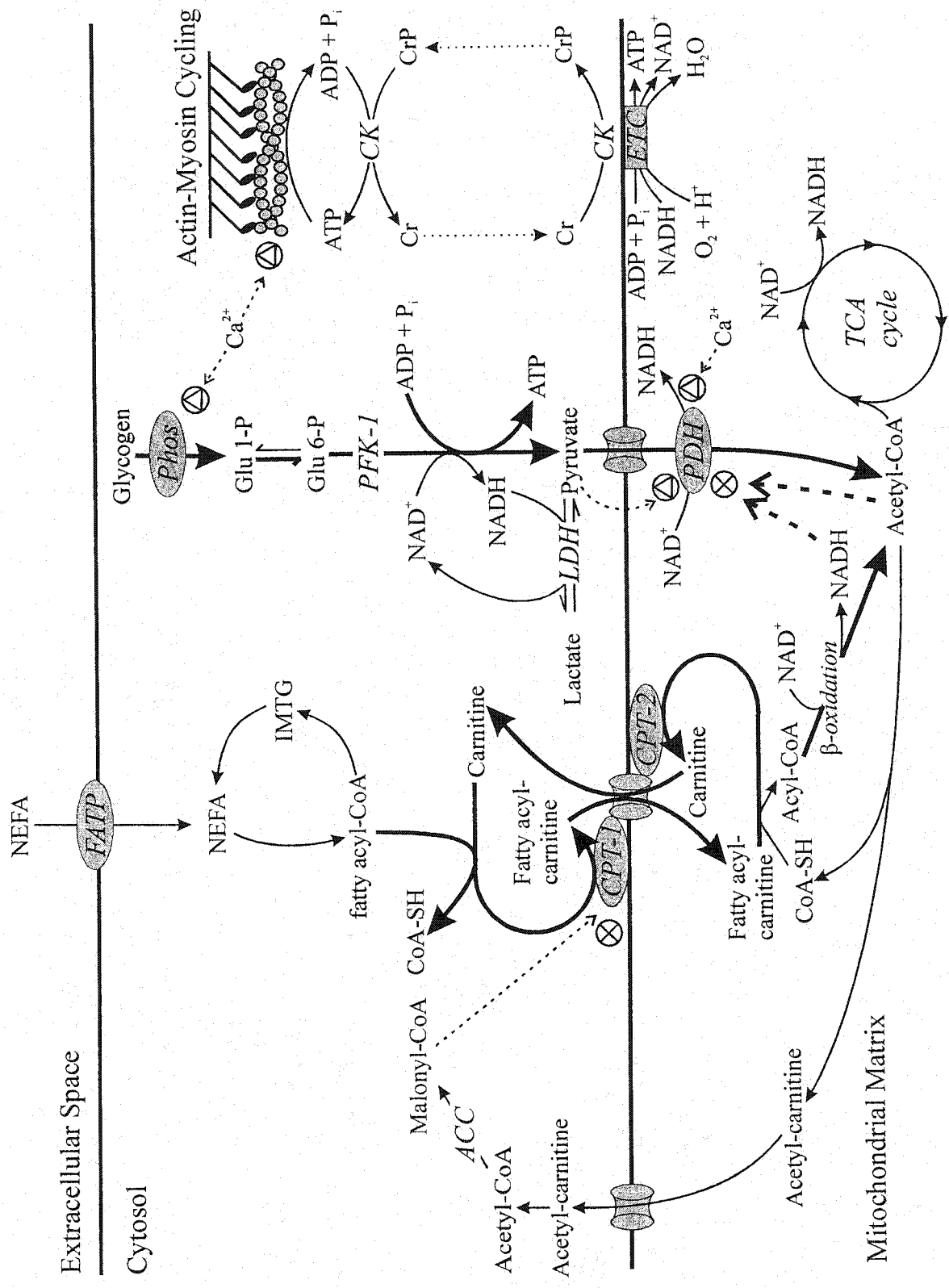
ATP Production during Recovery from High-Intensity Exercise

Upon exhaustion from high-intensity exercise there is a rapid activation of lipid oxidation, which becomes the major source providing the ATP needed for recovery metabolism. Enhanced lipid oxidation is achieved through an increase in CPT-1 activity as indicated by increases in white muscle fatty acyl-carnitine (Fig. 6-2). Products of fatty acid oxidation (NADH and acetyl-CoA) decrease the transformation of PDH, which prevents the oxidation of accumulated lactate *via* a mechanism similar to that proposed in mammals by the glucose fatty acid cycle (Randle *et al.*, 1963; Randle, 1998). Lactate is not oxidized but rather conserved as the substrate for *in situ* glycogen synthesis. The reason for the accumulation of malonyl-CoA in trout white muscle during the post-exercise period is equivocal and regulatory effects of these increases in malonyl-CoA on CPT-1 activity deserve further research attention.

ATP Production during Sustained Exercise

Substrate utilization in red muscle of trout during sustainable exercise can be divided into two temporal portions: an initial acclimatization period that relies on carbohydrate oxidation followed by a prolonged period that is characterized by enhanced lipid oxidation (Fig. 6-3). During the first two minutes of swimming at 30 and 60% U_{crit} ,





enhanced carbohydrate oxidation was achieved by a rapid transformation of PDH, initiated by Ca^{2+} release from the sarcoplasmic reticulum and accumulation of pyruvate from glycolysis. Substrate for PDH was supplied by an activation of glycolysis probably mediated through a small transformation of Phos. Within 15 min of sustainable swimming, the catalytic rate of PDH returned to control values and there was an increase in fatty acid oxidation as indicated by an increase in fatty acyl-carnitine and a decrease in red muscle malonyl-CoA. As proposed by the glucose fatty acid cycle (Randle *et al.*, 1963; Randle, 1998), the accumulation of products of lipid oxidation (NADH and acetyl-CoA) were likely the proximate cues for the deactivation of PDH and the resulting reduction in carbohydrate oxidation. Sustainable exercise at 30 and 60% U_{crit} is supported by approximately equal contributions of carbohydrate (~45%) and lipid (35%) with ~20% protein oxidation. Non-sustainable swimming at 90% U_{crit} is characterized by a sustained elevation of red muscle PDH activity despite the accumulation of high concentration of acetyl-CoA. The sustained elevation in PDH activity indicates that mechanisms exist to override the inhibitory effects of high acetyl-CoA on PDH transformation during periods of high ATP turnover and highlight the importance of carbohydrate oxidation in powering red muscle contraction during high-speed swimming.

LCFA Uptake by Red and White Muscle

Long chain fatty acid uptake by trout muscle cells is predominately *via* a carrier-mediated process (Fig. 6-2 & 6-3; FATP) and not by simple diffusion. Palmitate uptake by isolated red and white muscle vesicles occurs by a transport mechanism that

demonstrates saturation kinetics, is inhibited by pharmacological agents and other LCFA, and demonstrates a $Q_{10} \geq 2$. However, the carrier-mediated uptake of LCFA into red and white muscle does not appear to contribute to the regulation of lipid oxidation in trout muscle. Prolonged exercise and chronic elevations in cortisol, both known to enhance whole body lipid oxidation in trout had no effect on palmitate uptake into trout red and white muscle. This lack of regulation coupled with the very high V_{\max} of LCFA uptake into trout muscle compared to mammals, suggests that the cellular uptake process may not contribute to the overall regulation of lipid metabolism in trout muscle.

OVERALL CONCLUSION

The relative rates of lipid and carbohydrate oxidation by trout red and white muscle are, in part, dictated and coordinated by the regulation of rate-limiting enzymes (Phos and PDH). During periods of low ATP turnover (sustained “aerobic” exercise and recovery from high-intensity exercise), fatty acid oxidation predominates and reduces carbohydrate oxidation *via* a mechanism similar to that proposed by the glucose fatty acid cycle (Randle *et al.*, 1963; Randle, 1998). The products of fatty acid oxidation (NADH and acetyl-CoA) inhibit PDH through allosteric activation of PDH kinase, which transforms PDH_a into PDH_b , resulting in a reduction in the oxidative utilization of carbohydrate. Through the same inhibitory mechanism, glycogen entry into glycolysis is reduced through an inhibition of Phos. During periods where high ATP turnover rates are necessary (high-intensity, exhaustive exercise and prolonged swimming at 90% U_{crit}), ATP production by substrate level phosphorylation predominates and PDH activation

appears to be insensitive to the factors that regulate the glucose fatty acid cycle.

Carnitine palmitoyltransferase-1 appears to play an important role in regulating the rate of lipid oxidation in trout red muscle. As in mammals, malonyl-CoA allosterically regulates CPT-1 activity in trout red muscle but the role of malonyl-CoA in white muscle is equivocal. In contrast to mammals, the transport process involved in the uptake of LCFA into trout muscle cells does not appear to contribute to the regulation of lipid metabolism in fish.

CHAPTER 7

REFERENCES

- Abumrad, N. A., El-Maghrabi, M. R., Amri, E. Z., Lopez, E. and Grimaldi, P. A.** 1993. Cloning of a rat adipocyte membrane protein implicated in binding or transport of long-chain fatty acids that is induced during preadipocyte differentiation. Homology with human CD36. *J. Biol. Chem.* **268**: 17665-17668.
- Abumrad, N. A., Park, J. H. and Park, C. R.** 1984. Permeation of long-chain fatty acid into adipocytes. Kinetics, specificity, and evidence for involvement of a membrane protein. *J. Biol. Chem.* **259**: 8945-8953.
- Abumrad, N. A., Perkins, R. C., Park, J. H. and Park, C. R.** 1981. Mechanism of long chain fatty acid permeation in the isolated adipocyte. *J. Biol. Chem.* **256**: 9183-9191.
- Alsop, D. and Wood, C. M.** 1997. The interactive effects of feeding and exercise on oxygen consumption, swimming performance and protein usage in juvenile rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Biol.* **200**: 2337-2346.
- Anderson, G. W., McKinley, R. S. and Colavecchia, C.** 1997. The use of clove oil as an anaesthetic for rainbow trout and its effects on swimming performance. *N. Am. J. Fish. Manag.* **17**: 301-307.
- Awan, M. M. and Saggerson, E. D.** 1993. Malonyl-CoA metabolism in cardiac myocytes and its relevance to the control of fatty acid oxidation. *Biochem. J.* **295**: 61-66.
- Bassingthwaighte, J. B., Noodleman, L., Van der Vusse, G. J. and Glatz, J. F. C.** 1989. Modeling of palmitate transport in the heart. *Mol. Cell. Biochem.* **88**: 51-58.
- Bavenholm, P. N., Pigon, J., Saha, A. K., Ruderman, N. B. and Efendic, S.** 2000. Fatty acid oxidation and the regulation of malonyl-CoA in human muscle. *Diabetes* **49**: 1078-1083.
- Bergmeyer, H.** 1983. *Methods of Enzymatic Analysis*. New York: Academic Press.

- Bernard, S. F., Reidy, S. P., Zwingelstein, G. and Weber, J. M.** 1999. Glycerol and fatty acid kinetics in rainbow trout: effects of endurance swimming. *J. Exp. Biol.* **202**: 279-288.
- Black, E. C., Connor, A. R., Lam, K. C. and Chiu, W. G.** 1962. Changes in glycogen, pyruvate, and lactate in rainbow trout (*Salmo gairdneri*) during and following muscular activity. *J. Fish. Res. Bd. Can.* **19**: 409-435.
- Bonen, A., Clark, M. G. and Henriksen, E. J.** 1994. Experimental approaches in muscle metabolism: hindlimb perfusion and isolated muscle incubations. *Am. J. Physiol.* **266**: E1-E16.
- Bonen, A., Dyck, D. J., Ibrahimi, A. and Abumrad, N. A.** 1999. Muscle contractile activity increases fatty acid metabolism and transport and FAT/CD36. *Am. J. Physiol.* **276**: E642-E649.
- Bonen, A., Dyck, D. J. and Luiken, J. J.** 1998a. Skeletal muscle fatty acid transport and transporters. *Adv. Exp. Med. Biol.* **441**: 193-205.
- Bonen, A., Luiken, J. J., Arumugam, Y., Glatz, J. F. and Tandon, N. N.** 2000. Acute regulation of fatty acid uptake involves the cellular redistribution of fatty acid translocase. *J. Biol. Chem.* **275**: 14501-14508.
- Bonen, A., Luiken, J. J., Liu, S., Dyck, D. J., Kiens, B., Kristiansen, S., Turcotte, L. P., Van Der Vusse, G. J. and Glatz, J. F.** 1998b. Palmitate transport and fatty acid transporters in red and white muscles. *Am. J. Physiol.* **275**: E471-E478.
- Boutilier, R. G., Heming, T. A. and Iwama, G. K.** 1984. Appendix: physicochemical parameters for use in fish respiratory physiology. In *Fish Physiology*, vol. 10A, eds. W. S. Hoar and D. J. Randall, pp. 403-430. New York: Academic Press.
- Boyd, A. E., Giamber, S. R., Mager, M. and Lebovitz, H. E.** 1974. Lactate inhibition of lipolysis in exercising man. *Metabolism* **23**: 531-542.
- Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248-254.
- Brass, E. P.** 2000. Supplemental carnitine and exercise. *Am. J. Clin. Nut.* **72(suppl)**: 618-623.
- Brett, J. R.** 1964. The respiratory metabolism and swimming performance of young sockeye salmon. *J. Fish. Res. Bd. Can.* **21**: 1183-1226.

- Burgetz, I. J., Rojas-Vargas, A., Hinch, S. G. and Randall, D. J.** 1998. Initial recruitment of anaerobic metabolism during sub-maximal swimming in rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Biol.* **201**: 2711-2721.
- Cederblad, G., Carlin, J. I., Constantin-Teodosiu, D., Harper, P. and Hultman, E.** 1990. Radioisotopic assays of CoASH and carnitine and their acetylated forms in human skeletal muscle. *Anal. Biochem.* **185**: 274-278.
- Chapman, M. J., Goldstin, S., Mills, G. L. and Leger, C.** 1978. Distribution and characterization of serum lipoproteins and their apoproteins in the rainbow trout (*Salmo gairdnerii*). *Biochem.* **14**: 4455-4464.
- Chasiotis, D., Sahlin, K. and Hultman, E.** 1982. Regulation of glycogenolysis in human muscle at rest and during exercise. *J. Appl. Physiol.* **53**: 708-715.
- Chien, D., Dean, D., Saha, A. K., Flatt, J. P. and Ruderman, N. B.** 2000. Malonyl-CoA content and fatty acid oxidation in rat muscle and liver *in vivo*. *Am. J. Physiol.* **279**: E259-E265.
- Connett, R. J. and Sahlin, K.** 1996. Control of glycolysis and glycogen metabolism. In *Handbook of Physiology. Exercise: Regulation and Integration of Multiple Systems*, vol. 12, eds. L. B. Rowell and J. T. Shepherd, pp. 954-994. New York: American Physiological Society.
- Davidson, W. S., Birt, V. L. and Green, J. M.** 1988. Palmitate-binding, serum albumin-like proteins in salmonids. *FEBS Letters* **233**: 299-302.
- De Boeck, G., Alsop, D. and Wood, C.** 2001. Cortisol effects on aerobic and anaerobic metabolism, nitrogen excretion, and whole-body composition in juvenile rainbow trout. *Physiol. Biochem. Zool.* **74**: 858-868.
- Demoz, A., Garras, A., Asiedu, D. K., Netteland, B. and Berge, R. K.** 1995. Rapid method for the separation and detection of tissue short-chain coenzyme A esters by reversed-phase high performance liquid chromatography. *J. Chromatogr.* **667B**: 148-152.
- Denyer, G. S., Cooney, G. J., Storlein, L. H., Jenkins, A. B., Graegen, E. W., Kusunoki, M. and Caterson, I. D.** 1991. Heterogeneity of response to exercise of rat muscle pyruvate dehydrogenase complex. *Pflugers. Arch.* **419**: 115-120.
- Dobson, G. P. and Hochachka, P. W.** 1987. Role of glycolysis in adenylate depletion and repletion during work and recovery in teleost white muscle. *J. Exp. Biol.* **129**: 124-140.

Dobson, G. P., Parkhouse, W. S. and Hochachka, P. W. 1987. Regulation of anaerobic ATP-generating pathways in trout fast-twitch skeletal muscle. *Am. J. Physiol.* **253**: R186-R194.

Dreidzic, W. R. and Hochachka, P. W. 1987. Metabolism in fish during exercise. In *Fish Physiology*, vol. VII, eds. W. S. Hoar and D. J. Randall, pp. 503-543. New York: Academic Press.

Dudley, G. A., Tullson, P. C. and Terjung, R. L. 1987. Influence of mitochondrial content on the sensitivity of respiratory control. *J. Biol. Chem.* **262**: 9109-9114.

Dyke, D. J., Peters, S. J., Glatz, J., Gorski, J., Keizer, H., Keins, B., Liu, S., Richter, E. A., Spriet, L. L. van der Vusse, G. J., and Bonen A. 1997. Functional differences in lipid metabolism in resting skeletal muscle of various fiber types. *Am. J. Physiol.* **272**: E340-E351.

Eros, S. K. and Milligan, C. L. 1996. The effect of cortisol on recovery from exhaustive exercise in rainbow trout (*Oncorhynchus mykiss*): potential mechanisms of action. *Physiol. Zool.* **69**: 1196-1214.

Gibala, M., MacLean, D., Graham, T. and Saltin, B. 1998. Tricarboxylic acid cycle intermediate pool size and estimated cycle flux in human muscle during exercise. *Am. J. Physiol.* **275**: E235-E242.

Glatz, J. F., Luiken, J. J. and Bonen, A. 2001. Involvement of membrane-associated proteins in the acute regulation of cellular fatty acid uptake. *J. Mol. Neurosci.* **16**: 123-132.

Gregory, T. R. and Wood, C. M. 1999. The effects of chronic plasma cortisol elevation on the feeding behaviour, growth, competitive ability, and swimming performance of juvenile rainbow trout. *Physiol. Biochem. Zool.* **72**: 286-295.

Hamilton, J. A., Johnson, R. A., Corkey, B. and Kamp, F. 2001. Fatty acid transport: the diffusion mechanism in model and biological membranes. *J. Mol. Neurosci.* **16**: 99-108.

Hamilton, J. A. and Kamp, F. 1999. How are free fatty acids transported in membranes? Is it by proteins or by free diffusion through the lipids? *Diabetes* **48**: 2255-2269.

Harrington, A., Russel, K., Singer, T. and Ballantyne, J. 1991. The effects of tricane methanesulfonate (MS 222) on plasma non-esterified fatty acid in rainbow trout, *Oncorhynchus mykiss*. *Lipids* **26**: 774-775.

- Hassid, W. and Abraham, S.** 1957. Chemical procedures for analysis of polysaccharides. In *Methods Enzymology*, vol. 3, eds. S. Colowick and N. Kaplan. New York: Academic Press.
- Heigenhauser, G. J. and Parolin, M. L.** 1999a. Role of pyruvate dehydrogenase in lactate production in exercising human skeletal muscle. *Adv. Exp. Med. Biol.* **474**: 205-218.
- Heigenhauser, G. J. F. and Parolin, M. L.** 1999b. Role of pyruvate dehydrogenase in lactate production in exercising human skeletal muscle. In *Hypoxia: Into the Next Millennium*, eds. R. Roach P. Wagner and P. Hackett, pp. 205-218. New York: Kluwer Academic.
- Hill, A. and Lupton, H.** 1923. Muscular exercise, lactic acid and the supply and the utilization of oxygen. *Quart. J. Med.* **16**: 135-171.
- Howlett, R. A., Parolin, M. L., Dyck, D. J., Hultman, E., Jones, N. L., Heigenhauser, G. J. and Spriet, L. L.** 1998. Regulation of skeletal muscle glycogen phosphorylase and PDH at varying exercise power outputs. *Am. J. Physiol.* **275**: R418-R425.
- Hudson, R. C. L.** 1973. On the function of the white muscles in teleosts at intermediate swimming speeds. *J. Exp. Biol.* **58**: 509-522.
- Hultman, E., Bergstrom, J., and McLennan Anderson, N.** 1967. Breakdown and resynthesis of phosphoryl creatine and adenosine triphosphate in connection with muscular work in man. *Scand. J. Clin. Lab. Invest.* **19**: 50-66.
- Ibrahimi, A., Bonen, A., Blinn, W. D., Hajri, T., Li, X., Zhong, K., Cameron, R. and Abumrad, N. A.** 1999. Muscle-specific overexpression of FAT/CD36 enhances fatty acid oxidation by contracting muscle, reduces plasma triglycerides and fatty acids, and increases plasma glucose and insulin. *J. Biol. Chem.* **274**: 26761-26766.
- Jobling, M.** 1994. *Fish Bioenergetics*. London: Chapman and Hall.
- Johnson, I. A.** 1981. Structure and function of fish muscles. *Symp. Zool. Soc.* **48**: 71-113.
- Johnson, I. A. and Moon, T. W.** 1980. Endurance exercise training in the fast and slow muscles of a teleost fish (*Pollachius virens*). *J. Comp. Physiol. B* **135**: 147-156.
- Johnson, L. N.** 1992. Glycogen phosphorylase: control by phosphorylation and allosteric effects. *FASEB J.* **6**: 2274-2282.

- Juel, C., Kristiansen, S., Pilegaard, H., Wojtaszewski, J. and Richter, E. A.** 1994. Kinetics of lactate transport in sarcolemmal giant vesicles obtained from human skeletal muscle. *J. Appl. Physiol.* **76**: 1031-1036.
- Kasvinski, P. J. and Meyer, W. L.** 1977. The effect of pH and temperature on the kinetics of native and altered glycogen phosphorylase. *Arch. Biochem. Biophys.* **181**: 616-631.
- Katz, A., Spencer, M. and Sahlin, K.** 1990. Failure of glutamate dehydrogenase system to predict oxygenation state of human skeletal muscle. *Am. J. Physiol.* **256**: C26-C28.
- Keins, B. and Richter, E. A.** 1996. Types of carbohydrate in an ordinary diet affect insulin action and muscle substrates in humans. *Am. J. Clin. Nut.* **63**: 47-53.
- Keins, B. and Richter, E. A.** 1998. Utilization of skeletal muscle triacylglycerol during postexercise recovery in humans. *Am. J. Physiol.* **275**: E332-E337.
- Kieffer, J. D.** 2000. Limits to exhaustive exercise in fish. *Comp. Biochem. Physiol.* **126A**: 161-179.
- Kieffer, J. D., Alsop, D. and Wood, C. M.** 1998. A respirometric analysis of fuel use during aerobic swimming at different temperatures in rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Biol.* **201**: 3123-3133.
- Krauskopf, M., Amthauer, R., Araya, A., Concha, M., Leon, G., Rios, L., Vera, M. and Villanue, J.** 1988. Temperature acclimatization of the carp. Cellular and molecular aspects of the compensatory response. *Arch. Biol. Med. Exp.* **21**: 151-157.
- Krebs, E. G., Love, D. S., Bratwold, G. E., Trayserm, K. A., Meyerm, W. L. and Fishers, E. H.** 1964. Purification and properties of rabbit skeletal muscle phosphorylase b kinase. *Biochem.* **3**: 1022-1033.
- Krohn, M. and Boisclair, D.** 1994. The use of stereo-video system to estimate the energy expenditure of free-swimming fish. *Can. J. Fish. Aquat. Sci.* **51**: 1119-1127.
- Labree, K. and Milligan, C. L.** 1999. Lactate transport across sarcolemmal vesicles isolated from rainbow trout white muscle. *J. Exp. Biol.* **202**: 2167-2175.
- Lauff, R. F. and Wood, C. M.** 1996. Respiratory gas exchange, nitrogenous waste excretion, and fuel usage during aerobic swimming in juvenile rainbow trout. *J. Comp. Physiol. B* **166**: 501-509.

- Lauff, R. F. and Wood, C. M.** 1997. Effects of training on respiratory gas exchange, nitrogenous waste excretion, and fuel usage during aerobic swimming in juvenile rainbow trout (*Oncorhynchus mykiss*). *Can. J. Fish. Aquat. Sci.* **54**: 566-571.
- Legate, N. J., Bonen, A. and Moon, T. W.** 2001. Glucose tolerance and peripheral glucose utilization in rainbow trout (*Oncorhynchus mykiss*), American eel, (*Anguilla rostrata*), and black bullhead catfish (*Amerurus melas*). *Gen. Comp. Endocr.* **122**: 48-59.
- Lepage, G. and Roy, C.** 1988. Specific methylation of plasma nonesterified fatty acids in a one-step reaction. *J. Lipid. Res.* **29**: 227-235.
- Luiken, J. J., Glatz, J. F. and Bonen, A.** 2000. Fatty acid transport proteins facilitate fatty acid uptake in skeletal muscle. *Can. J. Appl. Physiol.* **25**: 333-352.
- Luiken, J. J., Schaap, F. G., van Nieuwenhoven, F. A., van der Vusse, G. J., Bonen, A. and Glatz, J. F.** 1999a. Cellular fatty acid transport in heart and skeletal muscle as facilitated by proteins. *Lipids* **34** (Suppl.): S169-175.
- Luiken, J. J., Turcotte, L. P. and Bonen, A.** 1999b. Protein-mediated palmitate uptake and expression of fatty acid transport proteins in heart giant vesicles. *J. Lipid. Res.* **40**: 1007-1016.
- Metcalf, V. J., Brennan, S. O., Chambers, G. and George, P. M.** 1999. High density lipoprotein (HDL), and not albumin, is the major palmitate binding protein in New Zealand long-finned (*Anguilla dieffenbachia*) and short-finned eel (*Anguilla australis schmidtii*) plasma. *Biochim. Biophys. Acta* **1429**: 467-175.
- Meyer, R. and Foley, J.** 1996. Cellular processes integrating the metabolic response to exercise. In *Handbook of Physiology. Exercise: Regulation and Integration of Multiple Systems*, vol. 12, eds. L. B. Rowell and J. T. Shepherd, pp. 841-869 New York: American Physiological Society
- Milligan, C. L.** 1996. Metabolic recovery from exhaustive exercise in rainbow trout. *Comp. Biochem. Physiol.* **113A**: 51-60.
- Milligan, C. L. and Girard, S. S.** 1993. Lactate metabolism in rainbow trout. *J. Exp. Biol.* **180**: 175-193.
- Milligan, C. L., Hooke, G. B. and Johnson, C.** 2000. Sustained swimming at low velocity following a bout of exhaustive exercise enhances metabolic recovery in rainbow trout. *J. Exp. Biol.* **203**: 921-926.
- Milligan, C. L. and Wood, C. M.** 1986. Tissue intracellular acid-base status and the fate of lactate after exhaustive exercise in the rainbow trout. *J. Exp. Biol.* **123**: 123-144.

- Mommsen, T. P. and Hochachka, P. W.** 1988. The purine nucleotide cycle as two temporally separated metabolic units: a study on trout muscle. *Metabolism* **37**: 552-556.
- Moyes, C., Buck, L., Hochachka, P. and Suarez, R.** 1989. Oxidative properties of carp red and white muscle. *J. Exp. Biol.* **143**: 321-331.
- Moyes, C. D., Buck, L. T. and Hochachka, P. W.** 1988. Temperature effects on pH of mitochondria isolated from carp red muscle. *Am. J. Physiol.* **254**: R611-R615.
- Moyes, C. D., Schulte, P. M. and Hochachka, P. W.** 1992. Recovery metabolism of trout white muscle: role of mitochondria. *Am. J. Physiol.* **262**: R295-R304.
- Moyes, C. D. and West, T. G.** 1995. Exercise metabolism of fish. In *Molecular Biology of Fishes*, vol. 4, eds. P. Hochachka and T. Mommsen, pp. 367-392. Amsterdam: Elsevier Science.
- Newsholme, E. A. and Crabtree, B.** 1979. Theoretical principles in the approaches to control of metabolic pathways and their application to glycolysis in muscle. *J. Mol. Cell. Cardio.* **11**: 839-856.
- Odland, L. M., Heigenhauser, G. J., Lopaschuk, G. D. and Spriet, L. L.** 1996. Human skeletal muscle malonyl-CoA at rest and during prolonged submaximal exercise. *Am. J. Physiol.* **270**: E541-E544.
- Odland, L. M., Heigenhauser, G. J. F. and Spriet, L. L.** 2000. Effects of high fat provision on muscle PDH activation and malonyl-CoA content in moderate exercise. *J. Appl. Physiol.* **89**: 2352-2358.
- Odland, L. M., Howlett, R. A., Heigenhauser, G. J., Hultman, E. and Spriet, L. L.** 1998. Skeletal muscle malonyl-CoA content at the onset of exercise at varying power outputs in humans. *Am. J. Physiol.* **274**: E1080-E1085.
- Okubo, M., Bogardus, C., Lilloja, S. and Mott, D.** 1988. Glucose-6-phosphate stimulation of human muscle glycogen synthase phosphatase. *Metabolism* **37**: 1171-1176.
- Parolin, M. L., Chesley, A., Matsos, M. P., Spriet, L. L., Jones, N. L. and Heigenhauser, G. J. F.** 1999. Regulation of skeletal muscle glycogen phosphorylase and PDH during maximal intermittent exercise. *Am. J. Physiol.* **277**: E890-E900.
- Peters, S. J. and Spriet, L. L.** 1995. Skeletal muscle phosphofructokinase activity examined under physiological conditions *in vitro*. *J. Appl. Physiol.* **78**: 1853-1858.

Pilegaard, H., Juel, C. and Wibrand, F. 1993. Lactate transport studied in sarcolemmal giant vesicles from rats: effect of training. *Am. J. Physiol.* **264**: E156-E160.

Ploug, T., Galbo, H., Ohkuwa, T., Tranum-Jensen, J. and Vinten, J. 1992. Kinetics of glucose transport in rat skeletal muscle membrane vesicles: effects of insulin and contractions. *Am. J. Physiol.* **262**: E700-E711.

Pörtner, H., Boutlier, R., Tang, Y. and Toews, D. 1991. The use of fluoride and nitriloacetic acid in tissue acid-base physiology. II. Intracellular pH. *Respir. Physiol.* **81**: 255-275.

Putman, C. T., Jones, N. L., Lands, L. C., Bragg, T. M., Hollidge-Horvat, M. G. and Heigenhauser, G. J. F. 1995. Skeletal muscle pyruvate dehydrogenase activity during maximal exercise in humans. *Am. J. Physiol.* **269**: E458-E468.

Randle, P. J. 1995. Metabolic fuel selection: general integration at the whole-body level. *Pro. Nutr. Soc.* **54**: 317-327.

Randle, P. J. 1998. Regulatory interactions between lipids and carbohydrates: the glucose fatty acid cycle after 35 years. *Diabetes Metab. Rev.* **14**: 263-283.

Randle, P. J., Garland, P. B., Hales, C. N. and Newsholme, E. A. 1963. The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *Lancet* **1**: 785-789.

Rasmussen, B. B. and Wolfe, R. R. 1999. Regulation of fatty acid oxidation in skeletal muscle. *Ann. Rev. Nut.* **19**: 463-484.

Ren, J. M. and Hultman, E. 1988. Phosphorylase activity in needle biopsy samples: factors influencing transformation. *Acta Physiol. Scand.* **133**: 109-114.

Rennie, P., Winder, W. and Holloszy, J. 1976. A sparing effect of increased plasma fatty acids on muscle and liver glycogen content in the exercising rat. *Biochem. J.* **156**: 647-655.

Richards, J. G., Heigenhauser, G. J. F. and Wood, C. M. 2002a. Glycogen phosphorylase and pyruvate dehydrogenase transformation in white muscle of trout during high-intensity exercise. *Am. J. Physiol.* **282**: R828-R836. (Same as chapter 3)

Richards, J. G., Heigenhauser, G. J. F. and Wood, C. M. 2002b. Lipid oxidation fuels recovery from exhaustive exercise in white muscle of rainbow trout. *Am. J. Physiol.* **282**: R89-R99. (Same as chapter 2)

- Richards, J. G., Heigenhauser, G. J. F. and Wood, C. M.** 2002c. Substrate utilization during graded aerobic exercise in rainbow trout. *J. Exp. Biol.* **In Press.** (Same as chapter 4)
- Richieri, G. V., Anel, A. and Kleinfeld, A. M.** 1993. Interactions of long-chain fatty acids and albumin: determination of free fatty acid levels using the fluorescent probe ADIFAB. *Biochem.* **32**: 7574-7580.
- Romijn, J. A., Coyle, E. F., Sidossis, L. S., Gastaldelli, A., Horowitz, J. F., Endert, E. and Wolfe, R. R.** 1993. Regulation of endogenous fat and carbohydrate metabolism in relation to exercise intensity and duration. *Am. J. Physiol.* **265**: E380-E391.
- Ruderman, N. B., Saha, A. K., Vavvas, D. and Witters, L. A.** 1999. Malonyl-CoA, fuel sensing, and insulin resistance. *Am. J. Physiol.* **276**: E1-E18.
- Saha, A. K., Kurowski, T. G. and Ruderman, N. B.** 1995. A malonyl-CoA fuel-sensing mechanism in muscle: effects of insulin, glucose, and denervation. *Am. J. Physiol.* **269**: E283-E289.
- Saha, A. K., Laybutt, D. R., Dean, D., Vavvas, D., Sebokova, E., Ellis, B., Klimes, I., Kraegen, E. W., Shafrir, E. and Ruderman, N. B.** 1999. Cytosolic citrate and malonyl-CoA regulation in rat muscle *in vivo*. *Am. J. Physiol.* **276**: E1030-E1037.
- Saha, A. K., Vavvas, D., Kurowski, T. G., Apazidis, A., Witters, L. A., Shafrir, E. and Ruderman, N. B.** 1997. Malonyl-CoA regulation in skeletal muscle: its link to cell citrate and the glucose-fatty acid cycle. *Am. J. Physiol.* **272**: E641-E648.
- Sahlin, K.** 1985. NADH in human skeletal muscle during short-term intense exercise. *Pflugers. Arch.* **403**: 193-196.
- Scarabello, M., Heigenhauser, G. J. and Wood, C. M.** 1991a. The oxygen debt hypothesis in juvenile rainbow trout after exhaustive exercise. *Respir. Physiol.* **84**: 245-259.
- Scarabello, M., Heigenhauser, G. J. and Wood, C. M.** 1992. Gas exchange, metabolite status and excess post-exercise oxygen consumption after repetitive bouts of exhaustive exercise in juvenile rainbow trout. *J. Exp. Biol.* **167**: 155-169.
- Scarabello, M., Wood, C. M. and Heigenhauser, G. J. F.** 1991b. Glycogen depletion in juvenile rainbow trout as an experimental test of the oxygen debt hypothesis. *Can. J. Zool.* **69**: 2562-2568.
- Schulte, P. M., Moyes, C. D. and Hochachka, P. W.** 1992. Integrating metabolic pathways in post-exercise recovery of white muscle. *J. Exp. Biol.* **166**: 181-195.

- Shanghavi, D. S. and Weber, J. M.** 1999. Effects of sustained swimming on hepatic glucose production of rainbow trout. *J. Exp. Biol.* **202**: 2161-2166.
- Soderling, T., Jett, M., Hutson, N. and Khatra, B.** 1977. Regulation of glycogen synthase: phosphorylation specificities of cAMP-dependent and cAMP-independent kinases for skeletal muscle synthase. *J. Biol. Chem.* **252**: 7517-7524.
- Soivio, A., Westerman, K. and Nyholm, K.** 1972. Improved methods of dorsal aorta catheterisation: haematological effects followed for three weeks in rainbow trout (*Salmo gairdneri*). *Finn. Fish. Res.* **1**: 11-21.
- Spector, A., John, K. and Fletcher, J.** 1969. Binding of long-chain fatty acids to bovine serum albumin. *J. Lipid. Res.* **10**: 56-67.
- Spriet, L. L., Howlett, R. A. and Heigenhauser, G. J.** 2000. An enzymatic approach to lactate production in human skeletal muscle during exercise. *Med. Sci. Sports Exerc.* **32**: 756-763.
- Spriet, L. L., Söderlund, K., Bergström, M. and Hultman, E.** 1987. Skeletal muscle glycogenolysis, glycolysis, and pH during electrical stimulation in men. *J. Appl. Physiol.* **62**: 616-621.
- Stewart, J. and Driedzic, W.** 1988. Fatty acid binding proteins in teleost fish. *Can. J. Zool.* **66**: 2671-2675.
- Turcotte, L. P., Petry, C., Kiens, B. and Richter, E. A.** 1998. Contraction-induced increase in V_{\max} of palmitate uptake and oxidation in perfused skeletal muscle. *J. Appl. Physiol.* **84**: 1788-1794.
- Turcotte, L. P., Swenberger, J. R., Tucker, M. Z., Yee, A. J., Trump, G., Luiken, J. J. and Bonen, A.** 2000. Muscle palmitate uptake and binding are saturable and inhibited by antibodies to FABPpm. *Mol. Cell. Biochem.* **210**: 53-63.
- van den Thillart, G., van Waarde, A., Muller, H., Erklens, C., Addink, A. and Lugtenberg, J.** 1989. Fish muscle energy metabolism measured by *in vivo* ^{31}P -NMR during anoxia and recovery. *Am. J. Physiol.* **256**: R992-R929.
- van der Vusse, G. J. and Reneman, R. S.** 1996. Lipid metabolism in muscle. In *Handbook of Physiology. Exercise: Regulation and Integration of Multiple Systems*, vol. 12, eds. L. B. Rowell and J. T. Shepherd, pp. 954-994. New York: American Physiological Society.

- Wang, Y., Heigenhauser, G. J. and Wood, C. M.** 1996. Lactate and metabolic H⁺ transport and distribution after exercise in rainbow trout white muscle. *Am. J. Physiol.* **271**: R1239-1250.
- Wang, Y., Heigenhauser, G. J. F. and Wood, C. M.** 1994a. Integrated responses to exhaustive exercise and recovery in rainbow trout white muscle: acid-base, phosphogen, carbohydrate, lipid, ammonia, fluid volume and electrolyte metabolism. *J. Exp. Biol.* **195**: 227-258.
- Wang, Y., Wilkie, M. P., Heigenhauser, G. J. F. and Wood, C. M.** 1994b. The analysis of metabolites in rainbow trout white muscle: a comparison of different sampling and processing methods. *J. Fish Biol.* **45**: 855-873.
- Wang, Y., Wright, P. M., Heigenhauser, G. J. F. and Wood, C. M.** 1997. Lactate transport by rainbow trout white muscle: kinetic characteristics and sensitivity to inhibitors. *Am. J. Physiol.* **272**: R1577-R1587.
- Wasserman, D. H. and Cherrington, A. D.** 1996. Regulation of extramuscular fuel sources during exercise. In *Handbook of Physiology. Exercise: Regulation and Integration of Multiple Systems*, vol. 12, eds. L. B. Rowell and J. T. Shepherd, pp. 1036-1074. New York: American Physiological Society.
- Weber, J. M.** 1991. Effect of endurance swimming on the lactate kinetics of rainbow trout. *J. Exp. Biol.* **158**: 463-476.
- Weber, J. M.** 1997. Oxidative metabolism in muscle cells. In *The Lung: Scientific Foundations*, eds. R. G. Crystal J. B. West and P. J. Barnes, pp. 1883-1888. Philadelphia: Lippincott-Raven Publishers.
- Weiland, O. H.** 1983. The mammalian pyruvate dehydrogenase complex: structure and regulation. *Rev. Physiol. Biochem. Pharm.* **96**: 123-170.
- Williamson, D., Lund, P. and Krebs, H.** 1967. The redox state of free nicotinamide-adenine dinucleotide in the cytoplasm and mitochondria of rat liver. *Biochem. J.* **103**: 514-527.
- Wilson, R. and Egginton, S.** 1994. Assessment of maximum sustainable swimming performance in rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Biol.* **192**: 299-305.
- Winder, W. W., Arogyasami, J., Barton, R. J., Elayan, I. M. and Vehrs, P. R.** 1989. Muscle malonyl-CoA decreases during exercise. *J. Appl. Physiol.* **67**: 2230-2233.
- Wokoma, A. and Johnson, I. A.** 1981. Lactate production at high sustainable cruising speeds in rainbow trout (*Salmo gairdneri* Richardson). *J. Exp. Biol.* **90**: 361-364.

Wolf, K. 1963. Physiological salines for freshwater teleost. *Prog. Fish-Cult.* **25**: 135-140.

Wood, C. 1991. Acid-base and ion balance, metabolism, and their interactions, after exhaustive exercise in fish. *J. Exp. Biol.* **160**: 285-308.

Wood, C. M. and Wang, Y. 1999. Lactate, H⁺, and ammonia transport and distribution in rainbow trout white muscle after exhaustive exercise. In *SEB Seminar Series: Regulation of Acid-Base Status in Animals and Plants*, eds. S. Egginton E. W. Taylor and J. A. Raven. London: Cambridge University Press.