THE DYNAMICS OF LACTATE AND AMMONIA IN RAINBOW TROUT (*Oncorhynchus mykiss*) WHITE MUSCLE

BY

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THE DYNAMICS OF LACTATE AND AMMONIA IN TROUT WHITE MUSCLE

ABSTRACT

Fish engage in intensive exercise, both naturally or through human intervention, resulting in an extensive series of physiological and biochemical disturbances. The first part of the study assessed a range of different sampling, processing, and analytical methods for determining selected metabolites in trout muscle. The goal was to provide a set of reliable methods to preserve the original metabolic state in muscle. The second part of this study employed these methods and used rainbow trout (*Oncorhynchus mykiss*) as an *in vivo* model to examine the *integrated responses* of white muscle and plasma (acid-base, electrolytes, fluid volume, and metabolic state) to severe exercise. The investigation also addressed the issues of transmembrane distribution and transport of lactate, metabolic protons, and ammonia. In the third and largest section, an isolated-perfusion tail-trunk preparation (*in vitro*) was developed to characterize mechanisms involved in the regulation of lactate, ammonia, CO_2, HCO_3^-, and proton movement across the muscle cell membrane in both resting and post-exercise muscle.

Together, these studies yielded an integrated picture of the responses to intensive activity in trout white muscle. Exhaustive exercise induced a massive depletion of muscle glycogen, the majority of which was converted to lactate. The retention of lactate, and good carbohydrate conservation, suggested that , *in situ* glycogenesis, not oxidation, was the major fate of lactate in exercised muscle. The post-exercise redox state of muscle remained oxidative, challenging the traditional concept of "anaerobic" lactate production. The unorthodox involvement of fatty acid oxidation in exercised muscle was indicated by the elevated acyl-carnitine and decreased free carnitine levels. Small amounts of lactate and metabolic acid (ΔH_m^+) were released slowly from exercised muscle into extracellular fluid, and the two fluxes were uncoupled. H^+ flux reacted to both pH and electrochemical gradients. Lactate distribution did not reach its electrochemical equilibrium, and changes in lactate efflux were more sensitive to the lactic anion (Lac^-) concentration gradient than to the lactic acid (HLac) gradient, suggesting the possible involvement of electro-neutral carrier-mediated lactate transport. For the first time, it was clearly demonstrated, *via* the application of the specific inhibitors, α-cyano-4-hydroxycinnamate (CIN) and 4-acetamido-4'-isothiocyanostilbene-2,2'-disulphonate (SITS) in conjunction with L- and D-lactate kinetics, that the carrier-mediated lactate transporters (Lac^-/H^+ symport and Lac^-/HCO_3^--Cl^- antiport), and free diffusion of HLac were involved in the transmembrane lactate exchange in both the resting and exercised fish white muscle (*in vitro*). The "lactate retention" theory was supported by this study.

The decrease of ATP in the post-exercise muscle was mirrored by the increase in total ammonia ($T_{Amm} = NH_3 + NH_4^+$) and IMP. This suggested that adenylate deamination was the souce of ammonia production, and that the retention of ammonia in muscle was used for adenylate resynthesis. Muscle membranes were permeable not only to NH_3, but also to NH_4^+, and the T_{Amm} distribution was affected by both pH and electrical gradients. Membrane potential may be more important in determining ammonia distribution (NH_4^+ permeable) during the post-exercise condition whereas the pH gradient may be more important (NH_3 permeable) at rest. The lack of change in ammonia flux in response to amiloride treatment revealed that the NH_4^+/Na^+ exchanger was not involved in ammonia efflux.

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Wow! I can't believe I am actually through this "roller-coaster ride". It is still like a dream - the "rainbows" I have been chasing for the last six years, oh no! I meant the rainbow trout I have been after all over the place (including the notorious stretch on the courageous Highway 6). I guess that persistance sometimes does pay off, and I was foolish enough to chase those fish so hard till their tails all fell off. After all, the "de-tailed" perfusion study did not come out of nowhere. The moment when someone mispronouced my name "Yu(3) Xiang(2)" with "Yu(1) Xiang(1)" (meaning fish flavor), I knew my academic life must be revolved by "fishy" smell. I have to admit that there was no lack of hardship along with the joy and excitement during this journey, but I am glad I had a chance to take on this challenge and was able to come through it. Of course, I did not fulfil my colorful "rainbows" journey at McMaster without many helping hands, and more importantly, many warm and caring hearts.

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THESIS ORGANIZATION AND FORMAT

With the recommendation of my supervisory committee, the thesis has been presented in the "open-faced" format approved by McMaster University. Therefore, this dissertation consists of a total of six chapters. Chapter 1 provides a general introduction and literature review of the current understanding of the investigated area, and a summary of the objectives and findings of the study. Chapters 2-6 are the manuscripts that have been published, accepted for publication, or submitted for publication in scientific journals.

Chapter 1: **General introduction, objectives of the investigation and summary of major findings.**

Chapter 2: **The analysis of metabolites in rainbow trout white muscle: a comparison of different sampling and processing methods.**

Authors: Y-X. Wang, M.P. Wilkie, G.J.F. Heigenhauser, and C.M. Wood (referred to as Wang *et al.*, 1994a).

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Journal: *Journal of Fish Biology* 45: 855-873 (1994).

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Chapter 3: **Integrated responses to exhaustive exercise and recovery in rainbow trout white muscle: acid-base, phosphagen, carbohydrate, lipid, ammonia, fluid volume and electrolyte metabolism.**

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Chapter 4: **Ammonia movement and distribution after exercise across white muscle cell membranes in rainbow trout: a perfusion study.**

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Chapter 5. **Lactate and metabolic H^+ transport and distribution after exercise across white muscle cell membranes in rainbow trout: a perfusion study.**

Authors: Y-X. Wang, G.J.F. Heigenhauser and C.M. Wood (referred as Wang *et al.*, 1996b).

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Chapter 6. **Lactate transport by perfused rainbow trout white muscle: kinetic characteristics and sensitivity to inhibitors.**

Author: Y-X. Wang, P.M. Wright, G.J.F. Heigenhauser and C.M. Wood (referred as Wang *et al.*, 1996c).

Date submitted: April, 1996.

Journal: *American Journal of Physiology*

Comments: This study is about to be submitted for publication. This work was conducted exclusively by Y-X.W. under the supervision of C.M.W., with important technical help from P.M.W. and logistical contributions from G.J.F.H.

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LIST OF ABBREVIATIONS

β	non-bicarbonate buffer capacity.
ΔH_m^+	metabolic proton (acid).
Acetyl-CoA	acetyl coenzyme A.
ADP	adenosine diphosphate.
Amm	ammonia.
AMP	adenosine monophosphate.
ATP	adenosine triphosphate.
CA	carbonic anhydrase.
$Carn_a$	acetyl-carnitine.
$Carn_f$	free carnitine.
$Carn_l$	long-chain acyl-carnitine.
$Carn_s$	short-chain acyl-carnitine.
CIN	α-cyano-4-hydroxycinnamate.
CoASH	coenzyme A.
Cr	creatine.
ECF	extracellular fluid.
ECFV	extracellular fluid volume.
E_m	transmembrane potential.
FFA	free fatty acid.
Glu	glucose.
GLDH	glutamate dehydrogenase.
Gly	glycogen.
GPs	glycogen phosphorylase.
HK	hexokinase.
HPLC	high performance liquid. chromatography.
ICF	intracellular fluid.
ICFV	intracellular fluid volume.
IMP	inosine monophosphate.
Lac	lactate.
LDH	lactate dehydrogenase.
Hb	hemoglobin.
MCHC	mean cell hemoglobin concentration.
Mo_2	oxygen consumption.
MS-222	tricaine methanesulfonate.
MT	mitochondria.
NAD	nicotinamide adenine dinucleotide.
NADH	nicotinamide adenine dinucleotide, reduced.
NDF	net driving force.
PCA	perchloric acid.
Pco_2	CO_2 partial pressure.
PCr	creatine phosphate.
PDH	pyruvate dehydrogenase.
PFK	phosphofructokinase.
pH_a	arterial pH.

pH_e	extracellular pH.
pH_v	venous pH.
pH_i	intracellular pH.
Pi	inorganic phosphate.
PNC	purine nucleotide cycle.
P_{NH3}	NH_3 partial pressure.
Po_2	O_2 partial pressure.
Pyr	pyruvate.
RBC	red blood cells.
SID	strong ion difference.
SITS	4-acetamido-4'-isothiocyano-stilbene-2,2'-disulphonate.
TAG	triacylglycerol.
T_{Amm}	total ammonia.
Tco_2	total CO_2.
U_{crit}	critical swimming speed.
$Vo_{2\,max}$	maximal O_2 consumption rate.

CHAPTER 1

GENERAL INTRODUCTION, OBJECTIVES & SUMMARY OF FINDINGS

Teleosts (bony fish), as the largest group of vertebrates, exist in almost all portions of the aquatic environment on earth (168). The rainbow trout, as a member of the family Salmonidae, is generally regarded as the fish equivalent of the white rat in mammalian research, and has been used extensively in research as a benchmark for several reasons: 1) it is widely available through aquaculture, and, therefore, accessible to researchers; 2) it is extremely responsive to slight alterations in its environment, and therefore a sensitive indicator of environmental changes; 3) it is a very active , and therefore a good model for exercise studies; 4) the segregated and homogeneous muscle fiber types lend themselves well to muscle metabolism studies (130).

To survive, fish face many variables in the surrounding water (*eg*. CO_2, O_2, temperature, salinity, osmolarity, alkalinity, acidity and anthropogenic pollution) and must be able to cope with these challenges accordingly. Locomotion to various degrees, namely exercise, is involved in survival strategies such as: seeking optimal water quality, foraging, predation, avoiding predation, and spawning migration (24,80). Meanwhile human activities (*eg*. catch-release sport fishing, commercial and scientific survey fishing and release, hatchery handling, transportation and stocking practices) inevitably impose further pressure upon natural challenges, thereby forcing fish to "involuntarily" undergo exhaustive exercise. This is defined as an inability to continue burst activity (4,6,9,12,38,132,158,187,193,199,203).

This type of exercise could become a stress factor and make fish more vulnerable to environmental conditions. Exhaustive exercise has been shown to cause high mortality rates in fish, and the products of "anaerobic" glycolysis (the huge accumulation of lactate and acidity), and their release into the blood stream, are considered as one of the causes (9,159, 180, 197). Along with the exercise-induced lactic acid build-up, the immediate increase of ammonia levels, as a result of deamination of adenylate or amino acid metabolism in muscle, may be a contributing cause to fatigue in animals (36,50,131).

Serving as both the "engine" and the "fuel tank" for locomotion, skeletal muscle has been a major focus of exercise physiology in the last three decades. Research efforts have resulted in an extensive understanding of carbohydrate metabolism and its regulation in mammalian muscle (58). Skeletal muscle comprises up to 70% of the fish's body mass, and in short-term and intensive exercise, it produces large amounts of lactic acid and ammonia (149,157,169,177,180). The dynamics of these two metabolites play an important role in regulating whole body metabolism and acid-base balance. The exhaustive exercise-induced physiological disturbances to the animal are multi-fold and differ substantially between fish and mammals. The differences in living environment (air *vs*. water) and modes of locomotion (swimming *vs*. walking or running) provide a good platform for comparative and environmental physiologists to establish the connections between the functional responses to the surroundings and their metabolic consequences. The following sections of the Introduction focus on the current understanding of selected aspects of exercise physiology in fish in comparison with mammals in order to establish the main theme of my research project.

I. EXERCISE PHYSIOLOGY IN FISH

1) Fish vs. Mammals

The very different physical and chemical properties of the media in which these two groups of animals live make their modes of locomotion completely different. The differences that have the greatest influence on the structural and functional design of the animal include:

a) The high density and viscosity of water compared with air which allows fish to live at neutral buoyancy, but greatly increases the cost of ventilation (1,11,184); therefore the muscular and axial systems are designed for swimming, whereas terrestrial animals have developed a locomotive system which maintains the body posture against gravity (184).

b) The availability of O_2 in water is much lower than in air (approximately 3%) which makes O_2 uptake *via* gills a most unfavorable factor for fish homeostatic control. In order to satisfy their metabolic demands, fish have a large gill surface area (60% of total body surface area) to facilitate a relatively high rate (10-30 times greater than in air-breathers) of gill ventilation for per unit of oxygen delivery. Therefore, about 10-20% of resting metabolic cost is dedicated to ventilating the gills, while 1-3% is directed to lung ventilation. Moreover, the high level of ventilation facilitates highly effective CO_2 "wash out" from blood to water which leads to a relatively low blood Pco_2. This leads to a series of challenges for the fish in terms of acid-base, osmotic, ionic, and metabolic regulation (33,60,63).

c) Poikilothermic animals such as fish have a much lower metabolic rate than homeothermic mammals. The combination of high thermal capacity of water and high ventilation rate through the gills subjects fish body temperature to the changes in environmental temperature. Thus, the metabolic and related respiratory systems exhibit significant differences from mammalian systems. The responses of fish and mammals to exercise could be distinctly different as a result of their different structural and functional design.

2) "Aerobic" vs. "Anaerobic" Exercise

Exercise is generally defined as a physiological state, induced by locomotive and higher isometric muscle activities, which causes an elevated metabolic rate as compared with the resting state. It can impose an enormous stress upon various physiological systems in the animal body. In order to maintain an optimal exercise performance, the body undergoes a series of physiological and biochemical adjustments such as: increased cardiac output (a combination of elevated heart rate and stroke volume), ventilation rate, and oxygen consumption (Vo_2, 94,160); changes in the utilization of different fuels (high energy phosphagens, carbohydrates, lipids and sometimes proteins, 35,49,53,58,64); and elevated neurohumoral activities to coordinate and control these changes. However, along with the above responses in the physiological system, there are many complex and profound consequences (*eg*. acid-base and electrolyte disturbance, 93) with which animals must deal both during exercise and during the recovery period.

Exercise is traditionally categorized as aerobic and anaerobic, depending on whether the intensity of activity has surpassed the capacities of the system to supply O_2. Long-term, moderate exercise (usually with Vo_2 less than 40% $Vo_{2\ Max}$) is considered "aerobic" exercise. In mammals, this type of exercise usually does not lead to lactate accumulation either in muscle or in blood, despite an up to 25-fold increase in glycolytic flux (16,77,165). Under these conditions, the increase in glycolysis is balanced by an increase in pyruvate oxidation *via* the Krebs cycle. It is also believed that free fatty acids (FFA) can provide 30-40% of the required energy in the first few hours of this type of activity, with the remainder coming from carbohydrate. Carbohydrate is gradually depleted and thereafter the exercise relies more heavily on FFA metabolism (up to 60% of energy expenditure). This type of aerobic activity can last for hours, or even days in some cases, and does not usually induce acidosis in the animal body.

In fish, swimming performance under 80% of the critical swimming speed (U_{crit}, maximum sustainable swimming speed) is regarded as a standard measure of aerobic performance (89,184). Lipid and protein are thought to be the main fuel supplies, although the evidence is controversial (109). However, in some species of fish (*eg*. rainbow trout), slightly elevated lactate levels in blood and skeletal muscle (red and white) were reported during the initial stage of the sustainable exercise (185,191). From production sites, metabolites, such as lactate, are released into extracellular fluid, reach the blood stream and are transported to other organs including muscle. Therefore, the substances undergo turnover (R_t) in the circulation. When the rates of appearance and disappearance are equal, the substances reach the "dynamic steady state" of metabolism. In general, the "aerobic exercise" is at this steady state with an elevated metabolic turnover rate.

At higher exercise intensity (over 80% $Vo_{2\,Max}$), an increasing proportion of the pyruvate produced is reduced to lactate. This type of exercise is defined as "anaerobic" activity due to the traditional belief that contraction of muscle is limited by O_2 supply and, therefore, part of the energy requirement must be met through anaerobically derived adenosine triphosphate (ATP). In contrast to aerobic exercise, anaerobic exercise can only be sustained for a very short period (minutes) and it causes severe acidosis (respiratory and metabolic) in both muscle and blood (44). Muscle glycogenolysis and glucose uptake (not in fish) from blood occurs at a high rate and exceeds FFA metabolism (14,163). Normally, there is a tremendous accumulation of lactate in the blood and skeletal muscle. The turnover rate of lactate is very much elevated. In terms of the enzymatic control of energy production, anaerobic metabolism is elevated by both quantity of enzymes and the presence of isozymes, as opposed to aerobic metabolism which depends only on up-regulation of the quantity of enzyme (135). Moreover, cellular concentrations of enzymes are far greater than the cellular concentrations of their substrates and products (by approximately 100-fold), as opposed to the *in vitro* measurement of enzyme kinetics. The compartmentation (*ie*. soluble and bound) and the spatial organization of enzymes are important regulating mechanisms for controlling the metabolic fluxes. In this regard, the glycolytic enzymes (glyceraldehyde 3-phosphate dehydrogenase, GPDH and lactate dehydrogenase, LDH) are shifted from a soluble to a bound form with increased glycolysis during anaerobic exercise. In contrast, conditions favoring aerobic activities result in a decreased binding of glycolytic enzymes (140).

Recently, the concept of "anaerobic" exercise at sub-maximal levels has been challenged by various research groups (14,22,23,165,166). The argument is concerned with whether lactate formation is dependent on the availability of O_2 supply, despite the existing large body of evidence showing that impaired O_2 supply results in increased lactate levels in blood and muscle, whereas a decrease of lactate occurs during exercise under hyperoxic conditions. These challenges are based upon:

(a) The measurement of the oxidative-reduction (O/R) state of mitochondria (MT) in muscle during contraction, which was first conducted by Jöbsis and Stainsby in 1968, using NADH fluorescence to indicate MT NAD/NADH (85). They concluded that, unlike the situation in the resting state, where muscle electron transport was blocked by the lack of adenosine diphosphate (ADP) and/or P_i which leads to relatively low NAD/NADH, repetitive twitch contraction of muscle caused a drastic decrease in fluorescence indicating an increase in NAD/NADH. This is due to the breakdown of ATP which produces adequate ADP and P_i to remove the barrier to electron transport. The higher ratio of NAD/NADH (more oxidative) also indicates that O_2, as a substrate of the respiratory chain, is adequate. In other words, O_2 supply may be one of the controlling factors, rather than a limiting one.

(b) Measurements of cytosolic Po_2, from estimates of myoglobin saturation in freeze-clamped contracting muscle, suggested that the upper limit of the critical intracellular Po_2 for respiration was 0.5 torr during Vo_{2Max}. With a minimum Po_2 of 2-3 torr during sub-maximal activity, lactate formation cannot be due to an O_2 limitation of respiration and/or metabolism (22,23,42).

(c) A rather philosophical approach to this issue was taken by Holloszy and Coyle (74) to argue against the textbook paradigm of the "anaerobic threshold" concept or that lower lactate production and improved endurance at the same absolute work load after training, compared with the untrained animal, are due to enhanced O_2 delivery. According to this logic, it is reasonable to assume that the trained individual with higher aerobic metabolism (less lactate production) would consume more O_2 (higher Vo_2) with the same work rate. In other words, the trained individual should have a lower work rate than the untrained one with the same O_2 consumption. However, this is not the case since O_2 consumption is the same in both the trained and untrained state, as long as it is measured at the same "sub-maximal" work rate (74).

(d) A recent study, using direct near-infrared spectrophotometric assessment of the O/R state of cytochrome oxidase, a-a_3, has revealed that the respiratory chains become more oxidized than in the resting state during repetitive twitching and tetanic contraction (166). This result seems to put an end to the on-going arguments that inadequate O_2 is a determinant of $Vo_{2\,Max}$ or lactate production.

Even with all these challenges, the classical terms of "anaerobic" and "aerobic" exercise are still conveniently and widely used.

1. SKELETAL MUSCLE

Fishes and mammals, due to their different forms of locomotion as mentioned above, have very different muscle arrangements. Mammalian skeletal muscles have long flexible tendons which maintain the flexible locomotion of the limbs. The elastic nature of the tendons allow the storage of a great proportion of the strain energy as the animal is moving against gravity, and most of this energy can be released during the propulsive phase of the stride. This structure provides terrestrial locomotion with a metabolic energy saving feature (19). Unlike mammals, most fish swim *via* the sequential contraction of the myotome on alternate sides of the body. This generates a lateral oscillation of the body which transfers the muscle contraction force to the central thrust from water (89). Most of the deeper fibers do not run parallel to the long axis, but instead are oriented at an angle of up to 40°, whereas the superficial sheet of fibers run parallel to the longitudinal axis of the body. The V-shape of the fibers allows the lateral flexibility. The 'helical arrangement' of the deeper fibers allows all of the fibers to contract at the same rate and by the same amount so that they may generate optimal force (87). Because fish do not have to move against gravity (neutral buoyancy), they can afford to carry a large muscle mass (60-70% of body mass, 118). This muscle mass provides the fish with the extra power to move against the high resistance of water during swimming, and store extra fuel in the form of glycogen, lipid, and protein. Fish muscle can contract without any volume change allowing the fish to maintain its stream-lined body shape. There is also no spindle in their muscle cell since the fish does not need to constantly hold its posture against the downward pull of gravity.

Swimming in water is associated with a high metabolic cost during burst activity, and more economic energy expenditure during low speed cruising. Yet, to cope with this style of locomotion, fish are required to have a more distinctive set of fibre types than mammals. Two main, and several minor, fiber types are found in salmon and trout. The fast twitch fibers (white, more glycolytic) constitute the majority (approx. 90%) of the already very large muscle mass, and are located mainly in the deeper zone. The non-twitch fibers (red, more oxidative) occupy no more than 10% of the total muscle mass and exist, generally, along the lateral line and as fin muscles (118). However, fish also have a pink muscle zone (fast oxidative fibers) which combines the properties of red and white fibers and is located between them (88,89). Although it is not clear whether red and white fibers can be inter-converted, since fish never cease to grow during their life, the proportion of the two types of fibers can vary rather significantly according to the life style of the fish. Exercise and training can certainly play an important part in this change. Multiple innervated red fibers are recruited to power low speed swimming with graded contraction. The number of active motor units can vary according to the resistance. White muscle fibers in primitive fish are focally innervated, while these fibers in more advanced teleosts have multi-terminal innervation. This feature allows fish with little red muscle to use white muscle to assist with cruising (11,91,92). In rainbow trout, the threshold swimming speed for recruitment of white muscle is 1.5 -2.5 body lengths s^{-1} (11,92).

White muscle includes fibers of various sizes. Commonly, this type of fiber has low mitochondrial (MT) content and capillary density (88). However, MT density varies among different species. For example, mean MT content in brook trout (9.3%) is five times greater than in plaice (92). Red fibers in fish have the highest MT content among any vertebrate skeletal muscle, and are highly vascularized (87).

2. RESPIRATORY AND METABOLIC ACIDOSIS

1) Definitions

Respiratory acidosis: generally refers to a marked depression in pH caused by Pco_2 elevation. H^+ and HCO_3^- concentrations both increase (193).

Metabolic acidosis: refers to the pH depression caused by metabolic proton loading (primarily a decline in HCO_3^- and an increase in H^+ concentration) which, for example, occurs with lactate production and ATP hydrolysis (67,196).

Blood acidosis occurs only during 'anaerobic', rather than 'aerobic', exercise. The exhaustive exercise-induced acidosis comprises both respiratory and metabolic components.

2) Differences between Fish and Mammals

Acid-base regulation is quite different in fish than in terrestrial mammals because of environmental and endogenous factors. Using water, rather than air, as a gas exchange medium, is the major cause of the differences:

(a) In fish, the counter-current water/blood flow over a very large gill surface area provides fish with an efficient gas exchange interface which facilitates O_2 uptake, and copes with their metabolic need. However, fish gills function as a site for both respiratory gas exchange and ion regulation, in contrast to air-breathing animals where lungs serve as specialized gas exchange devices only. Fish gills readily exchange ions; however, they are subjected to changes in water chemistry. Ionic exchange is tightly connected to acid-base regulation, and also has considerable impact on respiratory gas exchange.

(b) In fish, the Pco_2 gradient between arterial blood (2-4 torr) and water (less than 1 torr) is similar to that between arterial blood in mammalian lungs (42 torr) and alveolar air (40 torr). However, with Pco_2 in the atmosphere close to zero, a large Pco_2 gradient exists between the alveolar air and the tidal air. Water capacitance for CO_2 is greater than for O_2, and soluble carbonate and other non-carbonate buffering results in an even greater CO_2 capacitance. In other words, large changes in water CO_2 content will be reflected in only very small changes in Pco_2. Therefore, CO_2 washout can still be effective despite a very small trans-epithelium Pco_2 gradient.

(c) Non-bicarbonate buffer capacity (β) in blood and intracellular fluid is 1.5-4 fold lower in fish than in terrestrial mammals due to the lower protein contents. In an open system, pH equilibrium is very much dependent on β, as the dehydration of HCO_3^- to CO_2 requires a constant supply of H^+, while very low β limits the source of H^+ supply to carry on the HCO_3^- dehydration. In addition, β can directly facilitate carbonic anhydrase (CA) activity which catalyzes CO_2 hydration/dehydration reactions (8). This may explain why CA is present in the luminal surface of vascular endothelial cells such as mammalian lung, but not the vascular endothelium of fish gills (66).

(d) The bicarbonate buffering system (consisting of CO_2, H_2CO_3, HCO_3^-, and CO_3^{2-}) is also weaker in fish plasma ($\leq$ 10 mmol total concentration). The elimination of CO_2 contributes only a very small proportion of the change in total bicarbonate level. CO_2 moves across cell membranes largely in the form of molecular CO_2 because the plasma membrane is probably not very permeable to HCO_3^-, and HCO_3^-/Cl^- exchange constitutes only a small portion of total CO_2 excretion (61).

(e) Fish rely much more on transferring acid-base equivalents (H^+, OH^-, HCO_3^-, and other strong ions), rather than gas exchange across the gill epithelial surface, to adjust its acid-base state. In fresh water fish, metabolic proton movement across the gill branchial epithelium has been linked to strong ions (*eg*. Na^+/H^+ and Cl^-/HCO_3^- exchangers and NH_4^+/Na^+ exchange) (63,69,116,117,148,192). The electrogenic proton pump is also suggested in recent studies (81,110).

(f) During strenuous exercise, the red blood cells of some fish such as salmonids extrude H^+ *via* the catecholamine regulated Na^+/H^+ exchanger on the membrane (147). This is a protective process to raise intracellular pH and thereby prevent excessive acidosis caused by CO_2 and H^+ influx. Therefore, this mechanism assures protection of blood O_2 transport (147). Inevitably, this process also contributes to plasma acidosis (193).

3) The regulation of Acid-base Status in Aerobic and Anaerobic Exercise

In general, there are three pH restoring mechanisms during lactic acidosis:

(a) Adjusting Pco_2 by ventilation. At non-steady state (*eg*. highly intensive exercise over anaerobic threshold), hyperventilation causes respiratory alkalosis in the blood as CO_2 expiration exceeds its production. However, this is unlikely to happen in fish because ventilating water can be metabolically very expensive. Moreover, with already very low plasma Pco_2, hyperventilation in fish is not going to drive Pco_2 down to a great extent. In contrast to exhaustive exercise, low and moderate levels of exercise do not usually create acidosis in blood.

(b) Oxidation of lactate to CO_2 *via* the Krebs cycle, or resynthesis of glycogen *via* glyconeogenesis, will also consume protons to eliminate lactic acidosis. However, lactate oxidation could lead to respiratory acidosis as explained above. The oxidation of lactate usually occurs in sustained, low intensity exercise, and during the recovery period of intensive exercise. Lactate can be oxidized *in situ* or in other more oxidative tissues such as red muscle and heart (20,102,121,176). More recently, a number of studies have demonstrated that the resting, slightly exercised, and recovering white muscle fibers are also capable of carrying out lactate oxidation in higher vertebrates, but this role appears to be minimal in fish (115,121,129).

(c) Elimination of surplus H^+ from body fluid is another option for correcting acidosis, particularly in fish. There is evidence that fish excrete H^+ across the gill epithelium to avoid blood acidosis. Meanwhile the retention of lactic acid in the intracellular space also serves the same purpose (174,175,180), but does not favor intracellular acid-base regulation in the white muscle.

Catecholamine levels in arterial blood remain unchanged in both fish and mammals during sustained exercise (76,99). However, a large increase (10- to 100-fold) in circulating catecholamine concentrations occur at more intensive levels of exercise in fish (125,138) and mammals (103,104). The elevated catecholamines activate glycogen phosphorylase (GPs), phosphofructokinase (PFK), and pyruvate kinase (PK) which aids the increase in glycolytic flux (200). This could lead to lactic acidosis in blood, which is secondary to the lactate accumulation in muscle.

3. FUEL USE IN MUSCLE 'AEROBIC' AND 'ANAEROBIC' EXERCISE

The metabolism of various kinds of fuels during exercise and training has been studied extensively. Extensive reviews on this subject in both mammals and fish can be found in several papers (26,72,74,88,91,92,169).

The sequence of fiber recruitment is slow oxidative (type I), fast oxidative (type IIa), and fast glycolytic (type IIb), as the intensity of exercise increases. The most important feature of metabolic provision is the utilization of adenylates as the universal energy currency. Therefore, it is important to have an intimate coupling between various energy-producing pathways (*eg*. oxidative and glycolytic) and the respiratory chain in the mitochondria where ATP-energy conversion is conducted. In mammals, in both types of exercise, creatine phosphate can be depleted in all fibers, while ATP will seldom drop below 70% of its resting levels. However, the repletion of these high energy phosphagens is very rapid at the expense of the breakdown of energy substrates (muscle glycogen and triglycerides, plasma glucose and FFA). In fish, ATP can be depleted to a much lower level, and the replenishment takes a relatively long time (28,36,119,123,157).

1) "Aerobic" Exercise

In mammals, low intensity exercise mainly relies on free fatty acids (FFA) and carbohydrate (glycogen) oxidation (71,74,128). Amino acid deamination and transamination also contribute to the energy supplies, but to a lesser degree. Type I fibers are the first to lose glycogen at all submaximal working intensities. During a prolonged exercise period, these fibers can become glycogen-depleted while type IIa still contains moderate amounts of glycogen. Exogenous supplies of substrate (glucose) do not prevent glycogen depletion (25). Similarly, lipid infusion does not affect glycogen breakdown, although glucose uptake is inhibited (59). This result supports the notion that glycogen breakdown is not dependent on substrate availability, but is modulated by hormonal control (catecholamines) and the effects of contractile activity (151). The role of muscular FFA stores in exercise is not clear, but low intensity exercise increases the FFA pool several fold (78). The exogenous supply of substrates (FFA and glucose) becomes more important to sustain prolonged, low intensity exercise. This FFA supply can become successively more dominant as the glucose of hepatic origin is exhausted (1). However, at higher submaximal exercise, glucose uptake increases by 10- to 20-fold, thereby surpassing FFA oxidation in becoming the dominant energy supply (25).

In fish, unlike mammals, carbohydrates, lipid and protein are all used as aerobic fuels during low intensity exercise. Although the preferential use of fuels is not very clear, lipid and protein are considered the major carbon source for ATP synthesis (64). Amino acid oxidation is much higher in fish than in mammals. Davison and Goldspink (27) found that glycogen and lipid stores increased dramatically in both red and white muscle at low swimming speeds. At higher speeds, both lipid and glycogen dropped significantly. It is estimated that amino acid oxidation accounts for 10-20% of routine energy costs (86), and a large percentage of the amino acids are catabolized through gluconeogenesis to fuel the mucus production at gills and body surface (mucopolysaccharide) rather than being used for exercise directly (7). Various studies have reported that amino acid oxidation accounts for 15-85% of total Mo_2 depending on species, feeding status, and metabolic state (see review, 186). The level of amino acid catabolism may double after short and long term starvation (13,107,108). It has been found that proteins provide 30-60% of metabolic energy during migration (79). However, more recent work on juvenile trout indicated that lipid and carbohydrates contribute about 60% and 20% of the fuel, respectively, at the very beginning of the aerobic exercise, thereafter accounting for about 35% each (90,108). These studies also suggested that protein catabolism is highest in non-swimming fish (30-45%) and lowest in the higher speed swimmers (20%), and that lipid is mostly used in the resting and aerobically swimming fish (40-50%). As swimming speed increases, the contribution of carbohydrates also increases (108). The above finding was different from the result obtained by Van den Thillart (176) who estimated that 90% of the energy for aerobic exercise comes from lipid oxidation. The breakdown of protein can fuel the Krebs cycle directly with amino acids or increased carbohydrate stores *via* gluconeogenesis (35,51,137). In general, it is believed that the endogenous carbohydrate stores are mainly for use during anaerobic exercise in fish, and amino acid metabolism is of a much higher relative importance for fish than for mammals.

In fish, carbohydrates can fuel the exercising red muscle *via* the mobilization of stored intramuscular glycogen , and uptake of glucose and lactate from the circulatory system (liver and/or white muscle origin), although, in comparison with mammals, glycogen in red muscle and liver may play only a minor role in providing oxidative fuel for long-term sustainable swimming (130). The role of lactate as an oxidative fuel in fish, will be dealt with in a later section. Traditionally glycogen is considered a minor oxidative fuel for long term aerobic exercise, because red muscle glycogen storage only lasts for less than 1 h (123,130,137). However, a recent study in this laboratory has demonstrated that carbohydrates contribute a much higher portion of energy to aerobic swimming (108). This may be due to the recruitment of some white muscle during the higher aerobic swimming speed. The circulating glucose turnover rate of fish is generally 20-100 times lower than in mammals, and the glucose originated from hepatic glycogen alone can only support aerobic exercise for a short period. The slow release of hepatic glucose in this type of exercise further limits the importance of this substrate (41).

Lipid represent a critical source of energy in fish, as 6-20% of total wet body weight is fat, while the lipid content of muscle from some teleost species can range from 10% of the wet tissue weight in carp to over 20% in eels. A typical trout has body and muscle fat content of 2-10% and 2-3% (wet weight), respectively, of which approximately 90% are triacylglycerols (TAG, 30,64,109). This huge body store of lipid stands as the largest fuel reserve in fish which can be translated into over 100 h of aerobic activity in red muscle. In contrast to mammals, the liver, rather than adipose tissue, is the major lipid synthesis site in fish (53). Lipids are shuttled between deposit and metabolic tissues through the circulatory system as free fatty acids (FFA, fast delivery) and TAG (slow delivery). Upon aerobic exercise, teleosts mobilize hepatic and adipose fat storage and will rely on both exogenous FFA supply *via* the circulation and the *in situ* storage of TAG (186). The penetration of FFA through the interstitial space and muscle cell membrane is thought to be facilitated by albumin, while lipoprotein lipase facilitates TAG movement across the cell membrane (75). A hormone-sensitive lipase catalyzes intracellular hydrolysis of TAG to form FFA and glycerol. FFA are activated by ATP and Co-enzyme A (CoA-SH) to form Acyl-CoA which enters mitochondria *via* acyl-carnitine as a "vehicle". Acyl-carnitine can then be oxidized to Acetyl-CoA through β-oxidation and enters the Krebs cycle. The effect of exercise on plasma FFA varies among different species of fish, however, red muscle from teleosts demonstrates a high capacity of FFA utilization, suggesting that FFA are a potentially important fuel (130,162).

2) "Anaerobic" Exercise

In mammals, intensive exercise causes rapid glycogen depletion (to 10-50% of resting level), and massive lactate production (5- to 40-fold) in white muscle following a quick drop in PCr and ATP. Along with these changes, muscle contracting force decreases. However, in red muscle, glycogen is not greatly depleted (70% of resting level), while red muscle lactate also increases significantly (28), suggesting that this oxidative tissue acts as a lactate sink. White muscle generally has a much higher level of glycogen and PCr, and is the primary power generator during this type of exercise. Glycogen is believed to be the primary energy source for anaerobic exercise, especially during the first few minutes. The depletion of muscle glycogen is not the sole factor to cause fatigue, but very low glycogen might inhibit glycogenolysis and reduce the rate of ATP production leading to fatigue (36,58,139).

In fish, intensive exercise introduces similar changes in high energy phosphagen and endogenous carbohydrates (10,32,123). However, it takes longer for ATP and lactate to resume their resting levels compared to mammals (28,32,95). Recent data has also indicated that lipid metabolism may be involved in this type of activity in white muscle (121). Exogenous glucose uptake by muscle may be very limited in fish after exhaustive exercise as only 15% of post-exercise glucose turnover was accounted for by muscle disposal (188), and less than 10% of glycogen repletion was of circulatory glucose origin (130,133,134,180,188). In fish, the exogenous glucose uptake is not the major fuel since only 6-8% of circulating glucose was oxidized, indicating the role of the Cori cycle in post-exercise recovery is minimal (134). The relatively low hexokinase activity in comparison to extracellular glucose concentration, indicates that the slow glucose metabolism is not substrate limited (130,188).

In mammals, although significant glucose uptake occurs during intensive exercise, a large portion of it remains unphosphorylated. This causes muscle glucose levels to increase rapidly. The additive effect of insulin and muscle contractile activity stimulates glucose uptake. Although exercise-induced glucose uptake is insulin independent, its effect upon glucose uptake is mediated by the increased sensitivity of the transporter (GLUT-4) to insulin

(18). In direct conflict with this result, both Douen *et al.* (31) and Goodyear *et al.* (47) found that exercise enhanced, and insulin decreased the GLUT-4 density. Glucose uptake also varies among various fiber types. Henriksen (65) observed that insulin effect decreases in the sequence type I, type IIa, and type IIb, while the contraction-induced response is greatest in type IIa and lowest in type IIb.

Hepatic production of glucose consists of two components: glycolysis and gluconeogenesis. In humans, it is estimated that 70-80% of hepatic glucose originates from glycolysis, while gluconeogenesis accounts for the rest (151). Hepatic glucose production, and the uptake of glucose in muscle during exercise, are modulated by the interaction of insulin and glucagon. Insulin production is depressed during exercise by α-adrenergic stimulation. The depressed insulin causes an increase in hepatic glucose production and release. Although insulin can facilitate glucose uptake, contracting muscle is capable of glucose uptake without insulin appearance (73). In contrast, glucagon levels increase during exercise due to β_2-adrenergic stimulation upon the decrease of carbohydrate levels in muscle. The release of glucagon will stimulate glucose mobilization in the liver (34). FFA lipolysis is stimulated by the sympathetic nervous system, circulating catecholamines, and a reduction of insulin (56,182). However, glucagon has no influence on the control of lipolysis (84).

4. FLUID AND ELECTROLYTE BALANCE

Muscle contraction involves a series of membrane events: K^+ and Na^+ movements induce membrane depolarization to trigger an action potential; Ca^{2+} movement across the sarcolemmal membrane to form the crossbridge between actin and myosin to conduct contraction; elevation of energy production to provide ATP for the much needed ATPase reaction. In general, these events together rearrange inorganic and organic anions across the cell membrane. However, these rearrangements have to follow two principles: electro-neutrality must be maintained within each compartment, and osmolarity among the compartments must be equal. In fish, these matters could become complicated due to the large interface between water and animal, and homeostasis must be maintained against the large ionic and osmolarity gradients across the interface.

1) Roles of Selected Ions in Metabolism

A charged membrane is essential for membrane excitability. Na^+, Ca^{2+}, and Cl^- each exhibit a large concentration gradient across the cell membrane. In skeletal muscle, Cl^-, unlike the other two ions, is highly permeable. Therefore, its distribution is not far from its equilibrium potential. Similarly, K^+ permeability is relatively high in resting muscle. An action potential is propagated by the inward movement of Na^+ *via* a specific channel, which is followed by the outward movement of K^+ through its specific gated channel. After a train of action potentials, the muscle cell has gained Na^+ and lost K^+. The steady resting state condition is reestablished by a Na^+/K^+-ATPase pump. Although this pump is energy dependent, the relative energy expenditure is low compared to other cell functions. In the resting state, 5% of metabolic energy is spent in operating this pump. During highly intensive exercise, more than 10% of the total metabolic energy is utilized to operate this pump (55). Mg^{2+} and phosphate interact with this pump. Depressions in Mg^{2+} reduce the pumping rate, while elevated phosphate inhibits the reaction rate of this pump. The latter may be important when large amounts of PCr are broken down (161). Catecholamines activate this pump *via* β_2-adrenoceptors (21), while insulin also stimulates the Na^+/K^+-ATPase pump. Na^+/H^+ exchange has also been demonstrated in skeletal muscle and the exchange rate is pH dependent (95). The exchange is stimulated by intracellular acidosis and extracellular alkalosis (61,93,161).

2) Relationship between Metabolism and Electrolyte Balance

Electrolyte balance is crucial for maintaining metabolic flux for several reasons. Firstly, many ions such as Ca^{2+}, Mg^{2+}, and K^+ are essential for some metabolic enzymes to be active. Secondly, organic acids such as ATP, PCr, and lactate are substrates and/or products of important metabolic reactions. Lactate as an organic anion, connects glycolysis with ionic movement during exercise. Furthermore, ATP hydrolysis provides thermodynamic energy for those ATPase dependent ionic exchangers or transporters, thereby indirectly contributing to electrolyte balance.

3) The Effects of Exercise on Electrolyte Balance and Fluid Volume Distribution

The propagation of action potentials is caused by Na^+ influx and K^+ efflux during exercise. The loss of intracellular K^+ can exceed the gain of Na^+ by as much as a factor of 3. This significant decrease in intracellular $[K^+]$ could be the combined result of lost K^+, and cell swelling (112). This imbalance of electrolytes occurs as the Na^+/K^+-ATPase pump, in certain circumstances, is deactivated, therefore K^+ uptake can not match its efflux (161).

The effect of electrolyte changes on acid-base status can be tied to strong ion difference (SID). The change in pH can be attributed to one of three independent variables: Pco_2, SID, and sum of the weak acids and bases (167). The large efflux of K^+, and the formation of lactic acid reduce intracellular SID by 30-40 mmol l^{-1} and this represents a 0.5-0.6 unit drop in pH. However, the breakdown of PCr will cause a rise in SID and lead to temporary alkalosis.

The accumulation of metabolic products such as lactate, creatine and phosphate in the intracellular space could change the osmolarity of the cell fluid. This change may cause a transient fluid shift. Thus, high intensity exercise leads to rapid hemoconcentration, which is partially due to water shifting into the red blood cells (cell swelling) and muscle cells (125, 196). Elevated adrenergic activity also stimulates the spleen to release more red blood cells into the circulatory system, thereby contributing to the hemoconcentration (193).

In fish, exhaustive exercise causes vasodilation at the gills *via* the stimulation of β_1-adrenocepters by high levels of circulating catecholamines. The elevated catecholamines also cause an increase in gill surface area and a decrease in diffusion distance. Overall, this will increase gill permeability, cause a net loss of major electrolytes and a consequent gain in water. The transient extrusion of metabolic protons *via* the gills makes the matter even more complicated since this efflux of protons is usually coupled with enhanced Na^+ uptake and/or reduced Cl^- uptake. Later on, the extruded protons are recycled back for metabolic correction (70,116,117,192).

5. ROLES OF LACTATE AND AMMONIA

Both lactate and ammonia are produced in the white muscle with their levels increasing dramatically in the blood during, and after, intense exercise.

1) Ammonia

Ammonia (NH_3) metabolism is a vast and complex subject as there are numerous interactions with animo acid, fat, and carbohydrate metabolism. The focus here is its role in regulating acid-base balance and carbohydrate metabolism (51,74,120,127,177, 179).

As a respiratory gas, ammonia[1] represents a weak electrolyte in solution, and exists mainly as the ammonium ion (NH_4^+, over 97%) with a pK of 9.0 - 10.0 within the physiological pH range (17,82,106,126):

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[1] Ammonia will be used to represent total ammonia unless otherwise stated.

$$NH_3 + H_2O \rightleftarrows NH_4^+ + OH^-$$

where, NH_3 can be produced by both amino acid catabolism and adenylate deamination. During short and intensive exercise, ammonia production, in fast twitch skeletal muscle, is derived mainly from the deamination of adenylates:

$$ATP \rightarrow ADP \rightarrow AMP + H_2O \rightarrow IMP + NH_3$$

and/or *via* deamination of aspartate *via* purine nucleotide cycling (PNC):

$$\text{aspartate} + GTP + H_2O \rightarrow \text{fumarate} + GDP + P_i + NH_3$$

However, it is arguable whether the concomitant PNC functions in muscle under intensive activity. Amino acid transamination and deamination play an increasingly important role in resting and prolonged low intensity exercise; and liver, instead of skeletal muscle, is the major source of blood ammonia (119,127,179).

The majority of aquatic animals, like teleosts, are ammoniotelic, meaning ammonia is the predominant nitrogenous waste product (70% or more). The excretion of ammonia over urea, uric acid or other nitrogenous compounds (in other terrestrial vertebrates) is advantageous when considering that ammonia is metabolically efficient, as it is the product of many catabolic processes and needs no further conversion prior to excretion. NH_3 is small, lipophilic, yet very soluble in water, and its diffusivity is reported to be over 1000 times greater than CO_2 across the aqueous phase (62,106), which makes it easy to eliminate from the body. It has been suggested that ammonia, in the form of NH_4^+, moves between the compartments by competing with K^+ to diffuse through the channels which are subjected to the transmembrane electrical gradient, and/or by replacing H^+ in exchange for Na^+ (62). Ammonia can also substitute for the K^+ site on Na^+- K^+ ATPase (106). Ammonia (as NH_4^+)

movement is then accompanied by simultaneous H^+ removal (acid-base regulation) and ion regulation (50,62,105,106). However, it is worth mentioning that ammonia is toxic to cells and associated with peripheral and central fatigue (51,106,131,178).

The roles of AMP deamination are many-fold. In mammals, the maintenance of high energy charge (ATP/AMP ratio) and the prevention of adenine nucleotide from becoming depleted are paramount. The removal of free AMP promotes adenylate kinase activity in ATP synthesis, while the retention of IMP within the muscle intracellular space can conserve nucleotides for reamination of the adenine nucleotide pool. In fish, however, ATP was greatly depleted after exhaustive exercise and intramuscular ammonia concentration reached as high as 5-6 mmol kg^{-1} wet muscle (29,127,149,156,201,202). In contrast, muscle ATP level is very well defended in mammals (approximately 20% drop), and muscle ammonia never exceeds 1 mmol kg^{-1} (48,101,173). In fish, the production of ammonia *via* AMP deamination is high enough to play a role in buffering part of exercise-induced acidosis in muscle, whereas, in mammals, the role of ammonia buffering is very limited, indicated by the high ratio of intramuscular lactate/ammonia (48). In addition, ammonia can activate phosphofructokinase (PFK) and accelerate glycolytic production of lactate in muscle (51,163).

In general, transmembrane ammonia movement, and its regulation in fish muscle, are important issues in terms of the potential impacts they may have on metabolic and acid-base regulation. The current understanding of the issues remain controversial. This includes whether NH_3 or NH_4^+ is passively distributed across the muscle cell membrane, and whether the process is facilitated by the carrier-mediated transport of the ion (62,106,164,194,195, 201, 202). With regard to the passive diffusion of NH_3 or NH_4^+, cell membranes are usually considered very permeable to NH_3 (lipophilic). With a certain total ammonia concentration, the proportion of NH_3 is a function of pH (*ie.* higher pH results in greater NH_3 concentration levels). Thus, the transmembrane ammonia distribution should be dictated only by the pH gradient across the cell membrane assuming NH_4^+ is not permeable, and that the passive diffusion can reach its equilibrium very quickly. On the other hand, if NH_4^+ can also diffuse through the muscle cell membrane, ammonia distribution should be a function of the membrane potential E_m (*i.e.* a more polarized membrane results in more ICF [NH_4^+]). The rationale becomes that NH_3 diffusion should be a function of transmembrane pH gradient assuming NH_4^+ is not permeable, whereas the diffusion of NH_4^+ should be driven by its electrochemical gradient or the membrane potential (E_m, 201). The uncertainty over the question has evolved from the findings in mammalian muscle, where ammonia was distributed according to neither the pH gradient nor the membrane potential (48,50). In some cases, ammonia distribution ratio did not even alter between resting and exercise states (100). However, in fish, some have argued that the transmembrane distribution of ammonia was determined by the membrane potential rather than by the pH gradient (171,201,202). The search for the answers to whether ammonia distribution is determined by pH and/or membrane potential prompted part of the research theme in this study.

2) Lactate

In the past, lactate ($CH_3CHOHCOOH$, MW=89) was considered as the dead-end product of "anaerobic glycolysis" during exercise and was associated with the onset of fatigue and oxygen debt. As a metabolic acid (pK=3.75), lactate exists largely in the dissociated ionic form (Lac^-) at normal physiological pH. Traditionally, it has been believed that lactate is oxidized through the Krebs cycle during the recovery period when oxygen supply is not a limiting factor. However, recent data have supported a revolutionary concept that lactate represents an important metabolic intermediate which aids in the redistribution of carbohydrate energy. Because it can move across cell membranes with relative ease and the fate of lactate in skeletal muscle is multi-fold, lactate exchange between tissue compartments is of importance for the regulation of metabolism and acid-base balance (15,153).

The multiple fates of lactate in the white muscle are different among the vertebrates. In mammals, due to the paucity of mitochondria in white muscle, the Cori cycle as well as oxidation in more oxidative tissue (*i.e.* cardiac myocytes and slow oxidative fibers), was considered predominant. However, recent studies have suggested the existence of *in situ* oxidation and glyconeogenesis in white muscle (for review see, 44). At onset of exhaustive exercise, the liver, once regarded as the major site of lactate removal through the Cori cycle may contribute to the elevation in arterial lactate concentration (181,182). It is now believed that lactate acts as an important metabolic "shuttle" to coordinate the metabolism among diverse tissues (15). In mammals, lactate turnover rate is the highest among different metabolites ever reported in the resting state, and the turnover rate increases with animal activity levels. Hence, the release of lactate from muscle to blood has been demonstrated to account for a large portion of its total disappearance. However, in lower vertebrates such as amphibians, reptiles, and some species of fish, glycogenesis is predominant (37,43,46,190). In fish, lactate turnover rates are 26 (eels) to 290 (trout) times lower than mammals, and the lactate exchange rate between white muscle and the circulatory system is also very low (121, 130, 172), despite a 2 to 9 times increase in the turnover rate after sustainable and exhaustive exercise, respectively (89, 96,130). Recently, Rognstad and others (130,150) pointed out that the reversal flux *via* pyruvate kinase, an immense thermodynamic hurdle, needs to be addressed before the acceptance of this metabolic process as a fact, rather than a fiction.

Nonetheless, Gly repletion and the related Lac clearance are affected by many factors such as post-exercise metabolic state (active or inactive), training, initial Gly store, and the extent of Lac oxidation. It seems that some Lac oxidation must occur to supply ATP to satisfy various needs of energy demands, while trout white muscle tends to spare as much Lac as possible to replenish Gly.

The transport of lactate across the plasma membrane, including release and uptake, is of vital importance for replenishing the fuel energy and correcting the post-exercise acid-base disturbance. In this case, the large lactate gradient across the membrane existing during the post-exercise, and even resting conditions, suggests the sarcolemmal membranes act as a rate-limiting barrier for lactate translocation. Hence, the mechanisms of lactate movement across this plasma membrane became an important issue. Until 1974, when Halestrap and Denton (57) demonstrated the blockade of lactate transport into human erythrocytes by a specific inhibitor (α-cyano hydroxycinnamate), monocarboxylates, such as lactate, were assumed to be able to diffuse across phospholipid membranes as non-dissociated or dissociated forms, either directly or through a channel, at a rather fast rate (for review see 44,145). Since the identification, by Fishbein in 1986, of a lactate transporter defect disease in human muscle, a large body of evidence has revealed the existence of carrier mediated lactate transport in various types of mammalian cell membranes *i.e.*, erythrocytes, cardiac myocyte, hepatocyte, and skeletal muscle cells (141,142). Recently, studies utilizing the perfused skeletal muscle (45,95,98,183) and sarcolemmal vesicles (96,97,114,154,155) have identified a pH sensitive Lac^-/H^+ co-transporter and an anion exchanger (Band 3, $Lac^-/Cl^--HCO_3^-$ exchanger) as the major and minor lactate carriers, respectively. The physiological mechanisms of lactate movement across the plasma membrane have been characterized by using a variety of pharmacological inhibitors (49,95,114,143,154). The attempts to detect the lactate carrier have also evolved to the identification of a range of polypeptides in various tissues with molecular weight of 35-50 kDa (2,144), and these lactate carriers (34 kDa polypeptide) have now been partially purified and reconstituted into the rat skeletal-muscle sarcolemma vesicles (2,189). The Michaelis-Menten saturation kinetic properties were restored to the range comparable with native carriers ($K_m \approx 46$ mM), but the transport activity ($V_{Max} \approx 30$ nmol s^{-1} mg^{-1} protein) was 5-10 times greater (2,114,155,189). More recently, Garcia *et al.* (39,40) were able to use cDNA cloning to isolate monocarboxylate transporters I and II (MCT1 and MCT2) in various types of cells, including skeletal muscle. They pointed out that a family of monocarboxylate transporters exist in different types of tissues and play very different roles accordingly. Despite the well established evidence for the involvement of carrier-mediated transporters in Lac relocation suggested by many studies on mammalian muscle, there has also been evidence for the involvement of a non-saturable linear component (97,145,146,183), indicating the existence of lactate free diffusion. Apparently, this free diffusion component increases proportionally with the [Lac] gradient, while the carrier-mediated process reaches its plateau. In addition, some works have demonstrated that decreasing pH_i and/or increasing pH_e can lead to a rising Lac efflux from muscle, suggesting passive diffusion of un-dissociated lactic acid (113,153,155).

In fish, the mechanisms of lactate transport remain unclear. Unlike the situation in mammals, lactate is retained in fish white muscle for a much longer period (hours instead of minutes) after exhaustive exercise (5,43,95,170,174,175). White muscle, under this situation, acts as a "self-contained" metabolic system, and the slow recovery of ATP may be due to the low Lac oxidation and glucose uptake. The differences in the fate of lactate may be interrelated with its exchange behaviors between the compartments, and may further affect metabolic, ionic, and acid-base regulation in muscle. Batty and Wardle (1979) and Turner and Wood (1983) have suggested that an active retention mechanism is involved in preventing lactate release. To further identify the lactate transport mechanisms in fish muscle, more detailed kinetic and specific pharmacological blocking studies are required.

Metabolic acid (ΔH_m^+) release from white muscle appears to be uncoupled from lactate movement despite the well matched load of the two in muscle ICF (54,123). Intracellular [H^+] is too low to be passively distributed according the electrochemical gradient. Hence active extrusion must be involved, and the H^+/Lac^- cotransporter seems to fit this scenario. To the contrary, the differences in the transmembrane movement rates of these two ions suggest otherwise. However, one must bear in mind that ΔH_m^+ is a compound variable which constitutes H^+ flux in one direction and/or HCO_3^- flux in the opposite direction. Furthermore, any independent movement of H^+ and HCO_3^- or factors affecting the fluxes can complicate the net result of ΔH_m^+ flux. Therefore, it would be naive to reach any conclusion based only on the quantitative match-up of the two variables. A comprehensive analysis of the intra- and extracellular pH, CO_2, HCO_3^- and lactate status may help to gain a better understanding of this issue in fish white muscle.

II. OBJECTIVES

The ultimate goals of this thesis were:

1. To develop the optimal sampling, processing, and analytical methods for most faithfully preserving and measuring *in vivo* levels of a series of metabolites, at rest and post-exercise, in the white muscle of rainbow trout (Chapter 2).
2. To establish an integrated understanding of a wide spectrum of *in vivo* responses to exhaustive exercise (*ie*. acid-base status, a comprehensive set of metabolites, fluid volumes, and electrolytes) in a single batch of rainbow trout using the much improved sampling, processing and analytical techniques developed earlier; to characterize the influence of exhaustive exercise and recovery (within 4 h) on the utilization and replenishment of various fuel reserves, *ie*. high energy phosphates, carbohydrates, and lipid, in trout white muscle; to illustrate the ionic and fluid volume responses to exhaustive exercise in white muscle; and to evaluate the regulation of metabolic protons, lactate and ammonia distribution in post-exercise white muscle *in vivo* (Chapter 3).
3. To develop an *in vitro* isolated-perfusion tail-trunk preparation of rainbow trout which provides a reliable model of *white muscle* transport processes. The goal here was to examine the effects of transmembrane pH and electrical gradients on ammonia distribution in post-exercised trout white muscle, in comparison with the *in vivo* situation presented in the previous study (Chapter 4).
4. To use the *in vitro* isolated tail-trunk perfusion preparation to study the effects of pH and electrical gradients on the movement of lactate, metabolic protons, O_2, CO_2, and HCO_3^- in post-exercise trout white muscle, and to test the proposed "lactate retention" mechanism of earlier studies (Chapter 5).

5. To identify the roles of carrier-mediated transport and passive diffusion in lactate release and uptake in post-exercise and resting white muscle, respectively, using specific inhibitors, and L(+)- and D(-)-lactate (Chapter 6).

III. SUMMARY OF FINDINGS

Methods Development

Rainbow trout white muscle has become a much studied model system for investigating acid-base regulation and metabolic biochemistry of exercise. To assess whether the many quantitative and qualitative disagreements among various studies might be due to different sampling, processing, and analysis techniques, the first part of this project was designed to develop the most reliable protocols for studying these issues (Chapter 2).

The needle biopsy sampling method without anaesthesia was performed on resting fish and followed by two different freezing methods: blow-out of the muscle sample from the biopsy needle into liquid N_2 (BO, 175), and directly freezing the biopsy needle containing the muscle sample in liquid N_2 (DF, 123). This series of tests examined muscle metabolite levels, and demonstrated that the DF technique was better, because it resulted in lower muscle [Lac] and [Glu] when compared with the BD method. This suggested that a quick freezing process is crucial in effectively arresting glycolysis, and avoiding lactate elevation (136,170).

The second series of tests was conducted to compare the DF biopsy method with the MS-222 plus freeze-clamping method (170). The latter method resulted in a much lower [Lac] and a much higher [Gly] in the resting muscle. Similarly, muscle PCr level (33 mmol kg^{-1}) was significantly higher in the MS-222 method group, and indeed was the highest value ever reported for trout white muscle by either enzymatic or HPLC methods, indicating that muscle PCr level is sensitive to the sampling method used. In concert with the well preserved adenylate pool, there was a significantly lower muscle ammonia level. These results indicated that the MS-222 method is superior to the biopsy method in preserving the muscle adenylate and glycogen pools, and limiting lactate and ammonia production. In addition, the study revealed that muscle ATP level was not affected by the above sampling methods.

The implantation of a dorsal aorta catheter (DA catheter) had negligible influence on most muscle metabolites. This test provided justification for the application of a DA catheter in the *in vivo* study (chapter 3).

Based on the MS-222 sampling method, three post-sampling muscle tissue processing techniques were assessed using PCr, Cr, ATP, Amm, Gly, Lac, and Glu as the criteria. The glass homogenizer method (GH) and the mortar and pestle methods (MP) produced very similar values for all metabolites in both the resting and exercised muscle samples. However, the freeze-dry technique (FD) yielded significantly higher PCr, suggesting that lyophilization is an efficient way of preserving this labile high energy phosphate. The lack of change in ATP among the three processing methods supports the earlier conclusion that ATP is rather resistant to the sampling and processing protocol-induced variances. In parallel, the three processing methods did not result in differences in muscle Gly and Lac concentrations. It was concluded that the FD is the method of choice for post-sampling processing of muscle tissue, except for Amm measurements. Lyophilization has been suspected to cause extensive loss of ammonia in tissue (101), and this was proven to be the case in the present study. Swift freezing (*ie*. freeze-clamping in liquid N_2), and rigid temperature control prior to tissue breakdown (*ie*. the GH and MP method) are crucial for preventing the deamination of adenylates and amino acids, and for obtaining a reliable muscle tissue Amm concentration. In conclusion, the combination of MS-222 sampling method and the FD tissue processing technique, were recommended as the best set of methods to be used for muscle adenylate and carbohydrate measurement in my later studies. However, the MP method was preferred for tissue ammonia analysis.

An isolated-perfused tail-trunk preparation, with a one time flow-through protocol, was developed in order to study the transmembrane movement of lactate, ammonia, metabolic acid, respiratory gases and other electrolytes in trout white muscle. The tail-trunk preparation provides an excellent model for white muscle as it consists primarily of white fibers (over 90%) which are distinctively segregated from red fibers. The preparation is particularly suitable for flux studies since the conditions of inflowing saline can be manipulated, while the corresponding changes in the muscle trunk and venous outflowing saline can be monitored easily. Red blood cell (RBC) free perfusate was used to avoid potential complications despite some compromises involved in the O_2 and CO_2 carrying capacities. Meanwhile the isolated tail-trunk eliminated the influence of other organs on the substrate flux data, such as liver, gills, kidney, and heart. In general, the preparation mimicked the *in vivo* physiological conditions reasonably well, and yielded comparable results in terms of metabolic rates, substrate and ion flux, membrane potential, perfusion pressure, and flow rate.

Integrated Responses to Intensive Exercise in Trout

The patterns of acid-base and metabolic responses of fish muscle to strenuous exercise have been well studied owing to the efforts of many research groups (for review see 130, 193). However, the discrepancies in results of different studies are substantial, leading to uncertainties in mechanistic explanations. These differences may be due to a variety of factors such as body size, genetic strains, feeding and exercise regimes, and the physical condition of the fish. In addition, the different sampling, processing, and analytical techniques employed in various studies inevitably contribute to some of the differences. Hence, there was a clear need for integrating all the measurements of these responses in a single batch of fish in order to eliminate the artificial influences of inter-study variations.

Furthermore, it was necessary to establish a comprehensive picture of the *in vivo* responses to exercise in fish as a point of reference prior to the next stage, the *in vitro* studies.

In concert with many previous studies, blood gases and acid-base status responded to exercise in the expected fashion (83,117,123,172,174). The post-exercise acidosis in blood was a compound result of the mobilization of metabolic and respiratory acid; the metabolic component was relatively longer lasting than its respiratory counterpart. In conjunction with the contribution from protons released from muscle lactate production and ATP hydrolysis (29,124), proton extrusion from red blood cells (RBC) *via* the Na^+/H^+ exchanger plays an important role in causing post-exercise blood acidosis. The elevation in circulating catecholamine and Pco_2 accelerates Na^+/H^+ exchange, and facilitates the entry of Na^+ and Cl^- followed by inward fluid shift (117,198). The significantly decreased mean cell haemoglobin concentration (MCHC) and increased hematocrit suggest that both the size and concentration of RBC increased during post-exercise recovery.

This study offered no support for an earlier model of inward water, Na^+, and Cl^- shifts predicted for post-exercise white muscle (193). The relatively low muscle intracellular [Lac] in the present study (35 *vs*.50 mmol l^{-1}) was the likely explanation for the lack of fluid shift. Instead of the predicted lower white muscle intracellular K^+, higher plasma and muscle intracellular $[K^+]$ were observed, and gill tissue and swollen RBC's were suspected as the likely source. The muscle membrane potential estimated (E_m) by K^+, Na^+, and Cl^- distribution from the Goldman-Hodgkin-Katz equation (68):

$$E_m = \frac{RT}{F} ln \frac{PK^+[K^+]_{ECF} + PNa^+[Na^+]_{ECF} + PCl^-[Cl^-]_{ICF}}{PK^+[K^+]_{ICF} + PNa^+[Na^+]_{ICF} + PCl^-[Cl^-]_{ECF}}$$

was very similar to measured values in the white muscle of the teleost. Membrane depolarization was not observed after exercise.

Theoretically, muscle PCr depletion occurs prior to the beginning of ATP and Gly breakdown, as a result of the high affinity of creatine kinase for ADP. Unlike results from other studies, where PCr and ATP were depleted to very low levels (29,136,137,157,170), the present study indicated a reduction of only 40%. PCr recovered much more quickly than ATP (15 min *vs.* 2 h). The former was accompanied by a stoichiometric change in Cr, while the latter was accompanied by changes in IMP and Amm. Following the high energy phosphate breakdown, there was a 90% decrease in Gly and a corresponding elevation of Lac. The relationship among ATP, IMP and Amm suggests that adenylate deamination is the source of ammonia production in muscle. The long recovery period of ATP in fish muscle is in accord with the slow muscle Gly replenishment and Lac clearance. In contrast to mammals, where lactate clearance in muscle is very fast and predominantly *via* oxidation and the Cori cycle, glycogenesis *in situ* appears to be the major fate of muscle lactate, with the rate of oxidation being slow in mitochondria-poor fish white muscle. In addition to the low lactate oxidation rate, which produces less ATP, glycogen resynthesis is an ATP consuming process. Taken together, slow ATP recovery becomes unavoidable, and this may hinder any ATP related physiological functions such as ATPase-driven ion pumps and ATP dependent substrate transport.

The present study provided the first experimental evidence for the use of lipid as a fuel during and after strenuous exercise. Increases in long- and short-chain acyl-carnitine, along with the decrease in free carnitine, indicated the activation of free fatty acid (FFA) oxidation. In parallel with the above were significant increases in acetyl-CoA and acetyl-carnitine which indicated the possible role of FFA β-oxidation in providing ATP for glycogen resynthesis. It is possible that lactate oxidation was spared by FFA oxidation, as the increase in the availability of FFA depresses carbohydrate metabolism (*ie*. reduces Pyr utilization, 3,130). Moreover, glycerol, derived from triglyceride (TG, lipid) *via* lipolysis, can be phosphorylated to form glyceraldehyde 3-phosphate, and then enter the glycolytic pathway. The positive redox values (oxidative) recorded in the post-exercise muscle support this view, which challenges the traditional concept of "anaerobic" state in exhaustively exercised white muscle, and furthermore, make the scenario of lipid utilization credible.

Ammonia Dynamics*: *in vivo and in vitro approaches

As pointed out above, the dramatically elevated levels of intra- and extracellular Amm ($[\text{Amm}]_{ICF}$ and $[\text{Amm}]_{ECF}$, respectively) in fish white muscle after exercise, originate primarily from adenylate deamination. With a greater elevation of Amm in muscle ICF than ECF (5 *vs*. 0.2 mmol l^{-1}) after exercise, the measured transmembrane Amm distribution ratio increased from about 9.5 at rest to the range of 18.7-32.7. Assuming muscle cell membranes are diffusible to NH_3 and/or NH_4^+, then the total ammonia distribution is dictated by pH gradient and/or E_m, respectively, under equilibrium states. The post-exercise distribution ratios were much closer to that predicted by E_m (approximately 30), whereas the resting ratio was better represented by the pH gradient estimate (approximately 6). The *in vivo* results led to some clear conclusions: 1) An active retention mechanism can be ruled out because the measured Amm distribution ratios fell between the predicted limits set by the pH- and E_m-dictated ratio. Thus, passive diffusion is expected (152). 2) There appear to be an alteration of the relative permeabilities for NH_3 and NH_4^+ between the resting and post-exercise states, respectively, even though some earlier works suggested that the distribution was E_m-dictated regardless of its metabolic state (171,201,202), while others suggested it was pH-dictated regardless of metabolic state (62). The E_m-driven distribution of Amm in the post-exercise condition results in a greater retention of ICF Amm, which is advantageous since it enhances ICF buffering capacity, as well as ICF Amm reserve, for reamination of adenylates (29,50, 120).

To further elucidate the conclusions obtained from the *in vivo* study, the isolated-perfused tail-trunk preparation was employed to examine the effect of changing transmembrane pH gradient and E_m on Amm distribution in post-exercise fish muscle (Chapter 4). With experimental manipulation of pH_a (*via* changing HCO_3^-) from 7.4 to 8.4, a significant decrease in Amm efflux corresponded to an apparent drop in the outwardly directed NH_3 partial pressure (P_{NH_3}) gradient. In contrast, with a partial depolarization of the muscle cell membrane (*via* increasing extracellular K^+) from -92 mV to -60 mV, Amm efflux posted a marked rise (over 70%) in responding to a two-fold increase in the electrochemical gradient for NH_4^+, despite a large decrease in P_{NH_3} gradient (62%). The approach of predicting the Amm distribution from either the pH gradient or E_m, led to the same conclusions obtained from the *in vivo* study. The lack of amiloride effect on Amm flux rate lends further support to the conclusion that Na^+/H^+-NH_4^+ exchange is not involved in Amm transport.

Lactate Dynamics

The responses and the time courses in the post-exercise pH_i and pH_e obtained in the *in vivo* study were in accord with many similar studies (Chapter 3, for review see 130,193). The intracellular Lac and metabolic proton load were relatively large in comparison to the extracellular blood plasma (3 to 10 times). Despite the well coupled loading in the muscle ICF, the release patterns of Lac and metabolic acid differed significantly, and Lac unloading exceeded net metabolic acid efflux by more than 50%, suggesting the involvement of two different mechanisms. It is widely demonstrated that a large portion of lactate produced by trout white muscle during exercise never exits the cells, and the relatively slow clearance may be through metabolism *in situ* (*ie.* oxidation and glyconeogenesis), whereas, in higher vertebrates, Lac is regarded as a precursor for the Cori cycle and as a substrate for oxidation in more oxidative tissues. The stoichiometric match between the changes in muscle Gly and Lac provided quantitative, but circumstantial, evidence favoring the view of *in situ* glyconeogenesis as the major fate, but not the sole fate (Chapter 3).

The physiological rationales behind the slow release of Lac in fish muscle are many fold (see the Introduction above). However, the issue became pertinent as to how fish muscle is able to maintain Lac disequilibrium against the huge electrochemical gradient, and what the mechanisms are. Even though, Lac, as a "weak acid", may be distributed *via* passive diffusion according to the transmembrane pH gradient, the *in vivo* study demonstrated that the E_m and pH estimated Lac distribution ratio underestimated the measured one. Therefore, the free diffusion of Lac^- and HLac through the muscle cell membrane was ruled out as the sole mode of Lac redistribution at rest and post-exercise state. Therefore, metabolic energy expenditure is necessary to maintain the transmembrane gradients, and Lac must be actively retained. The retention rate becomes accelerated in the post-exercise situation to cope with the much elevated Lac "leakage". However, the *in vivo* results were obtained from intact fish where Lac in the blood plasma did not necessarily originate from white muscle, and Lac exchange was inevitable between tissues other than white muscle and blood plasma. Hence, an *in vitro* preparation was needed to isolate white muscle from other tissues in order to demonstrate the true Lac exchange across white muscle cell membranes. Taking this approach, in a perfused trunk study earlier, Turner and Wood (174) suggested that simultaneous Lac uptake could occur *via* the Lac-/HCO_3^- exchanger during the post-exercise period, based on the result of a 4-acetamido-4'-isothiocyano-stilbene-2,2'-dissulphonate (SITS)-induced increase in Lac efflux.

To further illuminate the above issues, the experimental changes in pH_e from 7.4 to 8.4 (*via* varying HCO_3^-) and partial depolarization of E_m (*via* increasing ECF $[K^+]$) were first employed in the isolated-perfused tail-trunk preparation (Chapter 5) to investigate the role of Lac passive diffusion in post-exercise white muscle. The *in vitro* results confirmed many of the *in vivo* findings. The Lac efflux rates (approx. 1.5 mmol kg^{-1} h^{-1}) were comparable to those estimated by an *in vivo* ^{14}C-Lac turnover study (122) and a perfused whole trunk study (174). This extremely slow Lac release, would take more than 20 h to clear the Lac load in white muscle (approx. 50 mmol l^{-1} ICF), without even considering the effect of a declining gradient or the possible continuing Lac production on site during this period, whereas, *in vivo*, it normally takes fish 8-10 h to finish the muscle Lac clearance. This provided quantitative evidence supporting the idea that a large portion of Lac produced during intense exercise never leaves fish white muscle. It was clearly demonstrated that Lac was retained in the white muscle against both the electrochemical gradient (net driving force, NDF) and the pH gradient in the post-exercise situation and even in the resting situation. An increase in pH_e did not result in a corresponding Lac efflux elevation suggested in an earlier study on frog muscle (113), while the over 100-fold increase in transmembrane HLac gradient from rest to post-exercise merely induced a 3-fold increase in Lac efflux. Thus, the influence of pH gradient on Lac efflux may be very limited, indicating that passive diffusion of HLac may not be the only Lac efflux mechanism. Moreover, rather than depressing Lac efflux, the partial depolarization of E_m accelerated Lac efflux owing to the increased intracellular [Lac] and depressed pH_i, which strongly discounts the possibility of Lac^- free diffusion according to the net driving force on Lac^- (NDF). The best possible correlation was between Lac efflux rates and the total Lac concentration gradient, suggesting the possible involvement of an eletroneutral carrier mechanism in Lac efflux. However, the possible involvement of a simultaneous Lac uptake mechanism could not be excluded. Furthermore, since active retention mechanisms (*ie*. H^+/Lac^- symport and Lac^-/HCO_3^- antiport) can also be influenced by factors such as pH gradient, pH_e, pH_i, extracellular buffer capacity, [Lac^-], [HCO_3^-], and even E_m (15,44,52,95, 111, 113,145,153), the situation could have been even more complicated.

Bearing these in mind, the next logical step was to use pharmacological inhibitors to identify the role of proposed carrier-mediated Lac transporters (Lac^-/H^+ cotransporter, Lac^-/HCO_3^--Cl^- exchanger) in the transmembrane movement of Lac, including influx and efflux at either the post-exercise or resting states (Chapter 6, for reviews see 15,44,145,153). The most important finding was that both carrier-mediated and passive diffusion processes are engaged in Lac release from the exercised white muscle. It was clearly demonstrated that the Lac^-/H^+ cotransporter is involved in Lac release in post-exercise white muscle, as Lac efflux was depressed by α-cyano-4-hydroxycinnamate (CIN), a competitive blocker of both the cotransporter and exchanger, while no significant effect of 4-acetamido-4'-isothiocyano-stilbene-2,2'-disulphonate (SITS), an anion exchanger blocker, was observed (see Fig. 6-1 for a schematic model). Thus, the role of the anionic exchanger in Lac *release* is considered minimal. Quantitatively, the remaining 60% of Lac efflux was through HLac free diffusion, assuming CIN completely blocked the carrier-facilitated Lac transport. If this is the case, the lack of a proportionate relationship between net lac efflux and the transmembrane HLac gradient found in Chapter 5 can only be explained by a *concurrent Lac uptake* mechanism, most likely via the Lac^-/HCO_3^- exchanger, proposed by Turner and Wood (174). Although an increase in net Lac efflux would be expected if there was a SITS blockade of the band 3 anion exchanger-facilitated Lac uptake, the lack of SITS effect in this study was not surprising due to the extremely low Lac concentration levels in the venous effluent. The SITS-induced elevation in ΔH_m^+ influx (equal to a decreased HCO_3^- efflux), supported the idea of Lac uptake mediated by the band 3 exchanger. With CIN blocking both the symport facilitated Lac efflux and the antiport-mediated Lac influx, the remaining 60% of Lac release represents the true "free diffusion portion" of Lac efflux.

Although both free diffusion and carried-mediated Lac transport are often affected by pH gradient, the Na^+/H^+ exchanger can act as an important co-regulator of pH_i to further influence the Lac^-/H^+ coupling. The application of amiloride in this study did not exhibit a significant impact on Lac efflux. Despite the amiloride-induced decrease in ΔH_m^+ flux, pH_i was hardly affected, owing to the high muscle ICF buffer capacity.

The study on Lac transport illustrated the potential uptake capability in the resting white muscle (Chapter 6). Despite the outwardly directed NDF on Lac^- and HLac concentration gradients (negligibly inward at very high extracellular [Lac]), net L-Lac influx occurred and surpassed the net Lac efflux rate measured in the post-exercise perfusion. This suggests that fish white muscle is capable of active Lac uptake. The hyperbolic L-Lac uptake curve (Fig. 6-4A) provides a strong case for saturation or partial saturation kinetics involved in Lac uptake in white muscle. However, because of the large linear component involved, conventional methods could not be applied to characterize the kinetics properties. The linear component was demonstrated by the D-Lac uptake curve which represents the Lac movement through Lac^-/HCO_3^- exchanger and/or by free diffusion since D-Lac does not move across the cell membrane by the Lac^-/H^+ cotransporter. Hence, the difference in Lac flux between the D- and -Lac isomer represents the portion of flux *via* the symport. At 16 mM of $[L\text{-}Lac]_a$, the difference between the 75% inhibition by CIN and the 45% inhibition by SITS represents the symport portion of Lac uptake (30%, Fig 6-4B). This is consistent with the 36% estimated by the different isomer fluxes. Taken together, the symport contributes 30-36%, band 3 antiport 39-45%, and free diffusion 19-25%. It should be pointed out that the diffusion and the symport portions were probably over- and under-estimated, respectively, as a result of the fact that [HLac] gradient did not exist in the L-Lac series, but did exist in the D-Lac series at 16 mM $[Lac]_a$ (Fig. 6-5B). The significant increase in ΔH_m^+ influx (16 and 32 mM $[L\text{-}Lac]_a$ series) and the elevated Tco_2 efflux also supports the carrier-mediated Lac uptake mechanisms.

In general, transmembrane Lac flux in fish white muscle is very slow in comparison with mammals and amphibians (1/10 and 1/5, respectively). Fish white muscle demonstrates the potential to actively retain Lac in white muscle *via* Lac^-/H^+ symport and Lac^-/HCO_3^- antiport. The uptake rate at rest greatly exceeds the efflux rate, even with the experimental conditions of an exaggerated transmembrane electrochemical gradient facing outward. The involvement of the band 3-mediated Lac uptake occurring in trout white muscle provides support for the "active lactate retention" mechanism.

ΔH_m^+ Flux in the Post-exercise and Resting Muscle

The uncoupling of Lac and ΔH_m^+ flux demonstrated in both the resting and post-exercise muscle were consistent between the *in vivo* and *in situ* perfusion studies (Chapter 3,5,6; and 123,174). Quantitatively, the post-exercise *in vivo* ΔH_m^+ loading rate in blood plasma remained lower than Lac efflux (approx. 50%), but was always higher than the corresponding *in situ* perfusion preparation with an equivalent pH gradient (Fig. 3-4B, Fig. 5-6). The lower flux of ΔH_m^+ than Lac *in vivo* was attributed to the temporary "storage" of excess H^+_m in the external water *via* the acid-base regulation mechanisms of the gills (70,193), while the higher than *in situ* ΔH_m^+ flux rate may be due to H^+ contributions of non-muscle origin such as H^+ released from RBC *via* catecholamine-induced Na^+/H^+ exchange. In contrast to the Lac movement, H^+ must be actively extruded from white muscle because the electrochemical gradient (NDF on H^+) is in favor of retaining it in the muscle cells. It is very important, however, to appreciate that ΔH_m^+ is a compound index, with influx suggesting a H^+ uptake and/or HCO_3^- efflux. Unlike Lac, the extrusion of H_m^+ is, apparently, affected by many factors, including the pH gradient, E_m (through its effect on the net driving force, NDF, for HCO_3^- and H^+), transporter activities, and ECF buffer capacity. Therefore, lower pH_e in the post-exercise situation may affect the H^+ extrusion mechanism, and the reversal of ΔH_m^+ flux from inward to outward with an increased pH_e supported the above conclusion (Chapter 5). In the perfusate, the relatively low β value and the lack of carbonic anhydrase resulted in a lower effective ECF buffer concentration than *in vivo*.

Hence, the low-pH$_a$ of the perfusate in the post-exercise and resting groups (Chapter 5,6) created an exceedingly low non-equilibrium pH$_v$, and resulted in transmembrane "equilibrium limitation" and the reversal of electrochemical gradients for ΔH_m^+ (*i.e.* NDF on H^+ and HCO_3^-). The higher pH$_a$ induced by elevated [HCO_3^-] in the perfusate indeed resulted in ΔH_m^+ efflux and supports the "equilibrium limitation" model (61).

The total CO_2 and ΔH_m^+ efflux rates remained unchanged, despite the increased D-Lac influx in the resting muscle (Fig. 6-6). Together with the unchanged HCO_3^- efflux indicated by the stable pH$_v$ and CO_2 efflux, the band 3-mediated Lac influx is regarded as limited when there is an elevated inward HLac gradient. The CIN and SITS-induced decrease in ΔH_m^+ influx were in agreement with the suggested carrier-mediated Lac uptake mechanisms.

This is the first study in fish clearly demonstrating the involvement of carrier-mediated transport in Lac movement across white muscle cell membranes and offering reasonable mechanisms to explain the current issue of how fish muscle retains Lac within the cells. This mechanism is of physiological importance, as fish white muscles are uniformly exercised during intensive exercise, and the Cori cycle plays very limited role in post-exercise clearance. Therefore, in contrast to mammals, there are no large Lac sinks (*e*.g. resting white muscle, large numbers of adjacent red fibers) to cope with the massive Lac washout from the white muscle. The Lac retention mechanisms also fit the proposed theory of *in situ* glycogenesis from Lac, and prevent the ECF from suffering from excessive acidosis as the result of lactic acid release which may, furthermore, impair blood O_2 carrying capacity and prolong the recovery process.

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CHAPTER 2

THE ANALYSIS OF METABOLITES IN RAINBOW TROUT WHITE MUSCLE: A COMPARISON OF DIFFERENT SAMPLING AND PROCESSING METHODS

ABSTRACT

I have investigated the effects of different sampling and processing methods on metabolite concentrations of glycogen (Gly), glucose (Glu), lactate (Lac), pyruvate (Pyr), ammonia (Amm), creatine phosphate (PCr), creatine (Cr), and adenosine triphosphate (ATP) measured in white muscle of rainbow trout at rest and immediately after exhaustive exercise. When samples were taken from resting fish by rapid needle biopsy (without anaesthesia), direct freezing of the needles in liquid N_2 yielded lower Lac and Glu levels than if the muscle cores were quickly blown out into liquid N_2. However, sacrifice of the fish by an overdose of MS-222 followed by freeze-clamping of excised muscle was superior to the biopsy method in preserving high levels of PCr and Gly (91% and 62% higher, respectively). In parallel, the MS-222 method also yielded lower levels of Amm (80%) and Lac (47%). Samples freeze-clamped by the MS-222 method were used to evaluate three methods of subsequent processing for enzymatic analysis of metabolites: classic glass homogenization (GH) in 8% perchloric acid (PCA) *versus* mortar and pestle (MP) pulverization or freeze-drying (FD) prior to PCA

extraction. For all metabolites, GH and MP methods produced similar values. However the FD technique yielded 20 % higher PCr levels which represented over 80% phosphorylation of the total Cr pool at rest, the highest ever reported via enzymatic analysis. Glu was also higher by FD, but Gly, Lac, and ATP were not affected. Indeed ATP was relatively stable throughout all sampling and processing procedures. MP, GH, MP&GH combination, and high speed motor driven grinding techniques all yielded similar Amm levels in resting muscle. However, tests demonstrated that even brief thawing of tissue greatly elevated Amm, while FD resulted in artificially low Amm values due to evaporative losses during lyophilization. Overall, muscle sampling by freeze-clamping on trout sacrificed by MS-222 overdose, followed by FD prior to PCA extraction, appears to be the best combination for the measurement of all white muscle metabolites except Amm, for which MP or GH are preferable.

INTRODUCTION

A great deal of effort has been devoted to assessing the metabolic and acid-base changes occurring in fish muscle during various types of exercise and recovery since the pioneering work of E.C. Black more than three decades ago (*eg*. Black *et al.*, 1962; see Wood, 1991 for a recent review). It is now clear that an issue of critical importance in interpreting these data is the actual method employed for sampling and subsequent processing of the tissue prior to biochemical analysis. Ideally, the methods used should provoke minimal disturbance to the metabolic and acid-base status present in the muscle *in vivo.*

Over the years, a great variety of sampling techniques have been employed. In early studies, the sampling procedure was often described as the excision of muscle from "freshly killed" fish followed by freezing or direct extraction without freezing (*eg*. Black *et al.*, 1962; Wardle, 1978). In later work, the benefits of rapid freezing of the samples in liquid N_2 were recognized. However in some studies biopsy needles were used to take samples from unanesthetized fish (*eg*. Turner *et al.*, 1983; Milligan & Wood, 1986), while in others the fish were first sacrificed by a cephalic blow (*eg*. Dobson & Hochachka, 1987; Girard & Milligan, 1993) or decapitation (*eg*. Dobson *et al.*, 1987). Excised samples were quick-frozen by various types of immersion in liquid N_2 or by freeze-clamping with liquid N_2 cooled tongs. Direct freeze-clamping of the whole body has been used in some studies on smaller fish (*eg*. Pearson *et al.*, 1990; Scarabello *et al.*, 1991). Various types of anesthetics (Johnston & Moon, 1980; Driedzic *et al.*, 1981; Davie *et al.*, 1986; Pearson *et al.*, 1990; Pörtner *et al.*, 1990; Tang & Boutilier, 1991; Schulte *et al.*, 1992) have also been employed to prevent excessive struggling prior to and during muscle sampling. Recently, by reviewing the literature on "resting" white muscle lactate in rainbow trout, Tang & Boutilier (1991) concluded that the sampling method had a major influence on the values obtained.

However, the methods of tissue processing *after* sampling and *prior* to biochemical analysis may be equally important in accurately determining the metabolite levels measured. Since metabolites like nucleotides are soluble and metabolic enzymes can be deproteinized in acid, tissue is usually extracted with a medium such as perchloric acid to terminate on-going metabolic reactions. Specific metabolites in the extract can then be assayed enzymatically or chromatographically. Previously, acid extractions were usually done via glass homogenization (*eg*. Black *et al.*, 1962; Wardle, 1978; Turner *et al.*, 1983; Milligan & Wood, 1986). More recently, pulverization of the tissue in liquid N_2 with a mortar and pestle, alone or in combination with glass homogenization, has become popular (*eg*. Driedzic *et al.*,1981; Dobson *et al.*, 1987; Tang & Boutilier, 1991; Girard & Milligan, 1993). In other studies, freeze-drying has preceded acid extraction (Pearson *et al.*, 1990; Scarabello *et al.*, 1991).

Two issues are particularly important: (1) How efficient is the extraction process in breaking up tissue cells to ensure full release of metabolites to the medium? (2) How stable are the metabolites prior to and during the extraction? Incomplete degradation of cell membranes could be a factor in the former, whereas incomplete denaturation of enzymes and/or warming of the tissue during extraction could depress metabolic substrates and elevate metabolic products. Accordingly, different combinations of sampling and tissue processing techniques could explain the great variability in fish muscle metabolite measurements reported in the literature.

The objective of the present study was to systematically assess the effects of various common sampling and processing techniques on adenylate and carbohydrate metabolites in rainbow trout white muscle. For comparative purposes, measurements of these same parameters in trout muscle were tabulated from other recent studies. Our measurements focussed on resting levels so as to provide a background for the changes occurring after exhaustive exercise. An additional objective was to determine whether prior implantation of a dorsal aortic catheter (Soivio *et al.*, 1972) influenced the metabolic picture in trout white muscle. This blood sampling technique is commonly employed in most modern studies. Finally, because of the recent controversy regarding the distribution of ammonia between white muscle and plasma in fish (Wright & Wood, 1988; Heisler, 1990; Tang *et al.*, 1992), we investigated the influence that various processing methods had on total ammonia concentrations in the white muscle of resting trout.

MATERIALS AND METHODS

Adult rainbow trout [*Oncorhynchus mykiss*], weighing 150-350g, were obtained from a local hatchery (Rainbow Springs Trout Farm, Ontario, Canada) and held in a 800 l tank for at least two weeks prior to the experiments. The fish were acclimated to 15±1°C in flowing dechlorinated Hamilton tap water (composition as in Milligan & Wood, 1986) without feeding for 5-7 days before use. Dorsal aortic catheters (DA) were then surgically implanted into selected fish (Soivio *et al.*, 1972); the fish were allowed to recover for a minimum of 48 h in darkened acrylic boxes supplied with 15°C water.

Resting fish (with or without DA) were kept in their boxes for 48 h prior to terminal sampling. Exercised fish were transferred to a 150L cylindrical tank at this time, then manually chased to exhaustion for 6 min followed by immediate terminal sampling. In DA implanted fish (rest or exercised), blood (2 ml) was sampled through the DA prior to muscle sampling for the analysis of arterial blood pH, Po_2, total CO_2, haematocrit (Hct), haemoglobin (Hb), and plasma protein. Plasma lactate (Lac), glucose (Glu), ammonia (Amm), pyruvate (Pyr), and inorganic phosphate (Pi) were also measured.

Experimental protocol

Series I

The needle biopsy sampling method of Turner *et al.* (1983) was followed by two post sampling protocols for freezing: blow-out (BO) of the sample from the biopsy needle into liquid N_2 (as used by Turner *et al.*, 1983), and direct-freezing (DF) of the sample while still in the biopsy needle by placing the latter in liquid N_2 (as used by Milligan & Wood, 1986). My goal was to compare the effect of post-sampling muscle tissue freezing methods on tissue Amm, Lac, Glu and Gly. In this series of studies, 36 uncannulated resting fish (18 per group) were used. The standard flux boxes were modified for biopsy sampling by adding a removable plastic sheet directly underneath the fish. During sampling, the box was rapidly drained via opening a large port in the bottom and the fish was simultaneously pulled quickly upward against the box lid by the plastic liner. This process usually prevented extraneous movement, as the fish was trapped side-ways against the sampling slit on the covering lid. Ten biopsy needles were then punched through the epaxial muscle posterior to the dorsal fin and above the lateral line to obtain white muscle. Despite our efforts to restrain the fish, this process sometimes induced struggling by the fish. The biopsy needles were stainless steel trocars (i.d.= 4 mm; C.D.M.V. Inc., St. Hyacinthe, Quebec) with the capability to take approximately 100 mg samples, though not all punctures were successful. Immediately after the sampling (about 5s), tissue samples were either mouth-blown out of the biopsy needles into liquid N_2 (BO) or directly frozen within the needle in liquid N_2 (DF). In the DF case, the frozen tissue samples were later punched out of the needle with a metal probe and stored in liquid N_2. In this series, all tissue extractions were performed by the glass homogenizer method (see Series III below).

Series II

Based on the results of Series I, the DF method was the superior technique for post-sampling freezing of tissue when the biopsy method was used for sampling. Therefore, the DF approach was chosen to compare biopsy sampling with the MS-222 anaesthesia plus freeze-clamping method of Tang & Boutilier (1991). These workers reported that rapid anaesthesia with a high dose of neutralized MS-222, followed by freeze-clamping of the excised muscle sample in liquid N_2-cooled aluminum tongs, yielded very low tissue lactate levels. Such a method might also keep "fast" metabolites, such as ATP and PCr, closer to true *in vivo* levels. An additional objective of this series was to test whether prior implantation of the DA would affect the metabolic status.

In this experiment, 50 resting fish in total were tested. However, not every metabolite was measured on each fish. In those without DA, 10 fish were sampled by DF and 13 fish were sampled by MS-222. In those with DA, 6 fish were sampled by DF and 21 fish were sampled by MS-222.

Slight modifications were made to the original MS-222 method of Tang & Boutilier (1991). The holding box (about 8 L) was closed 2-3 min before concentrated MS-222 was poured in. The fish usually lost balance within 1 min, without struggling. A concentration of 0.5 g l^{-1} MS-222, rather than 0.2 g l^{-1} was used as we found that the latter caused some struggling prior to anaesthesia; the MS-222 stock solution was neutralized (pH = 7) by NaOH to avoid acidifying the holding water; otherwise water pH would have dropped to pH = 2.5-3.0. NaOH, rather than $NaHCO_3$, was used to neutralize the MS-222 stock to avoid the complication of hypercapnia. Immediately after the fish lost balance, it was removed from the water, and a white muscle sample (5-10 g) excised between the dorsal fin and lateral line with a sharp scalpel. This sample was then freeze-clamped with liquid N_2 cooled aluminum tongs and stored in liquid N_2 before analysis. The entire process, from removal of the fish from the water until freeze-clamping, took about 10-15 sec. The glass homogenization method (see 3 below) was again employed for all analyses in this series.

Series III

In Series I and II, various sampling and freezing procedures were tested. According to the results, the MS-222/freeze-clamping method was determined to be the best technique. Post-sampling tissue processing, however, could also have a major influence on the metabolite analyses. Therefore, glass homogenizer (GH, the method used in Series I and II), mortar and pestle (MP), and freeze-drying (FD) techniques were tested after muscle samples were taken by the MS-222/freeze-clamping method from both resting and exercised fish. These three tissue processing methods were chosen due to their widespread use for tissue metabolite measurements (see Introduction).

a) Glass Homogenizer

In the case of samples obtained by the MS-222 method, frozen tissue fillets were broken into smaller pieces before processing, while tissue pellets obtained by biopsy needles were processed directly. A glass tissue homogenizer (Pyrex, No.2272, Vol. 7ml) containing 1.2ml of 8% $HClO_4$ (PCA) was placed in ice water. Approximately 150mg tissue was weighed in a pre-tared dish filled with liquid N_2. The tissue was weighed immediately after the liquid N_2 had evaporated, and then quickly transferred to the pre-cooled glass homogenizer and manually ground for 4 min. The homogenizer was submerged in ice-water during the entire grinding period. The homogenate was then centrifuged at 9000 g in a 1.5ml bullet tube for 5 min. The supernatant was then stored at -70°C until needed for further analysis.

b) Mortar and Pestle

Tissue was ground into very fine powder in a liquid N_2 cooled mortar and pestle. The tissue was always submerged in liquid N_2 during grinding to prevent moisture condensation. Connective tissue and bones were picked out and discarded during this process. About 150mg of frozen tissue powder was then transferred to a pre-weighed 1.5ml centrifuge tube containing 1.2ml ice-cold 8% PCA and the final weight was then determined. The tube was vortexed for 10 sec and set on ice for approximately 30 min. to allow the extraction to proceed. The homogenate was then centrifuged at 9000 g for 5 min, and the supernatant stored at -70°C for later use.

c) Freeze Drying (lyophilization)

The first step of this protocol involved the same process as the MP. However the frozen powder, instead of being transferred into PCA directly after grinding, was transferred to plastic vials partially filled with liquid N_2 These vials were then covered with perforated lids to allow N_2 to evaporate and to facilitate the freeze-drying process, which lasted 64 h. The lyophilized powder was then stored in a desiccator at -70°C until extraction. For the extraction, approximately 50mg of dry powder was weighed into a bullet tube with 1ml ice-cold 8% PCA, vortexed and set on ice for about 30 min to allow the extraction to proceed. The supernatant was then obtained and saved in the same fashion as above.

Series IV.

This final series compared four different processing methods for the measurement of white muscle ammonia levels: (a) manual grinding in a glass homogenizer (GH), (b) motor-driven grinding (MDG) using a Turrax Tissumizer with a microprobe head, (c) mortar and pestle (MP), and (d) MP and GH combination (MP&GH).

GH and MP data were obtained as described in Series III. In the MP&GH group, the muscle samples were first pulverized in a mortar and pestle under liquid N_2. They were then transferred to an ice-cold homogenizer containing 1 ml 8% PCA (approximately 10x dilution), and ground manually for 4 min as in the standard GH method. In the MDG treatment, approximately 0.5g of frozen tissue was weighed (under liquid N_2) and then transferred to about 10x volume of 8% PCA in a 17x100 polypropylene tube submerged in ice-cold water. The tissue was then ground by the Turrax Tissumizer at high speed for 2 min.

The homogenate was then centrifuged at 9000 g for 2 min and the supernatant stored at -70°C for later analysis.

The second part of this series evaluated the possible lability of measured muscle ammonia levels to thawing during processing. The MP&GH combination was used. White muscle samples (taken from one fish only by the MS-222 method) were ground into a fine powder under liquid N_2 by MP. They were then weighed, and allowed to sit at room temperature (20°C) for various time periods (0-60 min.) prior to acid extraction by GH in the normal fashion in an ice-cold glass homogenizer.

Analytical Techniques

The PCA extracted muscle supernatant was analyzed fluorometrically (Fluoro-micro-photometer, American Instrument Co.,Maryland, USA) for ATP and PCr (*cf.* Bergmeyer, 1983). Cr, Glu, Gly, Lac, and Pyr were assayed enzymatically (LKB UltralspecPlus 4053, LKB Ltd. Cambridge, UK) by methods described in Bergmeyer (1983). Muscle ammonia was measured by the glutamate dehydrogenase method of Kun & Kearney (1971) using spiked muscle tissue as internal standards. Muscle intracellular pH was measured by the homogenization technique of Pörtner *et al.* (1990), using the pH micro-electrode system described below.

Arterial blood Pa_{O2} was measured on a Po_2 electrode (Radiometer E5046, thermostatted to 15°C) connected to a Cameron Instruments (OM-200) oxygen meter. Arterial blood pH_a was determined with a thermostatted (15°C) Radiometer microelectrode (Type E5021) and a Radiometer PHM72 acid-base analyzer. True plasma total CO_2 was measured on a Cameron Instruments Capni-Con Total CO_2 analyzer (Model II). Pa_{CO2} and plasma HCO_3^- were calculated via manipulation of the Hendersen-Hasselbalch equation using appropriate constants for rainbow trout at 15°C, as described by Boutilier *et al.* (1984). True plasma was separated from red blood cells when hematocrit was measured by centrifuging 80μl of blood in a sealed capillary tube (Radiometer type D) at 5000 g for 5 min. Hemoglobin concentration was determined colorimetrically on whole blood via the cyanmethemoglobin method (Blaxhall & Daisley, 1973).

The remaining blood was centrifuged at 9000 g for 2 min to obtain plasma for other analyses. Total plasma protein was determined with an American Optical Goldberg refractometer (Alexander & Ingram, 1980). Plasma was deproteinized by adding 300 μl of plasma to 600 μl of 8% PCA and supernatant analyzed enzymatically for Lac, Pyr, Glu, and Amm (Bergmeyer, 1983). Plasma inorganic phosphorus was measured by the method of Fiske & SubbaRow (1925).

Statistical analysis

Data are reported as means ± 1 S.E.M. (N). The differences between data sets in Series I and II were tested by unpaired Student's two-tailed t-test ($P \leq 0.05$). In Series III and IV, the differences among the three or four treatment groups were tested by ANOVA ($P \leq 0.05$) followed by post-hoc comparison by mean of Duncan's multiple range and critical range test ($P \leq 0.05$) (Milliken & Johnson, 1984). The tests were performed on Statistica (Statsoft Inc., 1992).

RESULTS

Series I - Comparison of blow-out and direct freeze techniques.

Fig. 2-1 compares Amm, Lac, Glu, and Gly levels in white muscle samples of trout taken by needle biopsy and then frozen using either blow-out (BO) or direct-freezing (DF) techniques. The BO technique resulted in significantly greater resting Lac (53.6%) and Glu (88.7%) compared with the DF technique. White muscle Amm and Gly levels showed no significant differences between these two sampling methods. However there was greater variability in Gly levels in the BO treatment. The absolute Lac levels were relatively high (Tang & Boutilier, 1991; Parkhouse *et al.*, 1987), while Gly levels were relatively low (Milligan & Wood, 1986; Parkhouse *et al.*, 1987; Tang & Boutilier, 1991) in both sampling groups compared to the results of other *in vivo* studies.

Series II - Comparison of biopsy and MS-222 sampling methods.

In addition to the metabolites determined in Series I, ATP, PCr, and Pyr were also measured. With the exception of PCr and Pyr (see below), the fish with and without dorsal aortic catheters showed no significant differences in metabolite levels. Therefore these two treatment groups were combined to compare the biopsy (with DF) and MS-222 (with freeze-clamping) sampling methods (Fig. 2-2). The MS-222 group had significantly higher levels of PCr, Glu, and Gly than the biopsy group (91%, 402%, and 62%, respectively). The Amm and Lac concentrations in the biopsy group were 25% and 110% higher, respectively, than those in the MS-222 group. However ATP and Pyr levels were the same with the two treatments. In the biopsy group, Amm, Lac, Glu, and Gly levels were comparable to the levels measured in Series I (Fig.1).

The fish with DA, sampled by biopsy, demonstrated a significant 24% decrease in PCr compared to fish without DA. In contrast, the fish with DA sampled by the MS-222 method showed a significant 27% increase in PCr compared to the fish without DA (Table 2-I). Pyr levels followed a similar pattern; in the biopsy group, there was a 77% decrease in fish with DA compared to those without DA, while in the MS-222 group, fish with DA exhibited a 171% increase compared to those without DA (Table 2-I).

Series III - Comparison of glass homogenizer, mortar and pestle, and freeze-drying processing methods.

In this series, the three processing methods were tested on both resting and exercised fish. All samples were taken by the MS-222 method (with freeze-clamping). The same list of muscle metabolites as in Series II were measured, with the addition of Cr and the omission of Pyr. Basic blood gas, acid-base, and plasma metabolite levels were also measured in these fish (Table 2-II) in order to establish a good data base for this type of *in vivo* work.

The resting muscle pH_i of 7.253 and exercised pH_i of 6.645 are well within the respective ranges of other *in vivo* studies on trout (Milligan & Wood, 1986; Tang & Boutilier, 1991). A significant 0.6 unit decrease in the arterial pH of exercised fish, compared to the resting fish, corresponds with significant increases in both Lac and Pco_2 of arterial blood in the exercised fish (Table 2-II). In parallel, exercised fish exhibited significantly decreased arterial Po_2, elevated Hct, plasma protein, Pyr, Amm, and inorganic phosphorus, while HCO_3^-, Hb, and Glu did not change significantly (Table 2-II).

In resting fish, freeze-drying (FD) yielded 20% and 200% higher PCr and Glu, respectively, relative to either the glass homogenizer (GH) or mortar and pestle (MP) treatments (Fig. 2-3A). However ATP, Cr, Gly, and Lac remained unchanged among the three groups. The FD process significantly depressed Amm levels by about 50%, while GH and MP processes showed very similar Amm concentrations (Fig. 2-3A).

In exercised fish, there were marked depletions of ATP, PCr, and Gly, and substantial elevations of Cr, Amm, Glu and Lac in white muscle in comparison to resting fish (Fig. 2-3A,2-3B). Within the exercise treatment group, ATP, Gly, and Lac remained constant amongst the three processing methods (Fig. 2-3B). PCr was the same by GH and MP methods, but unfortunately was not measured by FD because of limited tissue supply. Cr, Amm and Glu were the same by GH and MP methods, but all showed significant differences when processed by FD (Fig.3B). In the FD treatment, Amm exhibited about a 70% decrease and Glu a 7-12 fold increase with respect to the other two treatments. Cr decreased significantly (about 50%) in the FD treatment. The lower Cr here suggests that PCr may have been higher than in the other two treatments, as at rest.

Series IV - The effect of various processing methods on resting muscle ammonia levels.

There were no significant differences (by ANOVA) in resting Amm levels in muscle samples processed by the four different extraction methods (Table 2-III). However, motor driven grinding (MDG) and the combination of glass homogenization and mortar and pestle (MP&GH) yielded slightly lower values than either GH or MP alone.

The thawing test demonstrated that resting Amm concentrations were extremely sensitive to even brief periods of thawing (Fig. 2-4). Samples that were ground into fine powder under liquid N_2 and then immediately extracted in PCA (*ie.* MP&GH) had Amm concentrations of approximately 0.7 mmol kg^{-1} wet weight, similar to the values in Table 2-III. When these samples were allowed to sit at room temperature for only 30 sec, Amm concentrations increased 3-fold. Amm concentrations increased progressively for the first 10 min, stabilizing thereafter at approximately 6 - 8 mmol kg^{-1} wet weight (Fig. 2-4). These were comparable to levels measured in exhaustively exercised fish by GH or MP (Fig. 2-3B).

DISCUSSION

To place the present data in context, Table 2-IV surveys other recent measurements of the same metabolites in the white muscle of rainbow trout at rest. The values were obtained by a variety of sampling, processing, and analytical methods, which are also summarized in Table 2-IV. The values tabulated for the present study are those thought to be most representative of the true situation *in vivo.*

The influence of the freezing method with the biopsy technique

Series I was designed to test whether the method of freezing after sampling could influence concentrations of Amm, Lac and other metabolites when samples were taken by needle biopsy. The similarity of Amm concentrations in the two freezing processes suggests that there was no elevation of adenylate deamination (Fig. 2-1; Mommsen & Hochachka, 1988). However, the BO method resulted in higher muscle Lac and Glu and greater variability in Gly than the DF method. The probable reason is that the tissue core blown into liquid N_2 will generate a vapor barrier at the surface which prevents the immediate freezing of the tissue. This short delay of freezing could postpone the arrest of on-going anaerobic glycolysis. In contrast, the biopsy needle will likely conduct heat away much faster and lead to quicker tissue freezing. In this regard, it is noteworthy that while Turner *et al.* (1983), who used the BO method, and Milligan & Wood (1986), who used the DF method, reported similar Lac levels in the muscle of resting trout (both of which were much higher than in the present study, Table 2-IV), Pyr levels measured in the former study were far greater.

The biopsy vs. the MS-222 technique

Recently Tang & Boutilier, (1991) have suggested, based on a review of the literature, that the MS-222 plus freeze-clamping method will produce lower and more realistic levels of muscle Lac than methods such as biopsy which may involve physical disturbance of the animal or struggling. In general, the results of Series II, showing much lower Lac and higher Gly levels with the MS-222 sampling method (Fig. 2-2), support this conclusion. Indeed the present Gly levels are amongst the highest ever recorded in this type of study (Table 2-IV). However, we were unable to obtain muscle Lac concentrations as low (Table 2-IV) as those reported by Tang & Boutilier (1991). Similarly, Milligan & Girard (1993) also did not obtain such low Lac levels. The reason for this difference remains unclear. Nevertheless the MS-222 method did demonstrate the great advantage of preserving high resting Gly and limiting Lac production (Fig. 2-2). In addition, the significantly lower muscle Amm in the MS-222 group indicates better preservation of the adenylate pool.

Muscle PCr and ATP, as high energy reserves, are often considered to be sensitive indicators of changes in metabolic status induced by sampling disturbances. In fact, this may not be true for ATP, because ATP levels were similar in the present study regardless of sampling or processing method (Figs. 2, 3). ATP levels reported by various methods in the literature were also fairly uniform (Table 2-IV). Indeed, a ^{31}P-NMR study of white muscle in goldfish has shown that the initial levels of ATP are maintained for 1 hour after excision of the tissue (Van den Thillart *et al.*, 1990). Because of the high affinity of creatine kinase for ADP and lower availability of ATP compared to PCr in the sarcoplasm, ATP hydrolysis usually takes place after the depletion of PCr (Driedzic & Hochachka, 1978).

However, PCr levels were clearly very sensitive to the method of sampling. In Series II, the PCr concentration of approximately 17 mmol kg^{-1} wet weight obtained by biopsy (Fig. 2-2) was comparable to values reported in studies in which fish were killed by disturbing methods such as cephalic blow or decapitation (Table 2-IV). Dobson & Hochachka (1987) have shown that 1-4 tail flaps prior to sampling could cause a 50-70% decrease in resting PCr. The PCr level of 33 mmol kg^{-1} wet weight obtained by the MS-222 technique in Series II (Fig. 2-2) was the highest ever reported by enzymatic or HPLC methods. While Cr was not measured in Series II, in Series III where the same technique was used, the PCr value was slightly lower (26 mmol kg^{-1} wet weight) but the sum of the PCr and Cr pool was 33 mmol kg^{-1} wet weight. Thus at least 80%, and possibly more, of the total creatine pool stayed in the phosphorylated condition with MS-222 sampling. A ^{31}P-NMR study on intact gold fish (Van den Thillart *et al.*, 1990) has indicated that 95% of the total creatine pool is phosphorylated under true *in vivo* conditions. Their study also suggested that 36% of white muscle PCr would break down within 6 seconds upon excision of muscle from fish.

Muscle Glu is usually elevated during exercise or under non-steady state conditions (Pearson *et al.*, 1990; Milligan & Girard, 1993). In this regard, the finding of higher muscle Glu with MS-222 sampling, versus biopsy (Fig. 2-2), appears to deviate from the conclusion that the MS-222 technique is superior in preserving *in vivo* metabolic status. However MS-222 induced anaesthesia is known to cause rapid plasma hyperglycaemia, probably associated with hypoxaemia and catecholamine release (Houston *et al.*, 1971). This mobilization of hepatic glucose will be reflected in white muscle by providing fuel for glycolysis (Moen & Klungsoyr, 1981; Walton & Cowey, 1982; Parkhouse *et al.*, 1988a).

The influence of dorsal aortic catheterization

Prior implantation of a DA catheter, with its attendant anaesthesia and handling stress, had a negligible influence on most muscle metabolites. Observed changes in the two metabolites which were affected, PCr and Pyr, are difficult to interpret because these effects were diametrically different, depending on whether sampling was via biopsy or MS-222 methods (Table 2-I). If I assume, based on the results of Series II (Fig. 2-2), that the MS-222 method yields values more representative of true *in vivo* conditions, then the higher Pyr could result from greater glycolytic flux, while higher PCr could reflect less sampling disturbance in fish with prior exposure to MS-222 and handling. Such a post-handling phenomenon would be similar to the higher PCr values seen during recovery from exhaustive exercise, and therefore result in differences from resting values reported in other studies (Milligan & Wood, 1986; Pearson *et al.*, 1990; Scarabello *et al.*, 1991).

Comparison amongst the three post-sampling tissue processing techniques

GH and MP methods yielded very similar values for all metabolites, both at rest and after exhaustive exercise (Fig. 2-3). This conclusion is in accord with the study of Lazzarino *et al.* (1989) on mammalian heart showing no significant differences in muscle PCr, ATP, Lac, and a range of other metabolites between a direct homogenization method and one with pre-pulverization under liquid N_2. However, in the present study, GH and MP methods yielded significantly lower PCr concentrations than with the FD approach (Fig. 2-3A). This finding suggests that the lyophilization method is more efficient in preserving this labile high energy phosphate store. As noted earlier, PCr and Cr concentrations by FD which represent over 80% phosphorylation of the total creatine pool are the highest ever reported by methods other than *in vivo* ^{31}P-NMR (Table 2-IV). The very similar ATP concentrations amongst the three processing methods support the conclusion reached earlier that ATP is quite stable. The processing method also had no influence on Gly or Lac concentrations, but surprisingly, Glu levels were higher in samples processed by FD than by GH or MP from both resting (Fig. 2-3A) and exercised fish (Fig. 2-3B). As I can see no reason why FD should artificially elevate Glu, we assume that FD is more effective in preserving Glu by arresting glycolytic processes.

Overall, my conclusion is that FD is the technique of choice for post-sampling processing of muscle tissue for all metabolites measured in the present study except Amm (see below). An additional benefit of the FD approach is convenience. A relatively large amount of tissue powder can be prepared at one time and stored in a desiccator -70°C. The powder is easy to aliquot and weigh, and quite stable during brief periods of handling at room temperature. The inconvenience of using liquid N_2 throughout tissue processing for multiple assays is avoided. The only practical disadvantage is invariable loss of small amounts of the very light tissue powder during handling, which may become a limitation if the total tissue sample size is small.

The influence of various processing methods on muscle ammonia measurements

The one metabolite for which FD processing does introduce artifact is Amm. Initially I believed that the much lower Amm concentrations with FD than with GH or MP (Fig. 2-4) were real, reflecting better preservation of true *in vivo* levels. However, I then conducted a detailed experiment following the time course of muscle Amm metabolism during recovery of trout from exhaustive exercise (Y. Wang, G.J.F. Heigenhauser, & C.M. Wood, unpubl. results). In contrast to all previously reported data (*eg*. Parkhouse *et al.*, 1987; Dobson & Hochachka, 1987; Mommsen & Hochachka, 1988; Wright & Wood, 1988; Tang *et al.*, 1992), when samples were processed by FD, muscle Amm remained low and more or less invariant throughout the recovery period. However, when samples were processed by MP, the standard pattern of large post-exercise elevation followed by gradual decline was seen. This led me to conduct a simple test. A series of known concentrations of Amm in solution were subjected to the same lyophilizing procedure for 66 h. They were then reconstituted to the original volume and analyzed for Amm. I found that over 70% of Amm was lost during lyophilization. The explanation for this is probably NH_3 evaporation. The FD process only drives tissue temperature to as low as -50°C, while the melting point of NH_3 is about -77°C and its boiling point is -33°C (Windholz & Budavari, 1983). Although less than 1% of Amm exists as NH_3 at physiological pH, with a continuous vacuum applied during the FD process, a significant amount of NH_3 will evaporate out of the tissue and drive more NH_4^+ toward NH_3 by dynamic equilibrium. As a consequence, tissue Amm measurements become artificially low. I conclude that Amm cannot be reliably measured on tissue samples processed by FD.

The test of the effect of tissue thawing on Amm levels (Fig. 2-3) indicated that any method which tends to warm up samples should be avoided. Such warming could accelerate adenylate deamination and lead to degradation of amino acids, resulting in an overestimate of muscle Amm. In fact, Kun & Kearney (1971) pointed out that even in acid, Amm can be liberated from amides within a very short period of time. As a result, they suggested that samples and extracts should be kept as cold as possible. Furthermore a "critical freezing zone" between -0.8 and -5°C has been defined by several research groups (Bito & Amano, 1962; Partmann, 1963; Norlan & Dyer, 1969, 1974). In this temperature "zone", glycolysis and ATP-catabolism may proceed at even higher rates than at room temperature.

MDG likely stands the greatest chance of entering this zone and warming the tissue up before the cells are broken down. In this regard, the resting muscle Amm levels obtained by Wright & Wood (1988) using this method were slightly higher than those reported by other methods (Table 2-IV). In the present study, however, there was no evidence that MDG caused any elevation in Amm (Table 2-III). It therefore seems unlikely that artifacts due to tissue processing methods provide the explanation for the current controversy about the distribution of ammonia between white muscle and blood plasma in fish (*cf.* Wright & Wood, 1988; Heisler, 1990; Tang *et al.*, 1992). Indeed our study showed that the Amm levels obtained are relatively independent of the grinding method, as long as warming is avoided (Table 2-III). MP and GH methods yield very similar values, and there appears to be no added benefit in combining the two (MP&GH).

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Figure 2-1.

Comparison of concentrations of ammonia (Amm), lactate (Lac), glucose (Glu), and glycogen (Gly) in resting rainbow trout white muscle sampled by needle biopsy and frozen in liquid N_2 by either the blow-out (open bars) or direct-freezing (hatched bars) methods. * indicates significant difference ($P < 0.05$) between the two treatments. Values are means ± 1S.E.M. (number of fish) in mmol kg^{-1} wet Wt.

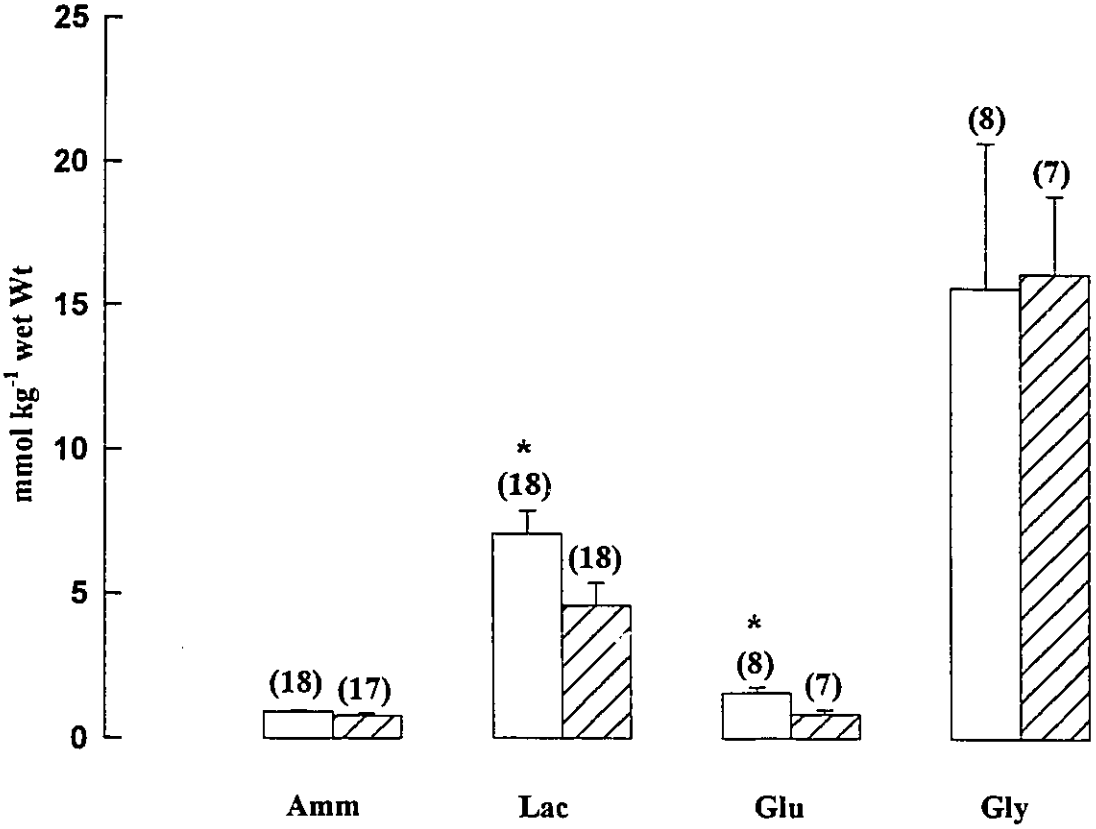

25
20
15
10
5
0
mmol kg^{-1} wet Wt
(18)
(17)
*
(18)
(18)
*
(8)
(7)
(8)
(7)
Amm
Lac
Glu
Gly

Figure 2-2.

Comparison of concentrations of ATP, creatine phosphate (PCr), ammonia (Amm), glucose (Glu), glycogen (Gly), lactate (Lac) and pyruvate (Pyr) in resting rainbow trout white muscle sampled by needle biopsy (with direct-freezing; open bars) or the MS-222 (with freeze-clamping; hatched bars) methods. * indicates significant differences ($P < 0.05$) between the two techniques. Values are means ± 1S.E.M. (number of fish) in mmol kg^{-1} wet Wt.

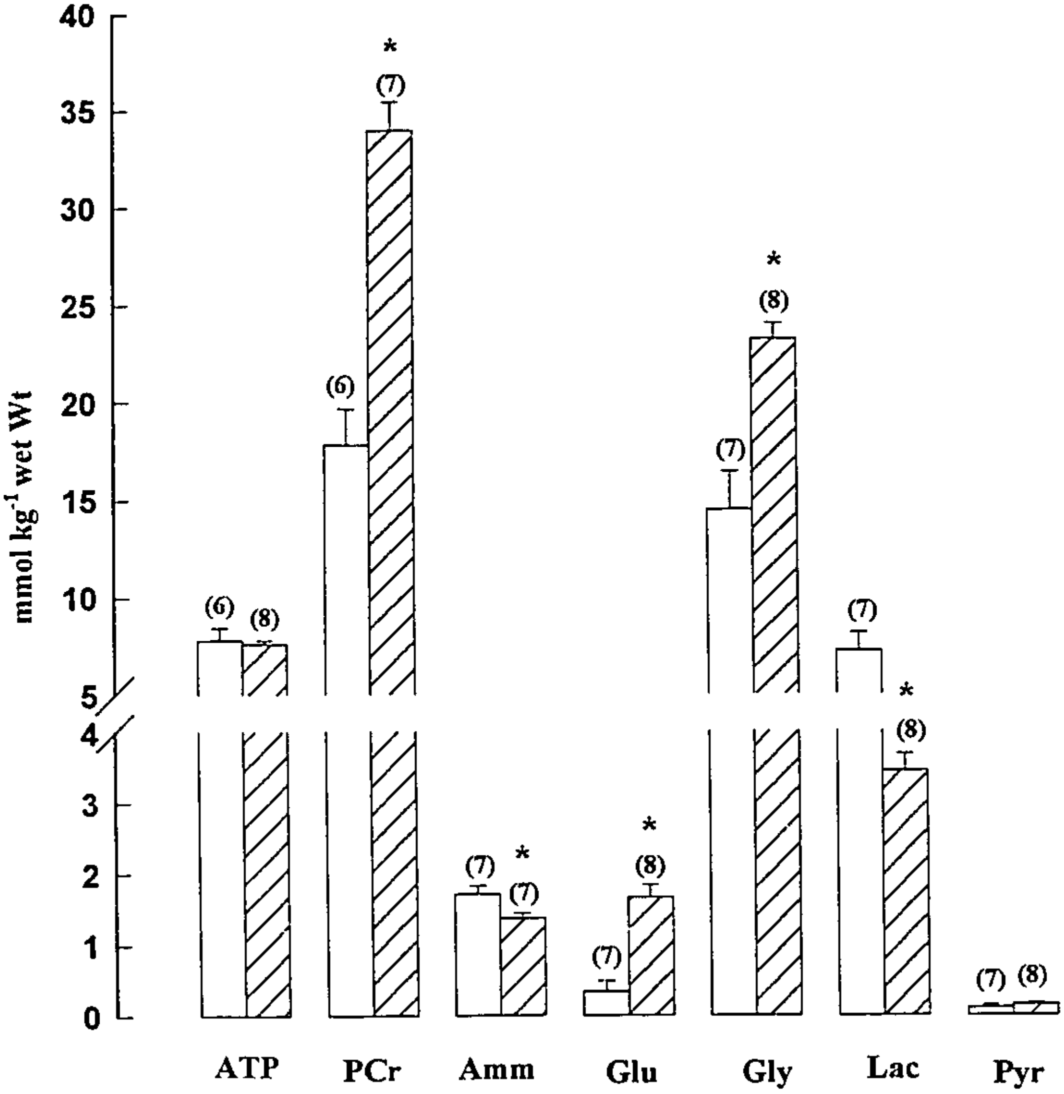

mmol kg-1 wet Wt
40
35
30
25
20
15
10
5
4
3
2
1
0
(6)
(8)
(6)
*
(7)
(7)
*
(7)
(7)
*
(8)
(7)
*
(8)
(7)
*
(8)
(7)
(8)
ATP
PCr
Amm
Glu
Gly
Lac
Pyr

Figure 2-3.

Comparison of concentrations of ATP, creatine phosphate (PCr), creatine (Cr), ammonia (Amm), glucose (Glu), glycogen (Gly), and lactate (Lac) in rainbow trout white muscle processed by glass homogenization (open bars), mortar and pestle (hatched bars), and freeze-drying (crossed bars) methods. (A) resting fish, (B) exhaustively exercised fish. * indicates significant differences ($P < 0.05$) amongst the three methods (ANOVA). (a) indicates no significant difference ($P > 0.05$) between the two groups, and (b) indicates significant differences ($P < 0.05$) from (a) (post-hoc comparison by Duncan's test). Values are means ± 1S.E.M. (number of fish) in mmol kg^{-1} wet Wt.

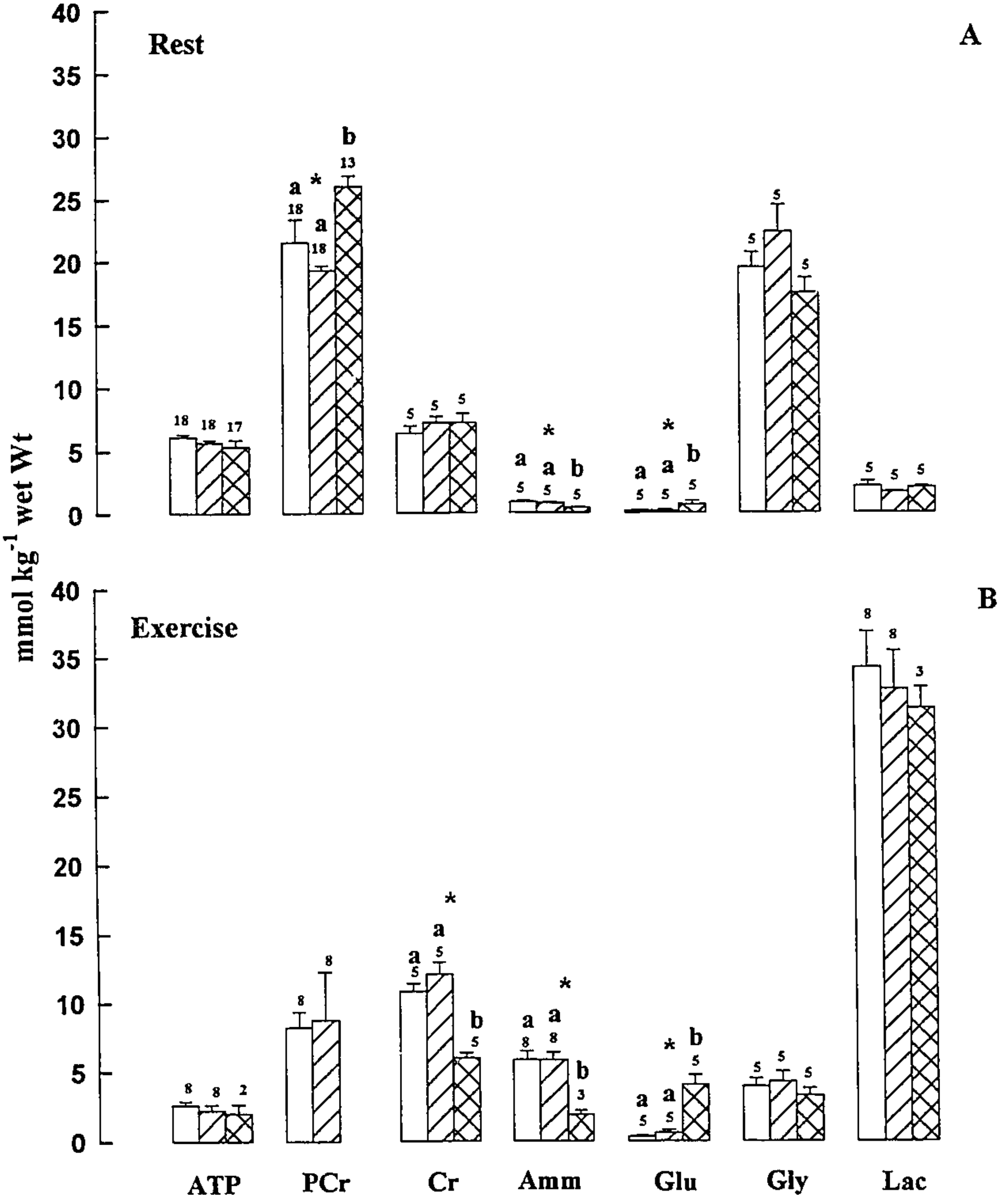
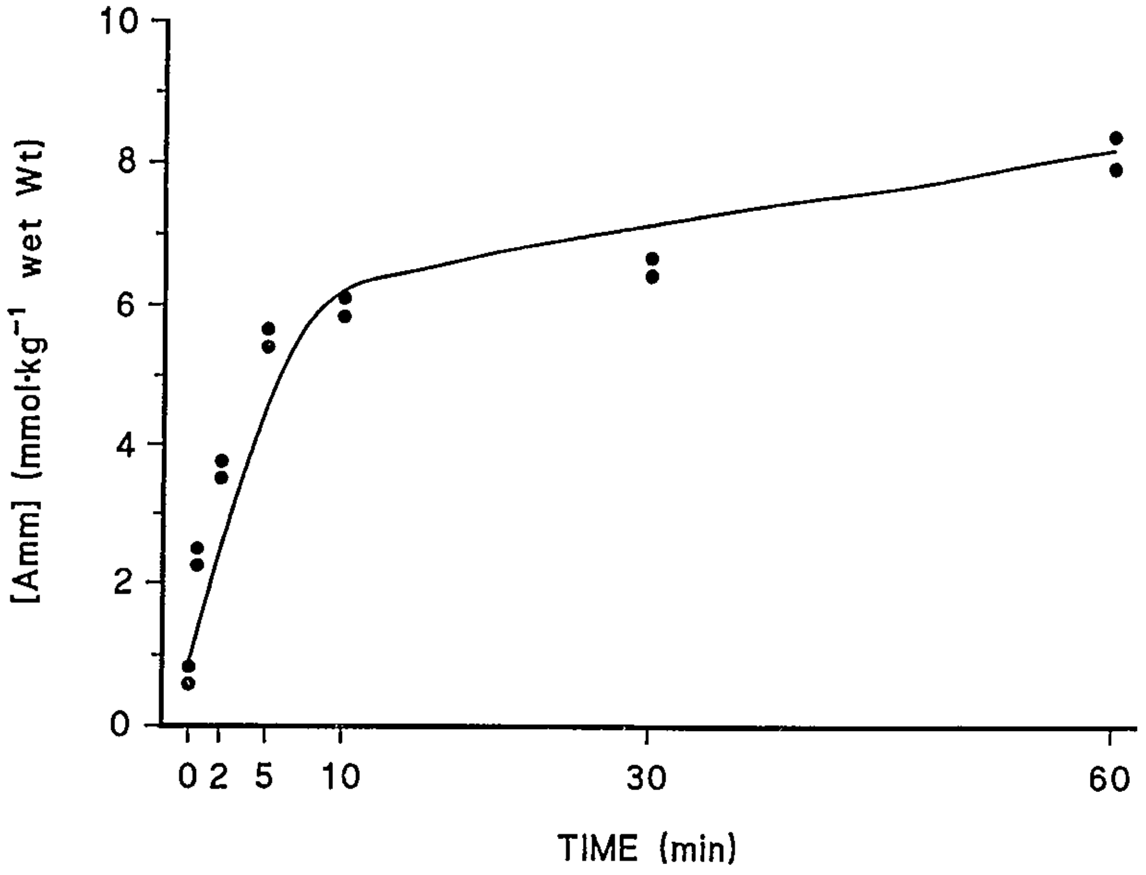

mmol kg^{-1} wet Wt
40
35
30
25
20
15
10
5
0
Rest
A
18
18
17
a
18
*
a
18
b
13
5
5
5
a
5
*
a
5
b
5
a
5
*
a
5
b
5
5
5
5
5
5
5
Exercise
B
8
8
2
8
8
a
5
*
a
5
b
5
a
8
*
a
8
b
3
a
5
*
a
5
b
5
5
5
5
8
8
3
ATP
PCr
Cr
Amm
Glu
Gly
Lac

Figure 2-4.

White muscle ammonia (Amm) concentrations after thawing at room temperature (20°C) for different time periods (0 - 60 min). Values are duplicate measurements on tissue from a single resting fish, expressed in mmol kg^{-1} wet Wt.



[Amm] (mmol·kg^{-1} wet Wt)
0
2
4
6
8
10
0
2
5
10
30
60
TIME (min)

Table 2-I.

Creatine phosphate (PCr) and pyruvate (Pyr) concentrations sampled by needle biopsy (with direct freeze) and the MS-222 (with freeze-clamping) techniques in white muscle of resting rainbow trout with and without dorsal aorta catheters (DA).

	Biopsy		MS-222	
	Without DA	With DA	Without DA	With DA
PCr	20.43 ± 2.27** (10)	15.58 ± 3.02* (6)	28.34 ± 2.30* (13)	36.01 ± 1.59 (21)
Pyr	0.190±0.044** (10)	0.044 ± 0.015* (6)	0.063 ± 0.022* (13)	0.171 ± 0.011 (21)

Values are means ± S.E.M. (number of fish) in mmol kg^{-1} wet weight. * indicates significantly ($P<0.05$) different from samples obtained from fish with DA. * indicates significantly ($P<0.05$) different from the samples obtained with MS-222 methods.

Table 2-II.

Blood gas and acid-base parameters and concentrations of metabolites in the blood plasma of resting and exhaustively exercised rainbow trout.

	Resting Fish	Exercised Fish
pH_i	7.235 ± 0.012 (18)	6.645 ± 0.042 (8)*
pH_a	7.968 ± 0.022 (18)	7.350 ± 0.040 (8)*
Po_2 (Torr)	117.8 ± 3.3 (18)	102.4 ± 4.9 (7)*
PCO_2 (Torr)	1.62 ± 0.14 (18)	5.32 ± 0.74 (7)*
$[HCO_3^-]$ (mM)	6.87 ± 0.47 (18)	7.39 ± 0.13 (8)
Hct(%)	21.0 ± 1.6 (18)	28.2 ± 3.2 (8)*
Hb (g $100ml^{-1}$)	6.56 ± 0.43 (13)	6.01 ± 0.59 (8)
Protein (g $100ml^{-1}$)	2.50 ± 0.27 (5)	3.17 ± 0.14 (8)*
Lac (mM)	0.85 ± 0.15 (5)	5.40 ± 0.63 (8)*
Pyr (mM)	0.032 ± 0.011 (5)	0.098 ± 0.007 (8)*
Glu (mM)	4.15 ± 0.81 (5)	4.16 ± 0.42 (8)
Amm (mM)	0.043 ± 0.004 (5)	0.298 ± 0.056 (8)*
P_i (mM)	1.06 ± 0.14 (5)	1.92 ± 0.21 (8)*

Values are means ± S.E.M. (number of fish). * indicates values that are significantly different ($P<0.05$) from corresponding resting values (see text for abbreviations).

Table 2-III.

A comparison of ammonia (Amm) concentrations in resting rainbow trout white muscle processed by manually driven glass homogenizer (GH), mortar and pestle (MP), combination of MP and GH (MP&GH), and motor driven grinding (MDG).

Processing Method	Muscle [Amm]
GH	0.89 ± 0.13 (5)
MP	0.80 ± 0.10 (5)
MP&GH	0.63 ± 0.09 (5)
MDG	0.53 ± 0.12 (5)

Values are means ± SEM (number of fish) in mmol kg^{-1} wet weight. ANOVA indicated no significant differences ($P > 0.05$).

Table 2-IV.

Resting levels of metabolites in rainbow trout white muscle determined by different sampling, processing and analytical methods.

	Concentration (mmol kg^{-1} wet weight)	Sampling Methods	Processing Methods	Analytical Methods	Source
PCr	17.47 ± 0.78	CB	MP & GH	Enzy	1
	19.83 ± 0.92	Decap	MP & GH	HPLC	2
	19.90 ± 1.60	Decap	MP & GH	HPLC	3
	13.05 ± 0.71	CB	GH	HPLC	4
	8.3	Diazepam	FD	Enzy	5
	22.62 ± 2.69	Somnotol Inj.	MP & GH	Enzy	6
	21*	Biopsy DF	GH	Enzy	7
	23	MS-222	MP	Enzy	8
	25.95 ± 0.87	MS-222	FD	Enzy	Present
Cr	27.52 ± 3.85	CB	MP & GH	Enzy	1
	31.5 ± 0.87	Decap	MP & GH	HPLC	2
	24.6 ± 1.0	Decap	MP & GH	HPLC	3
	19.36 ± 2.3	Somnotol Inj.	MP & GH	Enzy	6
	7.16 ± 0.14	MS-222	FD	Enzy	Present
ATP	5.24 ± 0.13	CB	MP & GH	Enzy	1
	7.33 ± 0.29	Decap	MP & GH	HPLC	2
	7.26 ± 0.11	Decap	MP & GH	HPLC	3
	6.65 ± 0.12	CB	GH	HPLC	4
	7.5	Somnotol Inj.	MP & GH	Enzy	6
	6.0	MS-222	MP	Enzy	8
	3.6*	Biopsy DF	GH	Enzy	7
	5.5	Diazepam	FD	Enzy	5
	5.30 ± 0.55	MS-222	FD	Enzy	Present

(Table 2-IV con't)

Amm	1.03 ± 0.04	CB	MP & GH	Enzy	1
	0.49 ± 0.06	CB	GH	Enzy	4
	1.61 ± 0.48*	CB	MDG	Enzy	14
	1.04 ± 0.05	Decap	MP & GH	Enzy	3
	1.03 ± 0.14	Decap	MP & GH	Enzy	2
	0.49 ± 0.04*	d-tubocurarine chloride	MP & MDG	Enzy	9
	0.80 ± 0.10	MS-222	MP	Enzy	Present

(Table 2-IV Con't)

	Concentration (mmol kg^{-1} wet weight)	Sampling Methods	Processing Methods	Analytical Methods	Source
Gly	22.4 ± 1.68	Decap	MP & GH	Enzy	2
	23.3 ± 1.0	Decap	MP & GH	Enzy	3
	8.4 ± 0.84	HB	GH	Enzy	4
	9.90 ± 0.87	CB	MP & GH	Enzy	1
	9.96 ± 1.58	CB	MP & GH	Enzy	11
	15	MS-222	MP	Enzy	8
	19.2	Diazepam	FD	Enzy	5
	33.90 ± 3.63	Somnotol Inj.	MP & GH	Enzy	6
	26.98 ± 1.32	MS-222	GH	Enzy	Present
	22.27 ± 2.14	MS-222	MP	Enzy	Present
Glu	1.56 ± 0.2	CB	MP & GH	Enzy	1
	1.02 ± 0.14	Decap	MP & GH	Enzy	10
	0.64 ± 0.05	CB	GH	Enzy	4
	0.3	MS-222	MP	Enzy	8
	0.26	Diazepam	FD	Enzy	5
	0.68 ± 0.29	MS-222	FD	Enzy	Present
Lac	6.62 ± 0.95	CB	MP & GH	Enzy	1
	3.0 ± 0.4	Decap	MP & GH	Enzy	10
	4.07 ± 0.31	CB	GH	Enzy	4
	5.76 ± 0.49	Decap	MP & GH	Enzy	2
	9.97 ± 1.12	Biopsy	GH	Enzy	7
	10 ± 1	Biopsy	GH	Enzy	12
	0.57 ± 0.06*	MS-222	MP & GH	Enzy	13
	1.28	Diazepam	FD	Enzy	5
	3.92 ± 0.94	Somnotol Inj.	MP & GH	Enzy	6
	4.26 ± 0.82	CB	GH	Enzy	11
	2.00 ± 0.16	MS-222	FD	Enzy	Present

	Concentration (mmol kg^{-1} wet weight)	Sampling Methods	Processing Methods	Analytical Methods	Source
Pyr	0.03 ± 0.005	Decap	MP & GH	Enzy	10
	0.13 ± 0.01*	Biopsy	GH	Enzy	7
	0.04	Diazepam	FD	Enzy	5
	0.19 ± 0.06	MS-222	MP & GH	Enzy	11
	0.6 ± 0.05	Biopsy	GH	Enzy	12
	0.11 ± 0.06	Somnotol Inj.	MP & GH	Enzy	6
	0.22 ± 0.09	MS-222	GH	Enzy	Present

means ±1 S.E.M. (expressed as mmol kg^{-1} wet weight).

* are corrected to units of mmol kg^{-1} wet weight. using either muscle water content or intracellular fluid volumes accordingly, from *in vivo* data of Y.Wang *et al.* (1994).

CB: Cephalic blow; Decap: decapitated; Somnotol Inj.: injection of somnotol; MDG: motor driven grinding; MP: mortar and pestle; GH: glass homogenization; FD: freeze-drying; Enzy: enzymatic analysis; HPLC: high performance liquid chromotography.

1. Dobson & Hochachka (1987)
2. Dobson *et al.* (1987)
3. Parkhouse *et al.* (1988a)
4. Mommsen & Hochachka (1988)
5. Pearson *et al.* (1990)
6. Schulte *et al* . (1992)
7. Milligan & Wood (1986)
8. Ferguson *et al.* (1993)
9. Tang *et al.* (1992)
10. Parkhouse *et al.* (1988b)
11. Milligan & Girard (1993)
12. Turner *et al.* (1983)
13. Tang & Boutilier (1991)
14. Wright & Wood (1988)

CHAPTER 3

INTEGRATED RESPONSES TO EXHAUSTIVE EXERCISE AND RECOVERY IN RAINBOW TROUT WHITE MUSCLE: ACID-BASE, PHOSPHAGEN, CARBOHYDRATE, LIPID, AMMONIA, FLUID VOLUME, AND ELECTROLYTE METABOLISM

SUMMARY

White muscle and arterial blood plasma were sampled at rest and during 4 h recovery from exhaustive exercise in rainbow trout. A compound respiratory and metabolic acidosis in the blood was accompanied by increases in plasma lactate (in excess of the metabolic acid load), pyruvate, glucose, ammonia, and inorganic phosphate, large elevations in haemoglobin and haematocrit, red cell swelling, increases in most plasma electrolytes, but no shift of fluid out of the ECF into the ICF of white muscle. The decrease in white muscle pH_i was comparable to that in pH_e; both recovered by 4h. Creatine phosphate and ATP were both reduced by 40% after exercise, the former recovering within 0.25 h, whereas the latter remained depressed until 4 h. Changes in creatine mirrored those in creatine phosphate, whereas changes in IMP and ammonia mirrored those in ATP. White muscle glycogen was reduced 90% by converting primarily to lactate; recovery was slow, to only 40% of resting glycogen levels by 4h. During this period, most of the lactate and metabolic acid were retained in white muscle, and there was excellent conservation of carbohydrate, suggesting

that *in situ* glycogenesis rather than oxidation was the major fate of lactate. The redox state ($NAD^+/NADH$) of the muscle cytoplasm, as estimated from ICF lactate, pyruvate, and pH_i, remained unchanged from resting levels, challenging the traditional view of the "anaerobic" production of lactate. Furthermore, the membrane potential, as estimated from ICF and ECF electrolytes via the Goldman equation, remained unchanged throughout, challenging the view that white muscle becomes depolarized after exhaustive exercise. Indeed, ICF K^+ was elevated. Lactate was distributed well out of electrochemical equilibrium with either E_m or pH_e - pH_i, supporting the view that lactate is actively retained in white muscle. In contrast, H^+ ions were actively extruded. Ammonia was distributed passively according to E_m rather than pH_e - pH_i throughout recovery, providing a mechanism to retain high ICF ammonia for adenylate resynthesis *in situ*. Although lipid is not traditionally considered a fuel for burst exercise, substantial decreases in free carnitine and elevations in acyl-carnitines and acetyl-CoA indicated an important contribution of fatty acid oxidation by white muscle during both exercise and recovery.

INTRODUCTION

As a sprint swimmer with over 60% of its body weight in white muscle (Johnston, 1980; Stevens, 1968), the rainbow trout provides an ideal model to study the exercise physiology of this tissue. Indeed, the acid-base, metabolic, and fluid volume responses induced by short term "anaerobic" exhaustive exercise in trout white muscle have been well studied in last three decades (*eg*. Black *et al.*, 1962; Driedzic and Hochachka, 1978; Johnston, 1980; Turner *et al.*, 1983; Turner and Wood, 1983; Wood and Perry, 1985; Milligan and Wood, 1986; Dobson and Hochachka, 1987; Dobson *et al.*, 1987; Parkhouse *et al.*, 1987, 1988; Mommsen and Hochachka, 1988; Pearson *et al.*, 1990; Tang and Boutilier, 1991; Storey, 1991; Pagnotta and Milligan 1991; Moyes *et al.*, 1992; Schulte *et al.*, 1992; Tang *et al.*, 1992; Ferguson *et al.*, 1993; Milligan and Girard, 1993). Wood (1991) has drawn attention to the need for integration of different responses, yet comparison amongst studies is difficult. Most investigations have focused on only one aspect of the exercise response, and trout of a variety of different sizes, strains, feeding regimes, and physical conditions have been used. In addition, a variety of different techniques have been employed to induce exhaustive exercise, to sample blood and muscle, to measure fluid volumes, and to assay muscle metabolites. The primary goal of the present investigation was to provide an integrated study in which a wide range of responses to exhaustive exercise (acid-base status, a comprehensive set of metabolites, fluid volumes, and ions) were characterized in a single batch of trout using standardized, improved sampling and processing methodology for muscle and blood (Munger *et al.*, 1991; Wang *et al.*, 1994).

A second goal was to characterize the influence of exhaustive exercise and recovery on the utilization and replenishment of various fuel sources, especially lipid, in white muscle.

It is widely accepted that high energy phosphogens (*ie*. creatine phosphate and ATP) are the fuel supply for strenuous exercise, while carbohydrates (*ie*. glycogen and glucose) provide fuel reserves available upon the depletion of high energy phosphogens (Milligan and Wood, 1986b; Dobson and Hochachka, 1987; Parkhouse *et al*., 1988; Pearson *et al*., 1990; Scarabello *et al*., 1991a; Schulte *et al*., 1992). Little is known about the effects of burst exercise on lipid metabolism in fish. Free fatty acids (FFA), together with proteins (amino acids), are traditionally considered as fuels only for long term aerobic swimming (Cowey *et al*., 1962; Driedzic and Hochachka, 1978; Mommsen *et al*., 1980; Walton and Cowey, 1982; Henderson and Tocher, 1987; Greene and Selivonchick, 1987). However, recent work by Milligan and Girard (1993) has indicated a sustained decrease in total lipid levels of white muscle after short term exhaustive exercise in trout. Earlier, Dobson and Hochachka (1987) reported a sustained decrease in plasma FFA levels. These findings led to inclusion of FFA metabolism in the present study.

It is possible that FFAs are a significant aerobic fuel during exhaustive exercise and/or a fuel supply for the ATP synthesis needed to replenish high energy phosphogens and glycogen during post-exercise recovery. FFAs cannot be oxidized (β-oxidation) unless they are transported into the mitochondria, for which carnitine is the vehicle through the formation of acyl-carnitine. β-oxidation produces acetyl-CoA to fuel the Krebs cycle. Determinations of total carnitine, free carnitine, short-chain acyl-carnitine, acetyl-carnitine, coenzyme A (CoASH), and acetyl-CoA provide far more information than do measurements of just the available FFA pool and the absolute triglyceride (TG) levels in muscle. Changes in acyl-carnitines in particular reflect the true level of FFA metabolism in white muscle.

A third goal was to specifically characterize the ionic responses to exhaustive exercise in white muscle. Wood (1991), on the basis of model calculations using plasma ion and fluid volume data from several studies, suggested that a large ionic disturbance must occur in the intracellular fluid compartment of white muscle, coincident with fluid volume shifts. In contrast, the only experimental study (Parkhouse *et al.*, 1987) reported small changes in intracellular electrolytes different from those predicted by Wood (1991), but assumed that fluid volume distribution remained unchanged. The present study therefore measured both fluid volume distribution and major intracellular electrolytes in white muscle after exhaustive exercise. The distribution of electrolytes between intra- and extra-cellular compartments also provided an estimate of the membrane potential of white muscle.

A final goal was to evaluate the control of H^+, lactate and ammonia distribution after exhaustive exercise. There is a growing body of evidence that lactate and H^+ are retained in white muscle for glycogenesis and oxidation *in situ* during recovery (*eg*. Turner and Wood, 1983; Milligan and McDonald, 1988, Wood, 1991; Schulte *et al.*, 1992; Milligan and Girard, 1993), but the mechanism is unknown. Ammonia, from the deamination of adenylates, also accumulates in large amounts, and has been implicated in metabolic regulation and acid-base balance (Dobson and Hochachka, 1987; Mommsen and Hochachka, 1988). Like lactate, much of the ammonia appears to be retained in white muscle to fuel adenylate resynthesis during recovery, but the retention mechanism is unclear. Indeed, the factors governing the distribution of ammonia in teleost muscle remain controversial (Wright *et al.*, 1988; Wright and Wood, 1988; Heisler, 1990; Tang *et al.*, 1992). In the present study, the two factors which could govern the *passive distribution* of lactate and ammonia were evaluated:

transmembrane pH gradient by measurement of intracellular and extracellular pH, and voltage gradient by estimation of white muscle membrane potential.

MATERIALS AND METHODS

Experimental animals

Adult rainbow trout (*Oncorhynchus mykiss*, 150-350g) were purchased from a local trout hatchery (Rainbow Springs Trout Farm, Ontario) and held in a 800l tank supplied with a continuous flow of aerated, dechlorinated Hamilton tap water (composition as in Milligan and Wood, 1986a) for at least two weeks before the experiment. During holding, fish were fed three times a week with commercial trout pellets (Aquaculture Zeibler Co.). Fish were acclimated to experimental temperature (15±1°C) without feeding for a period of 5-7 days before use to reduce possible dietary influences on acid-base and metabolic status. Dorsal aortic catheters (DA) were surgically fitted into fish while under MS-222 anaesthesia (Soivio *et al.*, 1972); the fish were allowed to recover for 48 h in darkened acrylic boxes supplied with 15°C water. DA catheters were flushed with heparinized Cortland saline (50 iu ml^{-1}; Wolf, 1963) twice a day to avoid blood clotting.

Experimental protocols

In this experiment, we compared acid-base and metabolic changes in blood and white muscle of trout at rest, immediately after 6 min of exhaustive exercise without recovery, and at various times during recovery (15, 30, 60, 120, and 240 min post-exercise). At each time, 8 - 13 fish were sampled.

In all fish, the extracellular fluid volume (ECFV) of white muscle was measured by the use of [^{3}H]labeled PEG-4000 (polyethylene glycol; New England Nuclear, NET-405, MW = 4000, 2 - 8 mCi mmol^{-1}). In a comparative study of different ECFV radiomarkers in trout, Munger *et al.* (1991) concluded that [^{3}H]PEG-4000 yielded the most conservative and reliable estimates. Intracellular fluid volume (ICFV) was measured as the difference between total tissue water content and ECFV. [^{3}H]PEG-4000 was dissolved in 140 mM NaCl and infused into the arterial bloodstream via the DA catheter (28 μCi ml^{-1} kg^{-1} body weight) 10-12 h prior to the experiment to allow the label to equilibrate throughout the ECFV (Munger *et al.*, 1991).

The resting fish were kept in darkened acrylic flux boxes with flowing water for about 48 h before the experiment. Trout in the exercised and recovery groups were transferred to a 150 l cylindrical tank and manually chased to exhaustion (6 min). The fish were returned immediately to their individual boxes after exercise and sampled at the appropriate time. At sampling, blood (2 ml) was taken from each fish *via* the DA catheter and replaced with Cortland saline to avoid potential disturbance caused by the decrease in blood volume. The blood sample was placed on ice immediately and the various blood analyses were carried out within 5 min of initial sampling. Methods for tissue sampling and processing were based on the conclusions of my comparative study of different methods (Wang *et al.*, 1994). The fish was sacrificed by adding a high concentration (0.5 g l^{-1}) of MS-222 (Sigma) to the surrounding water. The MS-222 stock solution was neutralized with NaOH to avoid acidifying the water. The fish lost balance in approximately 1 min. The fish was then immediately removed from the water; within 15 sec, a white muscle sample (3-5 g) was excised from between the lateral line and dorsal fin with a sharp scalpel. The muscle sample was immediately freeze-clamped with aluminium tungs pre-cooled in liquid N_2, and then stored under liquid N_2 for later analysis. A second muscle sample (2-3g) was taken from the same site to estimate white muscle ECFV, ICFV, water content, and ions.

Analytical protocols

Arterial blood pH_a was determined with a Radiometer microelectrode (E5021) and

PHM72 acid-base analyzer. Arterial blood Pa_{O2} was measured with a Radiometer Po_2 electrode (E5046) connected to a Cameron Instruments (OM-200) O_2 meter. The above measurements were conducted at 15°C. Haematocrit was determined by centrifuging 80 μl of blood at 5,000g for 5 min in a sealed haematocrit capillary tube (Radiometer type D). True plasma was obtained by breaking the tube and analyzed for total CO_2 on a Cameron Instruments Capni-Con Total CO_2 analyzer (Model II). Arterial plasma Pa_{CO2} and HCO_3^- were calculated by manipulation of the Hendersen-Hasselbalch equation using appropriate constants (α_{CO2} and pK') for rainbow trout at 15°C reported by Boutilier *et al.* (1984). Blood haemoglobin (Hb) level was assessed colorimetrically by the cyanmethaemoglobin method (Blaxhall and Daisley, 1973). Total plasma protein (C_{PP}) and plasma water content (C_W) were determined with an American Optical Goldberg refractometer (Alexander and Ingram, 1980).

The plasma used for analysis of metabolites was obtained by centrifugation at 9000 g for 2 min. Plasma (300 μl) was deproteinized with 600 μl of 8% perchloric acid (PCA). The supernatant was analyzed enzymatically for lactate (Lac), pyruvate (Pyr), glucose (Glu), and ammonia (Amm) by the methods described in Bergmeyer (1983). Plasma inorganic phosphate (Pi) was measured by the method of Fiske and Subba Row (1925).

For measurement of ECFV, 100 μl of plasma was added to 10 ml scintillation fluid (ACS; Amersham), and 50-100 mg of fresh muscle was digested in 2 ml NCS (Amersham) for about 12 h at 40°C in glass scintillation vials. The digests were then neutralized with 60 μl of glacial acetic acid. Organic scintillant (10 ml, OCS, Amersham) was added to the eutalized digests. The samples were counted on a LKB scintillation counter (Rackbeta 1217) with an on-board quench correction program for trout tissues (Munger *et al.*, 1991). White muscle water content was determined by drying fresh tissue (2 - 3g) to a constant weight at 85°C.

Part of the freeze-clamped white muscle tissue was ground into very fine powder in an insulated mortar and pestle cooled with liquid N_2. A portion of this frozen tissue powder was used to measure intracellular pH_i by the homogenization technique described by Pörtner *et a*l. (1990), employing the same pH electrode system as for blood at 15°C. The remainder of the frozen powder was lyophilized for 64 h, and then stored at -70°C in a desiccator. Subsamples of this lyophilized powder were later extracted with 8% PCA, 20 mg powder to 1 ml PCA, and used to measure selected metabolites, except Amm, which was measured on a direct extract of frozen tissue powder. The detailed processing and extraction methods, and the reasons for avoiding lyophilization for Amm measurements, have been outlined in our previous study (Wang *et al.*, 1994) .

ATP and creatine phosphate (PCr) were analyzed fluorometrically (Fluoro-microphotometer, American Instrument Co. Maryland, USA) on PCA extracted muscle supernatant. The supernatant was neutralized with 2.5 M K_2CO_3 prior to enzymatic ATP and PCr assays described by Bergmeyer (1983). Enzymatic assays (Bergmeyer, 1983) were also employed in analyzing creatine (Cr), Glu, glycogen (Gly), Lac, Pyr, and inosine monophosphate (IMP) in neutralized supernatant and read spectrophotometrically (LKB UltralspecPlus 4053). Total muscle phosphate was determined by the method of Fiske and Subba Row (1925). Total muscle Amm was measured by the glutamate dehydrogenase method as modified by Kun and Kearney (1971).

White muscle coenzyme A (CoA-SH) was determined by a modified radiometric (end-point) method described by Decker in Bergmeyer (1983), which includes two steps: (1)

phosphate acetyltransferase catalyzed acetylation of CoASH to acetyl coenzyme A (acetyl-CoA) with acetylphosphate as substrate, (2) citrate synthase catalyzed [1-^{14}C] citrate formation with [4-^{14}C] oxaloacetate as substrate. The CoA-SH assay therefore measures the sum of CoA-SH and acetyl-CoA. Acetyl-CoA alone was determined by using just the second step of the above method, and the true CoA-SH was obtained as the difference between these two measurements. In both of these assays, due to the instability of [4-^{14}C] oxaloacetate, it was freshly generated before use from L-[4-^{14}C] aspartate.

Acetyl-carnitine ($Carn_a$) was measured by another two-step reaction: (1) carnitine acetyltransferase catalysis of the reaction:

$Carn_a$ + CoA-SH $\rightleftarrows$ carnitine + acetyl-CoA

followed by (2) the radiometric assay of acetyl-CoA as described above (Cederlad *et al.*, 1990). No correction was made for endogenous acetyl-CoA as it was only 1-5% of acetyl carnitine - *ie.* within the variability of step 1. Muscle total carnitine ($Carn_t$) and free carnitine ($Carn_f$) measurement were achieved by using thiol-reagent (N-ethylmaleimide, NEM) to remove CoA-SH from the system to force the readily reversible reaction:

[^{14}C] $Carn_a$ + CoA-SH $\rightleftarrows$ carnitine + [^{14}C] acetyl-CoA

to proceed to completion in the direction right to left (McGarry and Foster in Bergmeyer, 1983). PCA (8%) extract supernatant neutralized with K_2CO_3 was used for the measurement of $Carn_f$. This extract contains $Carn_f$ and short-chain acyl-carnitine ($Carn_s$). Long-chain acyl-carnitines are insoluble in acid and are precipitated with denatured tissue protein; short-chain acyl-carnitines remain in solution but do not participate in the reaction. Therefore the neutralized extract was used to measure $Carn_f$ without further treatment according to the above radiometric method. To determine $Carn_t$ (free plus esterified short-chain), the neutralized PCA extract supernatant was mixed with KOH and incubated at 50°C for 2 h to hydrolyze all carnitine esters, then the carnitine assay described above was used to measure Carn_t (Cederlad *et al.*, 1990). Short-chain acyl-carnitine (Carn_s) level was calculated as the difference between Carn_t and Carn_f. In all these radiometric assays, ^{14}C label was counted by liquid scintillation counting (LKB Wallac 1217 Rackbeta) with an on-board quench correction program.

For the measurement of electrolytes, oven-dried white muscle tissue was digested in HNO_3 (1N) at 40-50° C for 48 h and the supernatant was then analyzed. Both plasma and muscle supernatant K^+, Na^+, Ca^{++}, and Mg^{++} were measured with flame atomic absorption spectrometry (Varian AA-1275), while plasma and muscle Cl^- was determined on a chloride titrator (Radiometer CMT10).

Calculations and Statistical Analysis

The levels of metabolites and electrolytes in ECF were expressed per l of plasma water (C_W). The detailed calculations of white muscle ICFV and ECFV have been described by Munger *et a*l. (1991). Due to the processing methods of this study, ion and metabolite concentrations of muscle were initially expressed per unit dry weight. However the final concentrations in muscle are presented per l of ICFV, incorporating appropriate correction for the amount in trapped ECFV in the muscle. The conversions were performed as follows:

$$Conc.(mmol\ kg^{-1}\ Wet\ Wt = Conc.(mmol\ kg^{-1}\ Dry\ Wt \times [1 - Tis\ H_2O\ (l\ kg^{-1}\ Wet\ Wt] \qquad (1)$$

For those metabolites or electrolytes which were measured in both plasma and muscle, then:

$$Conc.(mmol\ l^{-1}ICF) = \frac{Conc.(mmol\ kg^{-1}WetWt) - Conc.(mmol\ l^{-1}ECF) \times ECFV(l\ kg^{-1}WetWt)}{ICFV(l\ kg^{-1}WetWt)} \qquad (2)$$

For those metabolites which were not measured in plasma (and most likely to be negligible in ECF - *ie*. Gly, PCr, Cr, ATP, and IMP):

$$Conc.(mmol\ l^{-1}\ ICF) = \frac{Conc.(mmol\ kg^{-1}\ Wet\ Wt)}{ICFV\ (l\ kg^{-1}\ Wet\ Wt)} \qquad (3)$$

The "metabolic acid loads" (ΔH_m^+) during the post-exercise period in white muscle ICF and true plasma (ECF) were calculated according to the formula of Milligan and Wood (1986a):

$$\Delta H_m^+ = [HCO_3^-]_1 - [HCO_3^-]_2 - \beta\ (pH_1 - pH_2) \qquad (4)$$

where β, the non-bicarbonate buffer capacity of ECF, was taken as the same as for true plasma described by Wood *et al*. (1982):

$$\beta = -1.271 \times [Haemoglobin] - 2.31 \qquad (5)$$

The β value for rainbow trout white muscle (-73.59 mmol pH^{-1} l^{-1} ICF) was taken from Milligan and Wood (1986b).

The mean cell haemoglobin concentration (MCHC) was calculated as the ratio of Hb (g $100ml^{-1}$) and Hct (ml $100ml^{-1}$).

The membrane potential (E_M) of the white muscle was estimated from the intra- and extracellular concentrations of K^+, Na^+, and Cl^- according to the Goldman-Hodgkin-Katz equation:

$$E_m=\frac{RT}{F}ln\frac{PK^+[K^+]_{ECF}+PNa^+[Na^+]_{ECF}+PCl^-[Cl^-]_{ICF}}{PK^+[K^+]_{ICF}+PNa^+[Na^+]_{ICF}+PCl^-[Cl^-]_{ECF}} \tag{6}$$

where PK^+, PNa^+, and PCl^- are relative permeability coefficients taken from Hodgkin and Horowicz (1959).

In turn, the estimated membrane potential (E_M) was used to predict the distribution ratio of substances such as Amm, H^+, and Lac between intra- and extracellular compartments assuming that the distribution was governed solely by E_M (*ie.* a simple Nernstian distribution). For example for Amm (the sum of NH_4^+ and NH_3):

$$E_M=-\frac{RT}{zF}ln\frac{[NH_4^+]_{ICF}}{[NH_4^+]_{ECF}}=-\frac{RT}{zF}ln\frac{[Amm]_{ICF}-[NH_3]_{ICF}}{[Amm]_{ECF}-[NH_3]_{ECF}} \tag{7}$$

where R, T, z, and F have their usual meaning. For Amm and Lac, it was also possible to use measured pH_i and pH_e to predict the distribution ratio making an alternate assumption, that the distribution was governed solely by the difference in pH between intra- and extracellular compartments (*ie.* a simple distribution according to the laws of weak acids/bases; Jacobs and Stewart, 1936). For example, for Amm:

$$\frac{[Amm]_{ICF}}{[Amm]_{ECF}}=\frac{1+10^{(pK-pH_i)}}{1+10^{(pK-pH_e)}} \tag{8}$$

The measured distribution ratios for Amm, Lac, and H^+ could then be compared with those predicted by equations.

The redox state of the cytoplasmic compartment of white muscle (*ie.* $NAD^+/NADH$ ratio) was estimated from the apparent equilibrium of the lactate dehydrogenase reaction using measurements of intracellular Pyr, Lac, and pH_i (for $[H^+]$):

$$\frac{[NAD^+]}{[NADH]} = \frac{[Pyr]_{ICF}[H^+]_{ICF}}{[Lac]_{ICF}\,K} \qquad (9)$$

where K is the equilibrium constant of lactate dehydrogenase from Williamson *et al.* (1967).

Data are reported as means ± 1 S.E.M.(N). All of the exercise and recovery data were tested against the corresponding resting values by ANOVA followed by *post hoc* comparison by means of Duncan's multiple range and critical range test ($P \leq 0.05$) (Milliken and Johnson, 1984). The tests were performed on Statistica (Statsoft Inc.).

RESULTS

In the present study, exercised fish usually swam vigorously for approximately the first 2 min in response to manual chasing. Thereafter, they usually slowed or stopped for about 30 sec despite continued stimulation. The fish then generally resumed swimming for a further 2-3 min, but at a much lower intensity. By the end of the 6 min exercise period, most fish had stopped swimming entirely and were unable to respond to any stimulation.

Blood gases, extracellular and intracellular acid-base status, and haematology

Changes in blood gas and acid-base parameters were typical of exhaustive exercise. Pa_{O2} exhibited a significant 40% decrease immediately after exercise but returned to resting levels within 1 h (Fig. 3-1A). There were significant decreases in both arterial plasma (ie. extracellular) pH_e and white muscle intracellular pH_i (approximately 0.5 unit) after exercise and the correction of both acidoses was completed within 4 h (Fig. 3-1B). The pH gradient across the muscle cell membrane (0.6 unit) remained approximately constant throughout the 4 h recovery despite the acid-base disturbance. There was a three-fold increase in arterial blood Pa_{CO2} (1.73 to 5.85 torr) immediately after exercise, contributing a large respiratory component to the extracellular acidosis (Fig. 3-1C). Although pH_e was not completely corrected until 4 h, Pa_{CO2} returned to resting level within 1 h. In contrast to the rapid changes in Pa_{CO2}, arterial plasma HCO_3^- exhibited a delayed and relatively prolonged decline indicative of a more slowly developing metabolic acidosis in the extracellular compartment. Extracellular HCO_3^- reached its lowest value (67% resting level) at 1 h and returned to the resting value by 4 h (Fig. 3-1D).

Associated with this post-exercise acidosis was an almost 50% increase in haematocrit (Hct, Fig. 3-2A), a 30% increase in haemoglobin (Hb, Fig. 3-2B), and a 17% decrease in mean cell haemoglobin concentration (MCHC, Fig. 3-2B), the latter indicative of red blood cell swelling. By 4 h, MCHC had returned to resting levels, while Hct and Hb both stayed elevated.

Intracellular and extracellular metabolite status

Exhaustive exercise reduced muscle Gly reserves by more than 90% , from about 14.5 down to 1 mmol.l^{-1} (Fig. 3-3A), and post-exercise recovery was relatively slow. At 2-4 h, white muscle glycogen remained at only 40% of resting levels. White muscle Lac increased to about 35 mmol l^{-1} immediately after exercise, and changes in intracellular Lac almost mirrored those in Gly (Fig. 3-3B). Extracellular Lac increased at a slower rate and to a lesser extent (maximum about 10 mmol l^{-1}) than intracellular Lac after exercise; throughout recovery extracellular Lac remained significantly lower than intracellular Lac (Fig. 3-3B). Taking into account the relative sizes of the white muscle ICFV and the whole animal ECFV (Milligan and Wood, 1986a,b), the increase in ECF Lac by itself could account for no more than 25% of the clearance of Lac from white muscle. This was not the case with Pyr, where total levels were much lower in both compartments (0.1 - 1.5 mmol l^{-1} range; Fig. 3-3C). Both extracellular and intracellular Pyr continued to increase for some time after exercise; from 1-4 h, the extracellular level stabilized, whereas the intracellular declined at this time. In consequence, the ECF/ICF gradient for Pyr was reversed at 2h and 4h. Glu exhibited yet another pattern, with extracellular levels (4-6 mmol l^{-1}) consistently greater than intracellular levels (1-2 mmol l^{-1}; Fig. 3-3D). The amplitude of this gradient remained more or less unchanged throughout recovery despite elevations in both components. Intracellular Glu approximately doubled after exercise and remained significantly elevated until 4h; extracellular Glu was significantly elevated only at 1 h.

Calculation of ICF ΔH_m^+ and ΔLac indicated loads of similar magnitude, though the former was slightly greater from 0.5 through 2 h recovery (Fig. 3-4A). The lower initial ΔH_m^+ (0 h), compared with ΔLac, was in concert with PCr break-down during the same period (Fig. 5A). ΔLac and ΔH_m^+ declined more or less in parallel until 2 h and levelled off thereafter. ECF ΔH_m^+ and ΔLac were much lower than the ICF loads and followed different patterns (Fig. 3-4B). ECF ΔLac always exceeded ΔH_m^+ by at least 50%, except during first 15 min of recovery. Although the ECF metabolic acid load resumed its resting level within 4 h, the ECF Lac load remained elevated for the entire recovery period.

White muscle PCr was about 38 mmol l^{-1} at rest, fell by about 40% immediately after exhaustive exercise, but recovered within 15 min (Fig. 5A). Free Cr was about 24 mmol l^{-1} at rest, increased in mirror image to the decline in PCr, and recovered over a similar time course (Fig. 5). The increase in Cr stoichiometrically matched the decrease in PCr. Tissue total phosphate levels did not change after exercise (Fig. 5).

ATP levels in white muscle were about 7.5 mmol l^{-1} at rest, declined by about 40% immediately after exercise, and remained at a significantly depressed level (75%) for at least the first 2 h of recovery (Fig. 3-6A). Post-exercise reductions in ATP were mirrored by increases in IMP (Fig. 3-6A) and Amm (Fig. 3-6B). These two products of adenylate deamination demonstrated an almost identical pattern of changes, which was a 5-6 mmol/l increase immediately after exercise and a slow recovery which was not complete within 4 h. In parallel to the changes in ICF Amm, there was a 5-fold increase in ECF Amm immediately after exercise, followed by a slow recovery (Fig. 3-6C). However this ECF Amm surge (to about 0.2 mmol l^{-1}) was much lower, on an absolute basis, than the ICF surge (to about 5.5 mmol l^{-1}).

In addition to high energy phosphagens and carbohydrates, there was clear evidence of the use of free fatty acids as a fuel source during exhaustive exercise and recovery (Fig. 3-7). At rest, free carnitine ($Carn_f$) accounted for over 85% of the total carnitine pool ($Carn_t$) of about 3.2 mmol l^{-1} (Fig. 3-7A). Acetyl-carnitine ($Carn_a$) was extremely low (0.02 mmol l^{-1}) and made up only a small fraction of this difference, which is the short chain acyl-carnitine pool ($Carn_s$). $Carn_t$ did not change significantly during exercise or recovery. However $Carn_f$ decreased significantly (-40%) as a result of exhaustive exercise, and remained at this depressed level throughout the 4 h period. In concert with the decrease in $Carn_f$, there were significant increases in both the short-chain acyl-carnitine pool ($Carn_s$) and $Carn_a$ (4-fold and 6-fold, respectively). Both the $Carn_s$ and $Carn_a$ remained elevated throughout the post-exercise period.

In parallel with the constant total pool ($Carn_t$), there was a more or less constant total CoA pool of about 8-10 μmol l^{-1} (not shown). In this pool, about 90% was coenzyme A (CoASH) and 10% was acetyl-CoA at rest (Fig. 3-7B). The acetyl-CoA component doubled immediately after exhaustive exercise and stayed elevated at this level for the remainder of the recovery period. No significant changes in CoASH were observed.

Electrolyte and fluid volume distribution

There were no significant fluid shifts between the ICF of white muscle and ECF associated with exercise or recovery (Table 3-1). White muscle ECFV and ICFV remained at about 70 and 740 ml kg^{-1} wet weight, respectively. Muscle water content fluctuated slightly around 800 ml kg^{-1} wet weight. Plasma protein content (C_{PP}) also stayed unchanged at about 30 g l^{-1}

Despite the constancy of white muscle fluid volumes, a number of significant changes in intracellular electrolytes occurred (Table 3-2). There was a 10 - 15 mmol l^{-1} increase in the major intracellular cation K^+_{ICF} after exercise, which became significant within 15 min and persisted through 2 h; recovery was complete within 4 h. Ca^{2+}_{ICF} and Mg^{2+}_{ICF} also increased by 3 - 5 mmol l^{-1} after exercise; Ca^{2+}_{ICF} resumed its resting level within 30 min, while Mg^{2+}_{ICF} remained elevated throughout the recovery period. Na^+_{ICF} remained unchanged (Table 3-2). In contrast to the cations, Cl^-_{ICF} was not elevated until 2 h into the recovery period when it increased by about 3 mmol l^{-1} and had recovered to its resting level at 4 h (Table 3-2). Lac is included as an anion in Table 3-2 because with pK of 3.75, it exists largely (over 99%) as Lac^- at physiological pH. In the absence of ionic activity measurements, exact charge balance calculations are problematical. For example, most Ca^{2+}_{ICF} likely exists in the bound form, while the ratio of free Mg^{2+} to total is dependent on ATP levels. Nevertheless, it is evident from Table 3-2 that the increases in Lac^- and Cl^- anions were only partially balanced by the increases in K^+ and other cations in the intracellular compartment. Thus the "strong ion difference" (SID; Stewart, 1983) exhibited a sustained decrease after exercise, in accord with the elevation in ICF ΔH_m at this time (Fig. 3-4A).

Extracellular electrolytes also varied significantly during recovery (Table 3-3). Most inorganic ions increased after exhaustive exercise, though the time courses were variable and elevations ranged from about 7% (Na^+_{ECF}, Cl^-_{ECF}) to 100% (K^+_{ECF}, Mg^{2+}_{ECF}); increases in Ca^{2+}_{ECF} were not significant. Thus the major extracellular cation Na^+_{ECF} increased by about 11 mmol l^{-1} within 15 min and stayed elevated through 2 h into recovery. The major anion Cl^-_{ECF} increased by about 8 mmol l^{-1} immediately after exercise, but resting levels were re-established by 0.5 h, and a significant decline (-11 mmol l^{-1}) developed by 4 h. K^+_{ECF} also increased significantly immediately after exercise, but the increase (1 - 2 mmol l^{-1}) was sustained throughout the 4 h recovery period. Increases in Mg^{2+}_{ECF} (up to 0.5 mmol l^{-1}) and inorganic phosphate (Pi_{ECF}; up to 1.0 mmol l^{-1}) generally paralleled the time course of those in K^{+}_{ECF}. As in the intracellular compartment, charge balance calculations are problematical, but the increase in Lac^{-}_{ECF} anion was more or less balanced by the changes in inorganic electrolytes. There was no clear evidence of a decrease in SID to parallel the small ECF ΔH^{+}_{m} (Fig. 3-4B).

DISCUSSION

Blood gases, extracellular and intracellular acid-base status, and haematology

The pattern of blood gas and extracellular acid-base changes (Fig. 3-1) was very comparable to that observed in other studies on rainbow trout where exhaustive exercise was induced by chasing (Turner *et al.*, 1983; Milligan and Wood, 1986a,b, 1987; Nikinmaa and Jensen, 1986; Tang and Boutilier, 1988a,b; McDonald *et al.*, 1989; Wood *et al.*, 1990); similar explanations likely apply. The cause of Pa_{CO_2} elevation which contributes to the short-lived respiratory component of blood acidosis remains controversial (*eg.* Wood, 1991; Randall and Perry, 1992) and will not be dealt with here. The longer lasting metabolic component is generally attributed to a proton discharge into the ECF; the proton originates from lactic acid production and ATP hydrolysis in white muscle (Hochachka and Mommsen, 1983; Wood and Perry, 1985; Milligan and Wood, 1986b). The contribution of protons released from red blood cells may also be significant due to catecholamine induced stimulation of Na^+/H^+ exchange on the RBC membrane (*eg.* Nikinmaa and Jensen, 1986; Wood *et al.*, 1990). The significant decrease in MCHC (Fig. 3-2B) indicates RBC swelling as the result of large net entry of Na^+ and Cl^- followed by water into RBC triggered by Pco_2 and catecholamine elevation (Ferguson and Boutilier, 1989). This clearly contributed to the large increase in Hct (Fig. 3-2A). However the fact that Hb increased indicates that the concentration of circulating RBCs was also elevated. Inasmuch as there was no elevation in plasma protein concentration (C_{pp}) or other evidence of a fluid shift out of the ECFV into the white muscle ICFV in these experiments (Table 3-1), the increase in RBCs was likely the result of mobilization from the spleen (Yamamoto *et al.*, 1980; Pearson and Stevens, 1991).

Fluid and Electrolyte Distribution

In agreement with Milligan and Wood (1986b) and Parkhouse *et al.* (1987) there was no change in white muscle water content after exhaustive exercise (Table 3-1). However, in direct contrast to the results of Milligan and Wood (1986b), there was also no change in fluid volume distribution within the white muscle. Milligan and Wood (1986a,b), using [^{3}H]mannitol as an extracellular fluid marker, reported a 40 ml kg^{-1} shift of fluid out of the ECF into the ICF of white muscle which contributed to an even larger decrease (70 ml kg^{-1}) in whole body ECFV. One possible explanation for this disagreement is the different extracellular fluid marker ([^{3}H]PEG-4000) used in the present study. However, Munger *et al.* (1991) compared these two markers in rainbow trout, and concluded that [^{3}H]PEG-4000 was more conservative - *ie.* less permeant into the ICFV. It does not seem likely that a more permeant marker ([^{3}H]mannitol) would show a relative decrease in ECFV (and reciprocal increase in ICFV) after exercise when a less permeant marker ([^{3}H]PEG-4000) would not. Rather, we believe that the present batch of fish really did not undergo a fluid shift, whereas those of Milligan and Wood (1986a,b) actually did. In support of this view, the present fish exhibited no increase in C_{pp} (Table 3-1), while those of Milligan and Wood (1986b) exhibited a 30-40% increase in C_{pp}. Furthermore, in a preliminary experiment with a different batch of trout at a different time of year, we measured both an increase in C_{pp} (Wang *et al.*, 1994) and a fluid shift from ECFV to ICFV (detected with [^{3}H]PEG-4000) after exhaustive exercise. The reason for this difference is not known, but it may relate to the rather higher intracellular Lac concentrations after exercise in the trout of Milligan and Wood (1986b) and Wang *et al.* (1994) - 44 to 48 mmol l^{-1} *versus* 35 mmol l^{-1} in the present study (Fig 3B). Higher intracellular Lac in the white muscle would provide a greater osmotic gradient for fluid shift.

Changes in extracellular electrolytes after exhaustive exercise (Table 3-3) were qualitatively similar but quantitatively smaller relative to those measured in several previous studies (Turner *et al.*, 1983; Holeton *et al.*, 1983; van Dijk and Wood, 1988). This probably reflects the fact that a shift of fluid into the ICFV of white muscle did not occur in the present study, thereby reducing the extent of "haemoconcentration". Therefore the general increases in plasma electrolytes which did occur must have been due to fluid shifts into RBCs (Fig. 3-2B) and perhaps tissues other than white muscle, as well as ionic movements at the gills (*eg.* increased Na^+ uptake from the water in exchange for H^+; Holeton *et al.*, 1983; Wood, 1988) and perhaps the ICF/ECF boundaries of other tissues. The delayed fall in Cl^-_{ECF} was likely due to Cl^- losses across the gills (Holeton *et al.*, 1983; Wood, 1988) consequent to decreased Cl^-/HCO_3^- exchange for acid-base regulation.

As fluid shifts with white muscle did not occur in the present study, these results provide no support for the model calculations of Wood (1991) as to ionic redistributions between extracellular fluid and the intracellular compartment of white muscle. Indeed, rather than the loss of K^+_{ICF} predicted by Wood (1991), there was a significant increase (Table 3-2), and rather than the predicted elevation of Na^+_{ICF} and Cl^-_{ICF}, there was no change in the former and a delayed increase in the latter significant at only one sample time (Table 3-2). The rise in white muscle K^+_{ICF} (Table 3-2) at a time when K_{ECF} was elevated (Table 3-3) and branchial and renal K^+ losses were all also likely higher (Wood, 1988) was particularly intriguing. Parkhouse *et al.* (1987) reported similar increases in both plasma and muscle K^+ after exhaustion in trout. In contrast to earlier suggestions (Turner *et al.*, 1983; Wood, 1991), the white muscle must be a "sink" rather than a "source" for mobilized K^+ after exercise. Gill tissue (Wood and LeMoigne, 1991) and adrenergically stimulated and swollen red blood cells (Borgese *et al.*, 1987) remain likely "sources".

Measurements of intra- and extracellular Na^+, K^+, and Cl^- allowed estimation of the membrane potential (E_m) according to the Goldman-Hodgkin-Katz equation with relative permeabilities taken from frog white muscle (Hodgkin and Horowicz, 1959; Table 3-4). The resulting estimates were close to measured values in teleost white muscle (Hidaka and Toida, 1969; Yamamoto, 1972). Interestingly, if K^+ distribution alone were employed, more negative values (by approximately 20 mV) would result, while Cl^- distribution alone would yield values almost identical to those tabulated in Table 3-4. These data indicate that the muscle was not depolarized immediately after exercise contrary to earlier prediction (Wood, 1991), and may even have become slightly hyperpolarized, although none of the changes were statistically significant (Table 3-4).

'Metabolic Acid' and Lactate Dynamics

Observed changes in pH_i and pH_e after exhaustive exercise (Fig. 3-1B) were in very good agreement with other similar studies, as were the much larger ΔH^+_m and ΔLac loads in the intracellular fluid of white muscle relative to the extracellular blood plasma (Fig. 3-4; Turner *et al.*, 1983; Milligan and Wood, 1986b; Wright and Wood, 1988; Tang and Boutilier, 1991; Tang *et al.*, 1992; Schulte *et al.*, 1992). There is now a large body of evidence (reviewed by Wood, 1991; Schulte *et al.*, 1992; Milligan and Girard, 1993) that the majority of "lactic acid" produced by salmonids during strenuous exercise never leaves the white muscle, but rather is removed by metabolism *in situ*. Many questions remain, principal amongst which are: (i) what are the mechanism(s) of retention and why do the small amounts of H^+_m and Lac released apparently differ - *ie.* different patterns of ΔH^+_m and ΔLac in the

ECF (*eg*. Fig. 3-4); (ii) what are the relative fates of the metabolized "lactic acid" - *ie.* oxidation or glycogen resynthesis; and (iii) in the latter case, what are the enzymatic pathways by which this could occur. This last question awaits resolution to the problem of how the pyruvate kinase step of glycolysis can be reversed (Moyes *et al.*, 1992) but the present results do cast some light on the first two questions.

With respect to the retention and differential release of H^+_m and Lac, it is likely that the mechanisms governing the two ions are entirely different. The calculations of Table 3-4 demonstrate that even at rest, both H^+ and Lac are distributed well out of electrochemical equilibrium with the white muscle membrane potential (E_m), and that the deviations are in opposite directions. Thus the measured H^+ distribution ratio (H^+_{ICF}/H^+_{ECF}) is far lower (*ie.* pH_i higher) and the measured Lac distribution ratio (Lac_{ICF}/Lac_{ICF}) far higher than predicted by the resting E_m; in both cases, distribution of these ions must be maintained by the expenditure of metabolic energy. While it is theoretically possible that Lac might passively distribute according to the pH_i - pH_e gradient rather than E_m (*ie.* as a "weak acid"; Jacobs and Stewart, 1936), Table 3-4 illustrates that this is not the case. The measured Lac distribution ratio at rest was again far different from that predicted by the measured pH_i - pH_e gradient.

After exercise, the measured H^+ distribution ratio did not change substantially and remained far lower than predicted by the E_m (Table 3-4). There is therefore no need to postulate a retention mechanism for H^+; on the contrary, H^+ ions must be actively extruded from white muscle, just as at rest, though the turnover rates are likely higher because of the higher absolute H^+ concentrations in both ICF and ECF. It is possible that this extrusion mechanism may start to fail at very low pH_e. The observed stabilization of ΔH^+_m at a lower value than ΔLac in the extracellular compartment (Fig. 3-4B) may reflect this phenomenon.

There is also a temporary "storage" of excess H^+_m (but not Lac) in the external water via the acid-base regulatory mechanisms of the gills (Holeton *et al.*, 1983; Milligan and Wood, 1986a, Wood, 1988). The situation is very different for Lac. The measured Lac distribution ratio increased significantly after exercise, thereby deviating to an even greater extent from that predicted by either E_m or the pH gradient (Table 3-4). The conclusion is that Lac must be actively retained, and the extent of this active retention must become even greater after exhaustive exercise, especially since the passive rate of Lac "leakage" is reported to increase up to 9-fold at this time (Milligan and McDonald, 1988). Maintenance of high Lac_{ICF} may be particularly important to drive the process of glycogen resynthesis (see below). Like the present study, Batty and Wardle (1979), Turner and Wood (1983), and Milligan and Girard (1993) have all provided circumstantial evidence for such an inward Lac "pump" in trout white muscle, but proof of its existence remains elusive.

The high levels of Lac_{ICF} (Fig. 3-3B) may be contrasted with the low levels Glu_{ICF} which remained far lower than even Glu_{ECF} after exercise (Fig. 3-3D). Glu appears to play a very minimal role in both Lac production in muscle during exercise and in Gly resynthesis during the recovery (Batty and Wardle, 1979; Pagnotta and Milligan, 1991). Indeed functional hepatectomy, which presumably would block Glu mobilization from the liver did not affect exercise performance or muscle Lac accumulation, and in fact tended to accelerate its metabolic removal during recovery (Pagnotta and Milligan, 1991).

With respect to the disposition of Lac retained within the muscle, the present results suggest that Gly resynthesis is quantitatively more important than oxidation as the major fate (Fig. 3-3). This finding agrees well with some recent studies on exhaustively exercised trout (Schulte *et al.*, 1992; Milligan and Girard, 1993) but not others (Milligan and Wood, 1986b;

Pearson *et al.*, 1990). Glycogenesis *in situ* is therefore an important fate, though not necessarily the sole fate, of retained Lac (Wood, 1991; Schulte *et al.*, 1992; Milligan and Girard, 1993). It is now apparent that both the extent and time course of Gly repletion relative to Lac clearance are altered by factors such as training (Pearson *et al.*, 1990; Scarabello *et al.*, 1992), the initial Gly store, and the extent of its depletion by aerobic and anaerobic metabolism during exhaustive exercise (Pearson *et al.*, 1990; Scarabello *et al.*, 1991a,b). While some oxidation of Lac may also occur to supply ATP for the various components of "excess post-exercise O_2 consumption" (EPOC; Scarabello *et al.*, 1991a), trout white muscle seems to be designed to "spare" as much Lac and Pyr as possible for this Gly resynthesis. Confirmation of this hypothesis must await direct measurements of relative Lac flux rates through the oxidative and Gly resynthesis pathways.

Ammonia Dynamics

The present results also illuminate the distribution of Amm in white muscle. Wright *et al.* (1988), Wright and Wood (1988), and Tang *et al.* (1992) have all concluded that Amm distribution is dictated largely by the membrane potential (E_m) in fish muscle. Heisler (1990), on theoretical grounds, has argued that such a mechanism would be too costly as it would create an additional inward H^+ shuttle to tax the active H^+ extrusion mechanisms of the cell. Instead, Heisler argues that Amm should distribute according to the pH_i - pH_e gradient - *ie.* as a typical "weak base", a pattern seen in many higher vertebrates. Table 3-4 compares the measured distribution ratio (Amm_{ICF}/Amm_{ECF}) with that predicted by the pH gradient and by E_m respectively. In resting fish, Amm levels were low in both plasma and muscle (Fig. 3-6B), and the measured distribution ratio (about 9.5) was only slightly and non-significantly higher than predicted by the pH gradient (6.0). However, after exhaustive exercise and throughout recovery, both intracellular and extracellular concentrations were elevated, undoubtedly due to the deamination of adenylates during anaerobic activity (Mommsen and Hochachka, 1988). This is well illustrated by decreased ATP and stoichiometrically increased levels of IMP and Amm in muscle, the two end products of the AMP deaminase reaction (Fig. 3-6). The Amm_{ICF} increased to a much greater extent than Amm_{ECF} such that the measured distribution ratio (18.7-32.7) became much closer to that predicted by E_m (22.6 - 45.9) and significantly greater than that predicted by the pH gradient (5.0 - 8.5; Table 3-4).

These results lead to several conclusions. Firstly, there is no need to postulate any active retention mechanism for Amm comparable to that for Lac, for at all times the measured Amm distribution ratio fell in between the limits set by pH-dictated and E_m-dictated distributions. As first pointed out by Boron and Roos (1976), any ratio between these bounds can occur passively, simply depending on the relative permeabilities of NH_3 (favours a pH-dictated ratio) and NH_4^+ (favours a E_m-dictated ratio). Secondly, while the situation postulated by Heisler (1990) may apply in truly resting animals, E_m appears to dictate the passive distribution after exercise, suggesting that fish cell membranes have a significant NH_4^+ permeability. This raises the intriguing possibility that exercise-induced changes (*eg.* acidosis) may in some way alter the relative NH_3/NH_4^+ permeability of the cell membranes. However, it should be noted that previous experimental studies found an E_m-dictated distribution at rest as well as after exercise (Wright *et al.*, 1988; Wright and Wood, 1988; Tang *et al.*, 1992), and resting ratios are subject to the greatest error due to the very low plasma Amm concentrations. Thirdly, it is apparent that an E_m-dictated distribution ratio will ensure a more efficient passive retention of Amm_{ICF} for greater intracellular buffering (Dobson and Hochachka, 1987; Mommsen and Hochachka, 1988) and for stoichiometric balance with IMP for ATP resynthesis (Fig. 3-6). IMP is not lost across the cell membrane (Meyer *et al.*, 1980) so it would seem adaptive to retain Amm in similar concentration. At a pH-dictated ratio of 6.0, rather than an E_m-dictated ratio of ~30, much more Amm would have been lost to the blood and subsequently across the gills.

The Redox State of White muscle

The Pyr/Lac ratio is commonly employed to calculate the redox state of a tissue; Table 3-5 demonstrates that in the intracellular fluid of white muscle, Pyr/Lac fell by about 50% after exhaustive exercise, in agreement with many previous studies (*eg*. Turner *et al.*, 1983; Pearson *et al.*, 1990; Schulte *et al.*, 1992). At first glance, this would suggest a shift to a more reduced state throughout this period. However, the true redox state of the cytoplasm is represented by the NAD^+/NADH ratio which is determined not only by Pyr and Lac, but also by the free H^+ concentration - see equation (9). When the increase in intracellular [H^+] (*ie*. decline in pH_i) was additionally factored into the calculation, there was no significant change in the redox state after exercise and during recovery. This result suggests that the cytoplasmic compartment of white muscle remained well oxygenated, and challenges the traditional view of the "anaerobic" production of Lac. Indeed, it is in agreement with the emerging and controversial view in mammalian physiology that limitation of O_2 supply is not a determinant of Lac production during intense muscular exercise (*eg*. Stainsby *et al.*, 1989). However, an important *caveat*, first pointed out by Williamson *et al.* (1967), must be noted: the Pyr - Lac system is representative only of the redox state of the cytoplasmic compartment, and may be uncoupled from that of the mitochondria.

Fuel Shifting between High Energy Phosphogens and Carbohydrates

It is generally believed that PCr should be depleted prior to ATP and Gly depletion because of the high affinity of creatine kinase for ADP, and its high activity. Once PCr is depleted, ATP should start to fall, and depressed ATP along with increasing Amm, Pi, ADP, and AMP should activate glycolysis (*ie.* phosphofructokinase) despite an inhibition caused by increasing H^+ loading in the sarcoplasm (Dobson and Hochachka, 1987; Mommsen and Hochachka, 1988; Parkhouse *et al.*, 1988). Unfortunately there are no *in vivo* studies in fish during this brief period of dynamic change to confirm this scenario. Most studies, like the present, document the situation only once exhaustion has occurred and then follow the much slower changes which occur during recovery. In the present study, both PCr and ATP were reduced by only about 40% at the end of exercise (Fig. 3-5, Fig. 3-6A), yet Gly reserves were more than 90% reduced by equivalent conversion to Lac (Figs. 3A,B), so glycolysis was fully activated.

The depressed PCr recovered rather quickly (within 15 min) after exercise (Fig. 3-5), whereas ATP remained depressed for at least 2h (Fig. 3-6A), and the slowly recovering Gly was still depressed by 60% at 4h (Fig. 3-3A). This differential time course agrees with most but not all previous studies (reviewed by Scarabello *et al.*, 1991a). Difficulties in preserving true *in vivo* PCr levels during sampling and analysis may explain some discrepant studies; in the present investigation, resting PCr concentrations were the highest ever reported for trout muscle (reviewed by Wang *et al.*, 1994). In concert with changes in PCr were stoichiometric changes in Cr level which validated the PCr measurement (Fig. 3-3). The total creatine pool (about 70 mmol l^{-1} ICF) was comparable to that demonstrated in other similar studies (Dunn and Hochachka, 1986; Dobson *et al.*, 1987; Pearson *et al.*, 1990; Schulte *et al.*, 1992), but the percentage of phosphorylation (65%) was higher in the present study. This higher PCr/Cr ratio was probably due to a better preservation of PCr with improved sample handling (Wang *et al.*, 1994).

Neither PCr nor ATP were depleted to the extents reported in many previous studies (Milligan and Wood, 1986b; Dobson *et al.*, 1987; Dobson and Hochachka, 1987; Pearson *et al.*, 1990; Schulte *et al.*, 1992). In addition to improved phosphagen preservation during sampling and analysis, two other explanations may be involved: i) PCr may already have partially recovered (to 60% resting level) from a larger depletion immediately at the end of exercise because blood sampling and anaesthesia took 3-5 min to complete before the actual muscle sampling; ii) the fish may not have exercised as intensively as in some previous studies. Both explanations would be in accord with the unchanged redox state of white muscle after exercise (Table 3-5). Nevertheless, activation of glycolysis clearly occurred, and stoichiometric increases in Amm and IMP accompanied the significant depletion of ATP (Fig. 3-6).

The Role of Free Fatty Acid Metabolism

The traditional scheme of fuel utilization for exhaustive activity and recovery in white muscle does not include lipid, which is normally considered a fuel for long term sustainable "aerobic" activity (see Introduction). However, the results of Fig. 3-7 together with the "aerobic" redox state of the tissue (Table 3-5) argue strongly for an important contribution by FFA, especially in combination with other recent data. Dobson and Hochachka (1987) reported a 35% decrease in plasma FFA and doubled level of plasma glycerol in exhaustively exercised trout. Moyes *et al.* (1989) reported that white muscle mitochondria of another teleost, the carp, are capable of oxidizing fatty acyl-carnitines. Milligan and Girard (1993) reported large, highly variable decreases in white muscle total lipid which persisted through 6h of recovery.

Lipid is stored as triglyceride (TG); lipolysis of TG forms glycerol and FFA. Glycerol may be phosphorylated and oxidized to form glyceraldehyde-3-phosphate which can enter the glycolytic pathway. However FFA's, especially the long chain forms (C_{10}-C_{18}), are not very permeable through the inner mitochondrial membranes. In general, FFA's are transported in combination with carnitine, as acyl-carnitines, before undergoing β-oxidation. On the inner mitochondrial membrane, carnitine is regenerated and activation by CoA-SH occurs. Fatty acyl-CoA's then enter β-oxidation to produce acetyl-CoA as a substrate to fuel the Krebs cycle. Each shortening of acyl-CoA by 2 carbons via β-oxidation generates 17 ATP (Hochachka and Somero, 1984).

In the present study, I elected to measure indirect indices of FFA metabolism rather than changes in absolute TG levels in muscle for several reasons. Firstly, Milligan and Girard (1993) were unsuccessful in demonstrating significant changes in absolute levels because of great variability. Secondly, the observations of Dobson and Hochachka (1987) suggest that much of the FFA usage would represent increased uptake from the blood, rather than necessarily a net depletion of endogenous TG stores. Indeed, in mammals, intramuscular TG is a dynamic pool, and the combination of endogenous (intramuscular TG stores) and exogenous lipid (plasma FFAs and TG) makes up the total fat fuel source for muscle (Oscai *et al.*, 1990). Aerobic exercise enhances the uptake of plasma FFAs and TG by a great margin, while a high percentage of FFAs (70-90%) entering muscle are first esterified to TG in the sarcoplasmic reticulum.

Total carnitine ($Carn_t$) measured in the present study comprises short chain acyl-carnitines ($Carn_s$) and free carnitine $Carn_f$, but not long-chain acyl-carnitines ($Carn_l$). The well conserved $Carn_t$ (Fig. 3-7A) was expected since carnitine serves only as a vehicle to shift FFA into the mitochondrial matrix. However exercise caused a sharp 40% fall in $Carn_f$ which was sustained throughout recovery. There was a significant rise in $Carn_s$ which was greatest at 0.5 h but sustained throughout the recovery period (Fig. 3-7A). Although $Carn_l$ was not directly measured in this study, the decrease in $Carn_f$ should be equivalent to the combined increase of $Carn_s$ and $Carn_l$. At most times post-exercise (except 0.5h), the elevation in $Carn_s$ was substantially less than the decrease in $Carn_f$ suggesting that both long chain ($Carn_l$) and short chain acyl-carnitines ($Carn_s$), were elevated (Fig. 3-7A). These increases in acyl-carnitines point to the utilization of FFA's as an oxidative fuel source for ATP generation during both exhaustive exercise and post-exercise recovery. The elevated acetyl-CoA levels observed throughout the post-exercise period (Fig. 3-7B) reinforce this conclusion since the β-oxidation spiral will generate acetyl-CoA. Acetyl-carnitine ($Carn_a$), the shortest of the short chain acyl-carnitines, increased dramatically after exercise (Fig. 3-7B), but quantitatively accounted for less than 20% of the absolute elevation in $Carn_s$.

Although there was excessive Lac accumulation and a relatively high redox state in the ICF, both of which would favour Lac oxidation, the low activity of PDH in fish white muscle likely resulted in a low flux from Pyr to acetyl-CoA. It has also been reported in mammals that an increase in the availability of FFA actually depresses carbohydrate utilization (glucose uptake, glycolysis, and glycogenolysis; Neely and Morgan, 1974; Randle *et al.*, 1976; Rennie *et al.*, 1976). FFA β-oxidation, therefore, probably plays a major role in the high rate of acetyl-CoA production. While the free carnitine pool is always high compared with CoASH in white muscle, carnitine acts as a receptor for acetyl groups to form acetyl-carnitine and to release CoASH (see Materials and Methods). The advantages of carnitine acting as an acetyl unit buffer are two-fold: i) maintaining the available CoASH pool at certain level which is essential for FFA to form acyl-carnitine, then being transported across MT membrane; ii) keeping available CoASH at a sufficient level in order to carry on the oxidation from oxaloacetate to succinate to prevent acetyl-CoA accumulation due to lack of oxaloacetate. The relatively stable CoASH pool throughout the entire time course also supports the above theory. In mammals, with acetyl-carnitine taking up to 85% of the total carnitine after exercise, acetyl-carnitine can also act as a reservoir for Pyr. This acetate sink provides a temporary store which can shunt acetate groups back to acetyl-CoA in a very quick fashion and re-enter the Krebs cycle just like glycerol-3-phosphate (Pearson and Tubbs, 1967; Harris *et al.*, 1987). However this is unlikely in the case of trout white muscle, where Carn_a was less than 10% of the carnitine pool.

The two times daily injection of heparin may well have elevated the basal level of available FFA in plasma. Lipoprotein lipase (LPL) attached onto the luminal surface of endothelial cells of capillaries can be released by heparin administration (Rennie *et al.*, 1976), and the elevated plasma LPL may enhance the hydrolysis of plasma TG and therefore increase FFA uptake (Scow and Blanchette-Mackie, 1985). Despite this possible complication introduced by heparin administration, there was still a clear trend of enhanced FFA utilization in exercised fish in comparison with resting fish in the present study (Fig. 3-7). In mammals, the exercise-induced FFA utilization is facilitated by hormone-sensitive lipase (HSL) and the epinephrine sensitive fraction of LPL in muscle intracellular fluid. The activation of these two enzymes is believed to function as a coordinated unit in providing both endogenous and exogenous FFAs for β-oxidation and is regulated through a cAMP cascade (Oscai *et al.*, 1982).

The present results provide clear evidence of FFA utilization in white muscle of trout during both exercise and recovery. The use of lipid as an energy source under both circumstances is documented in humans (Essen, 1978; McCartney *et al.*, 1986; Bangsbo *et al.*, 1991), and the same now appears true in fish. While the mechanisms of FFA utilization (outlined above) are presumably similar during exercise and recovery, the functions of the fuel supplies (as ATP) are probably different. During exercise, the ATP likely helps power the contracting muscle, while during recovery. the ATP should be directed towards restorative processes. In light of Lac conservation for Gly resynthesis, lipid oxidation may be particularly important in providing ATP for the processes of EPOC, which include GLY resynthesis (Scarabello *et al.*, 1991a). In this regard, it is noteworthy that Moyes *et al.* (1992) reported that physiological levels of FFA's inhibit the oxidation of Pyr by white muscle mitochondria of trout *in vitro* via allosteric inhibition of pyruvate dehydrogenase. This would provide a mechanism by which FFA oxidation could "spare" Pyr and Lac, while providing the ATP needed for Gly resynthesis. A recent study in human muscle (Bangsbo *et al.*, 1991) also demonstrated that FFA's are the major substrate oxidized during recovery from high intensity exercise, despite the availability of high levels of Lac. However at this stage of present study, it is difficult to partition the FFA pool utilized for muscle activity and Gly replenishment. There is a clear need for further studies on lipid utilization in fish in relation to exhaustive exercise.

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Figure 3-1.

Changes in (A) arterial Pa_{O2}, (B) arterial plasma pH_e and white muscle intracellular pH_i, (C) Pa_{CO2}, and (D) HCO_3^- prior to and after exhaustive exercise in the rainbow trout. Bar indicates 6 min. of exercise, 0 immediately after exercise. Dashed line represents the mean of resting value. N = 8 at rest, 10 at 0 h, 11 at 0.25 h, 9 at 0.5 h, 13 at 1 h, 11 at 2 h, and 8 at 4 h. * indicates a significant ($P < 0.05$) difference from corresponding resting value. Values are means ± S.E.M.

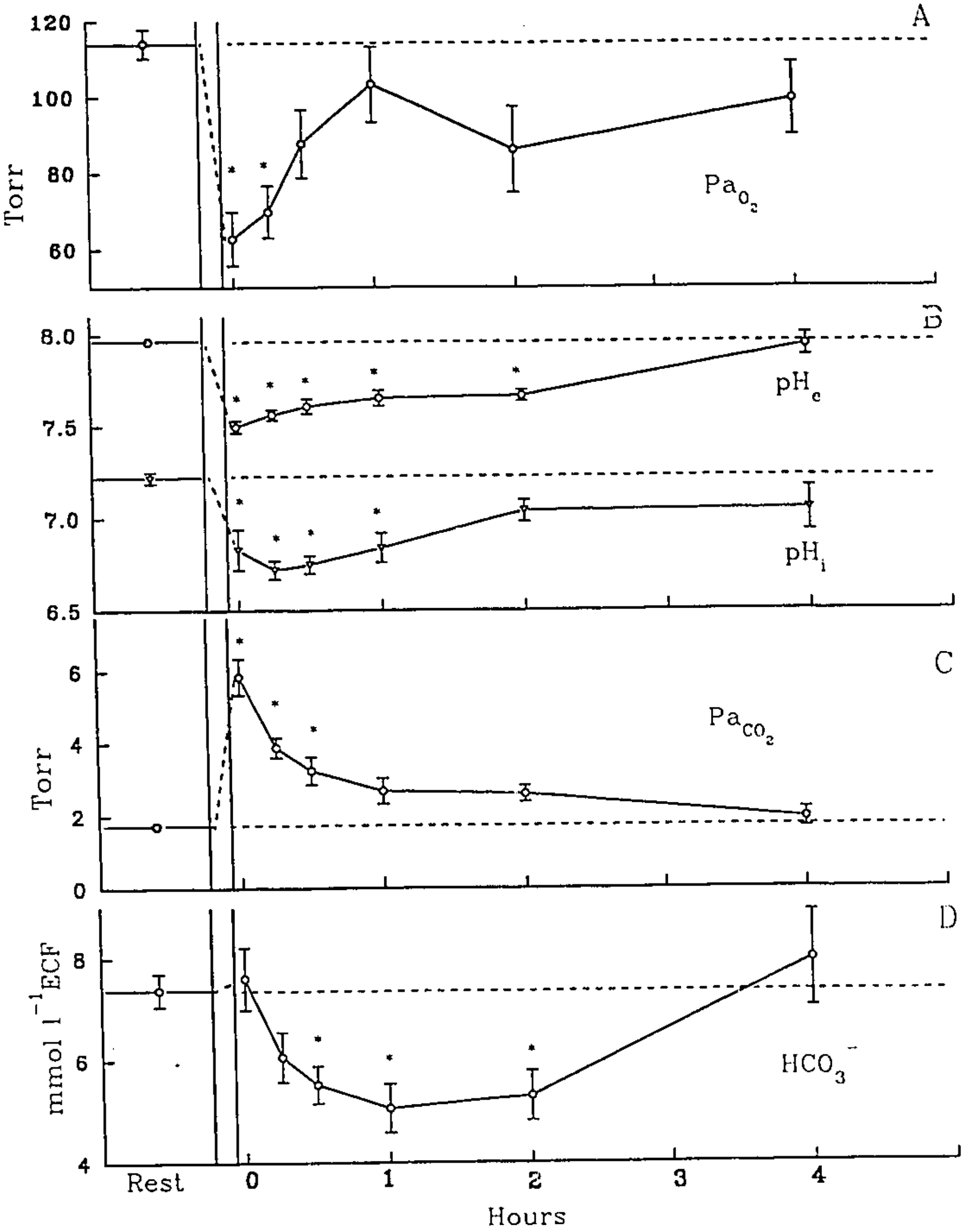

A
120
100
80
60
Torr
Pa_{O_2}
*
B
8.0
7.5
7.0
6.5
pH_e
pH_i
C
6
4
2
0
Torr
Pa_{CO_2}
D
8
6
4
mmol l^{-1} ECF
HCO_3^-
Rest
0
1
2
3
4
Hours

Figure 3-2.

Changes in (A) arterial blood haematocrit (Hct) and haemoglobin (Hb), and (B) mean cell haemoglobin concentration (MCHC) prior to and following exhaustive exercise. Other details as in the legend of Fig. 3-1.

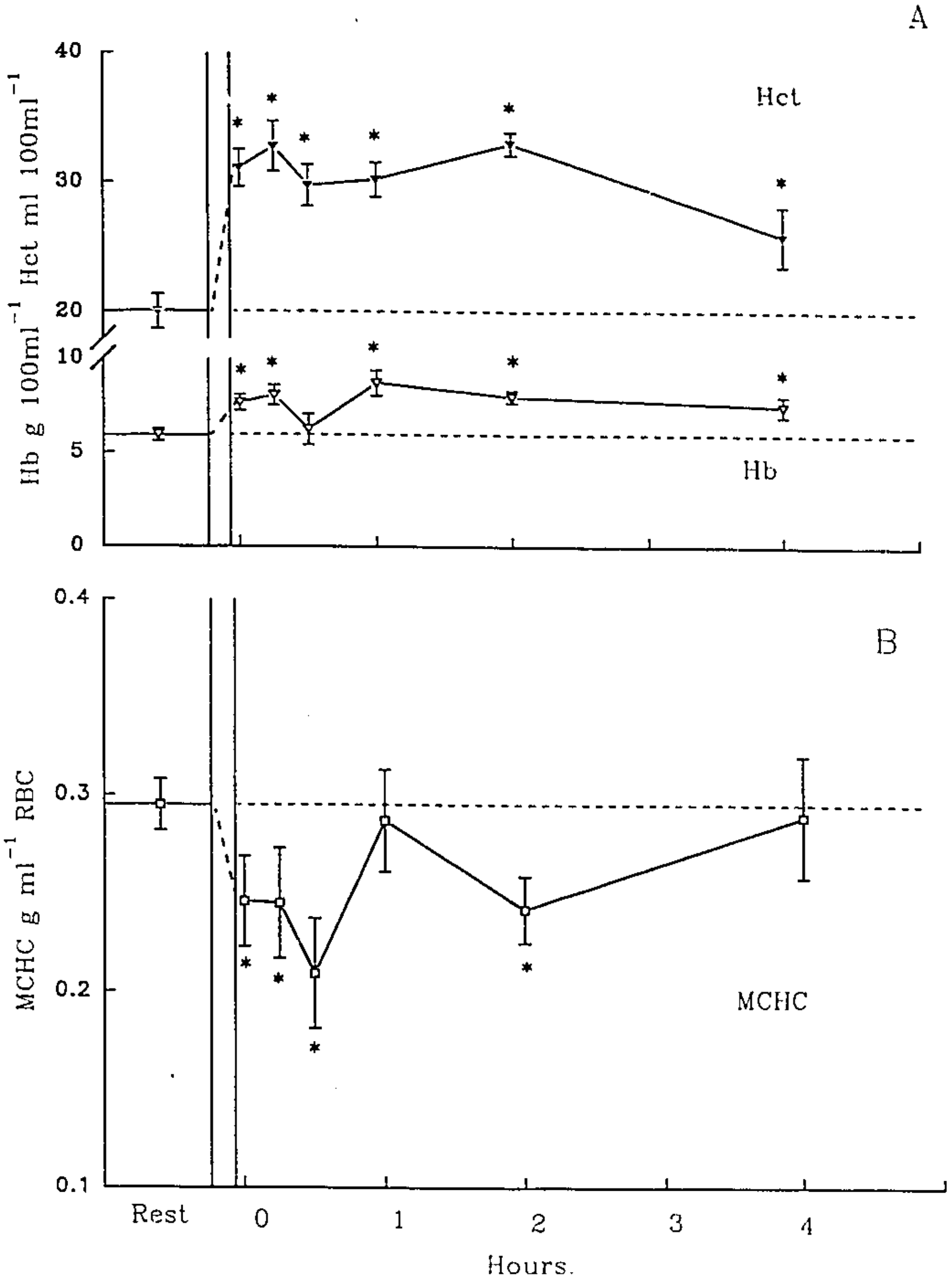

A
Hct
Hb
Hb g 100ml⁻¹ Hct ml 100ml⁻¹
40
30
20
10
5
0
B
MCHC
MCHC g ml⁻¹ RBC
0.4
0.3
0.2
0.1
Rest
0
1
2
3
4
Hours.
*

Figure 3-3.

Changes in concentration of (A) white muscle intracellular glycogen (Gly), (B) intra- and extracellular lactate (Lac), (C) pyruvate (Pyr), and (D) glucose (Glu) prior to and following exhaustive exercise. Values are means ± S.E.M. in mmol l^{-1} ICF or ECF. Other details as in the legend of Fig. 3-1.

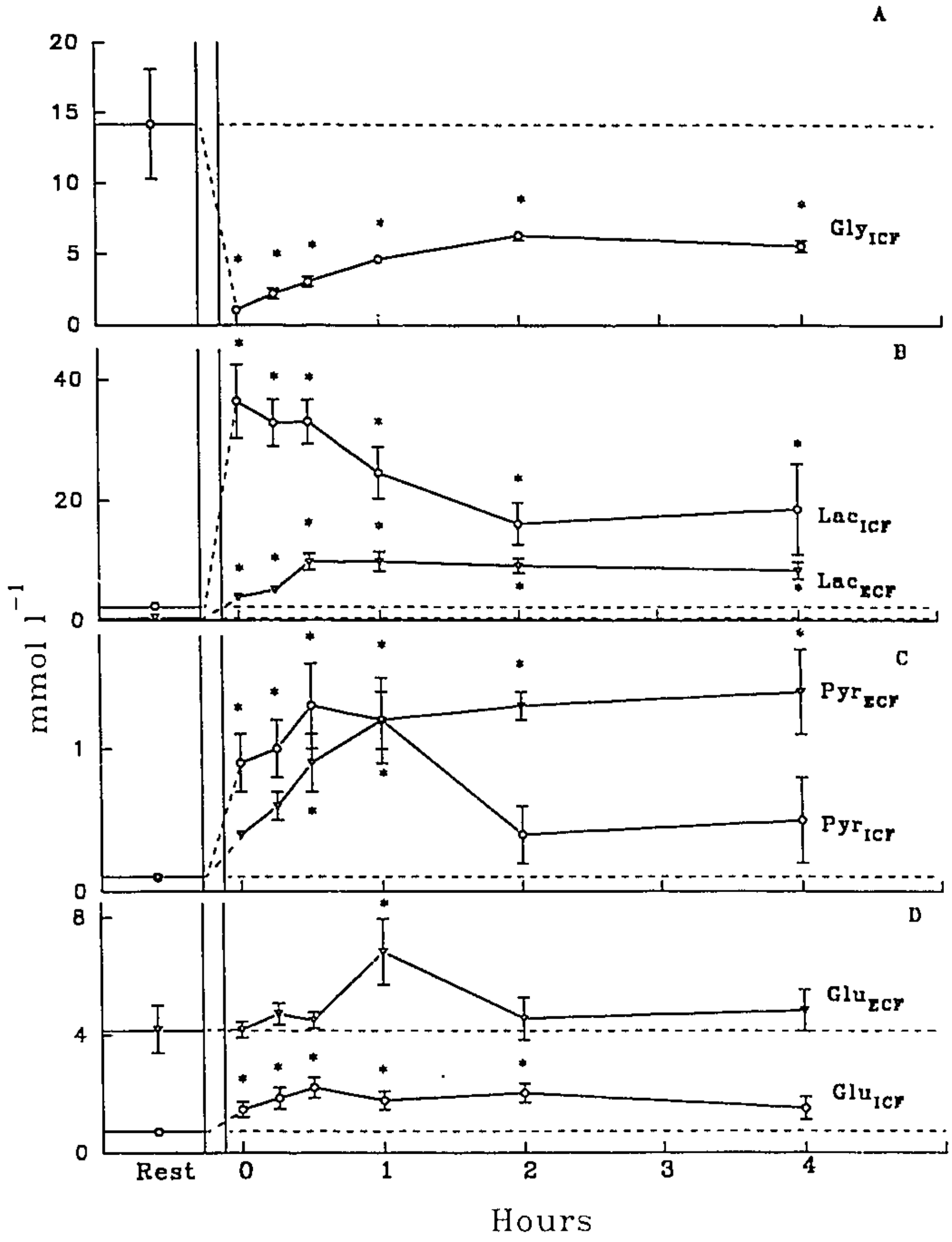

A
B
C
D
20
15
10
5
0
40
20
0
1
0
8
4
0
mmol l^{-1}
Gly$_{ICF}$
Lac$_{ICF}$
Lac$_{ECF}$
Pyr$_{ECF}$
Pyr$_{ICF}$
Glu$_{ECF}$
Glu$_{ICF}$
Rest
0
1
2
3
4
Hours

Figure 3-4.

(A) White muscle ICF and (B) ECF lactate (ΔLac) and metabolic acid (ΔH_m^+) loads after exercise. ΔLac and ΔH_m^+ are zero, by definition, at rest. See text for detailed calculation. Values are calculated from mean at each time interval. Bar indicates exercise period.

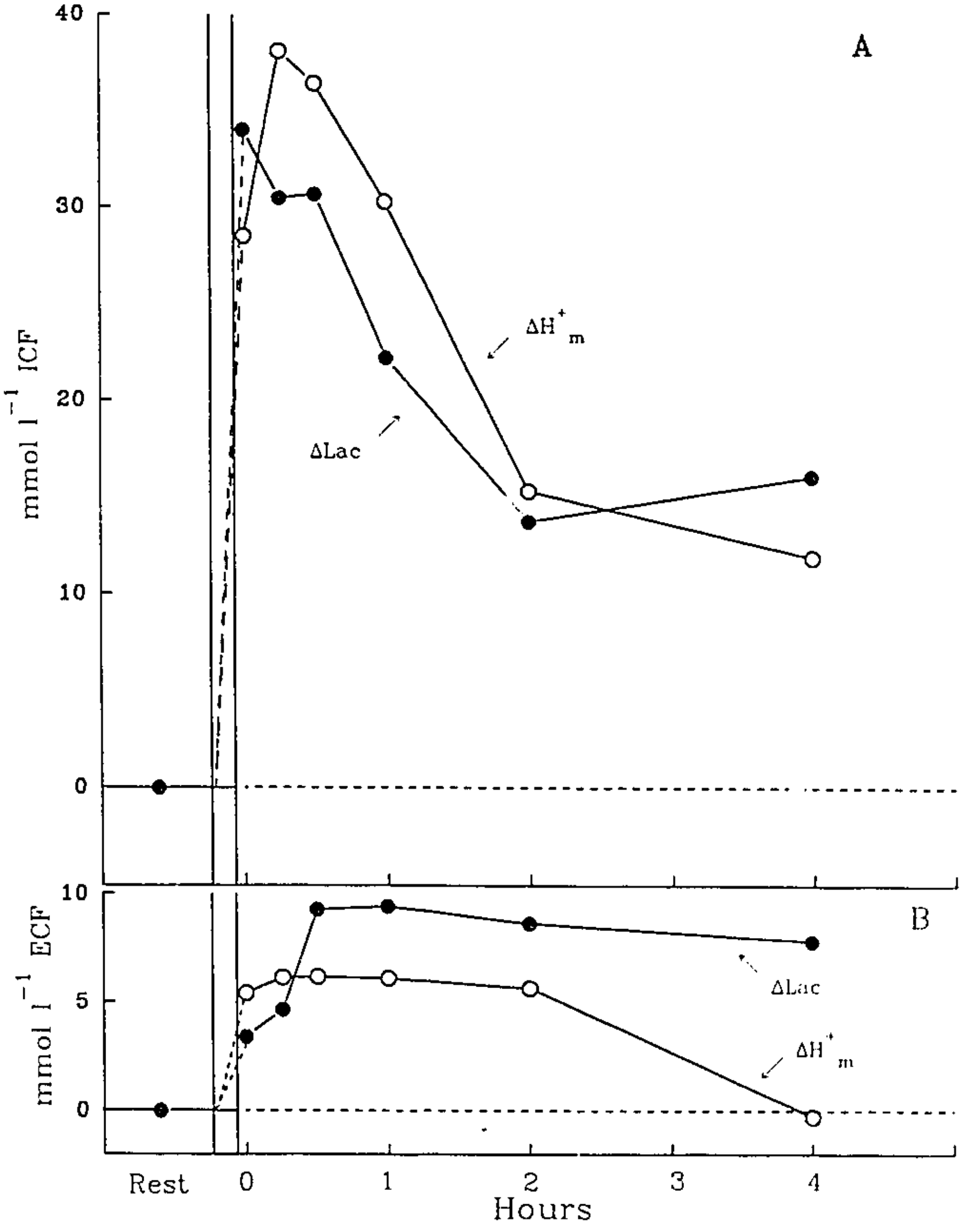
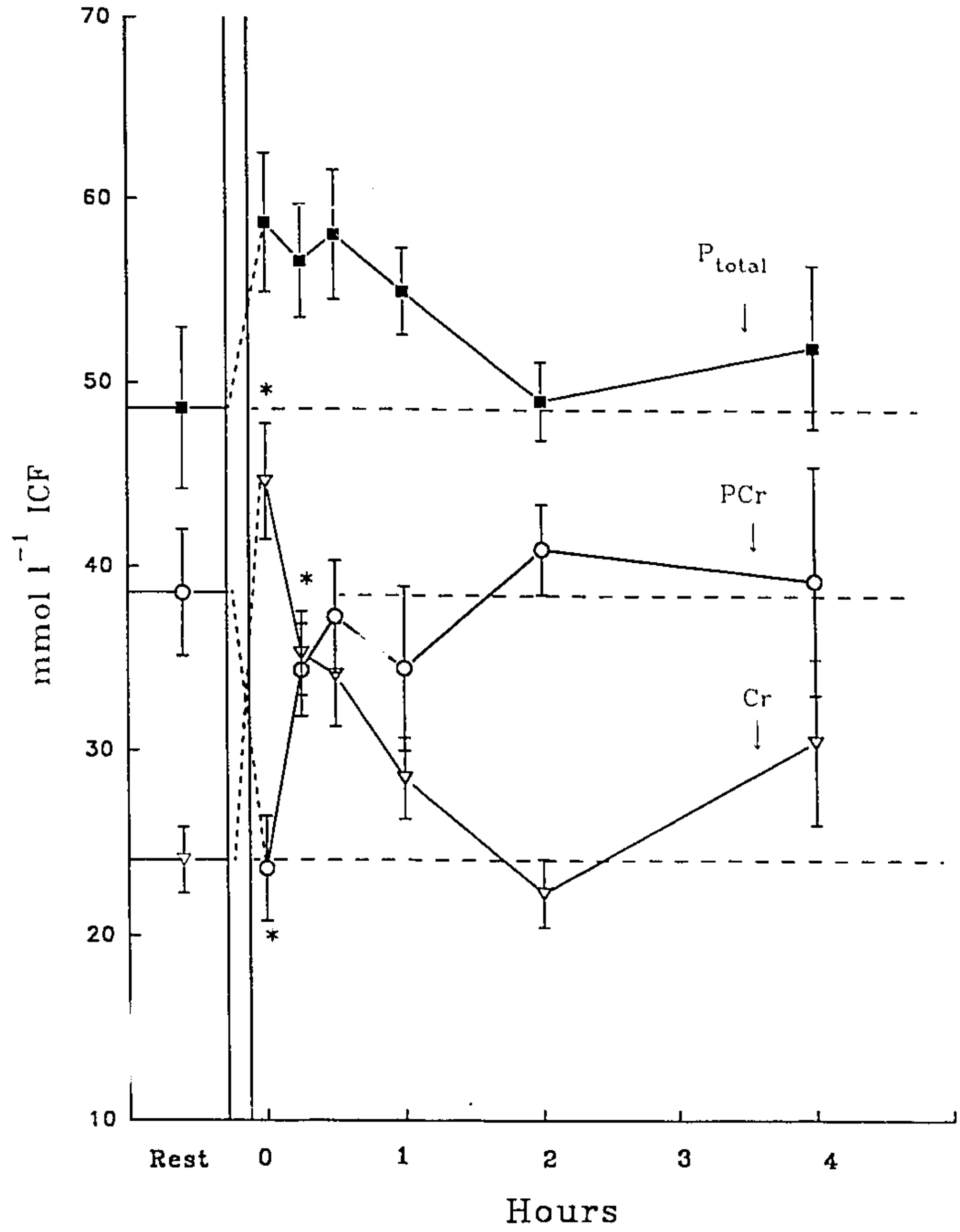

A
B
mmol l^{-1} ICF
mmol l^{-1} ECF
ΔH$^{+}_{m}$
ΔLac
ΔLac
ΔH$^{+}_{m}$
40
30
20
10
0
10
5
0
Rest
0
1
2
3
4
Hours

Figure 3-5.

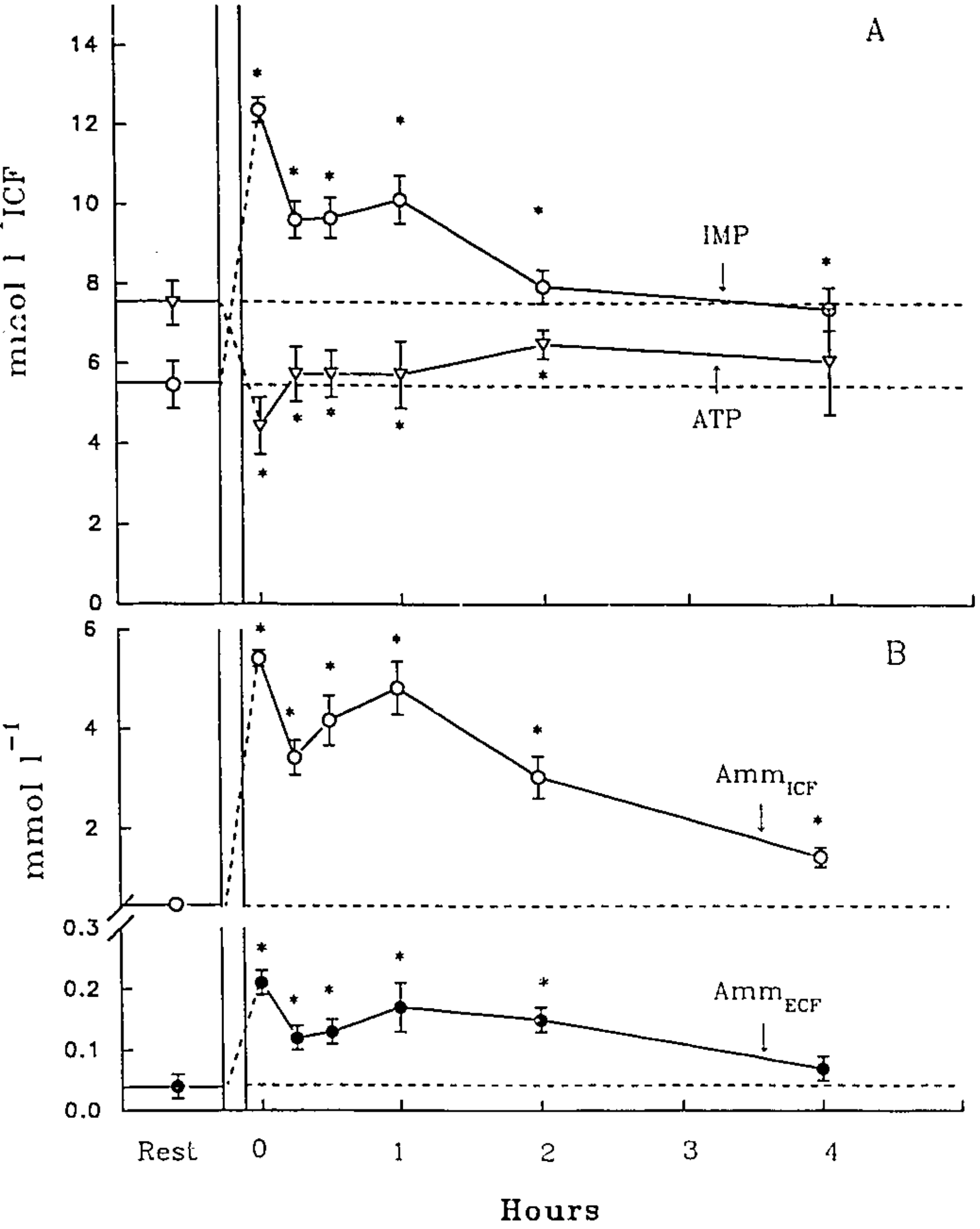
Changes in white muscle intracellular total phosphate (P_{total}), creatine phosphate (PCr), and creatine (Cr) prior to and following exhaustive exercise. Other details as in the legend of Fig. 3-1.



70
60
50
40
30
20
10
mmol l^{-1} ICF
Rest
0
1
2
3
4
Hours
P_{total}
PCr
Cr
*
*
*

Figure 3-6.

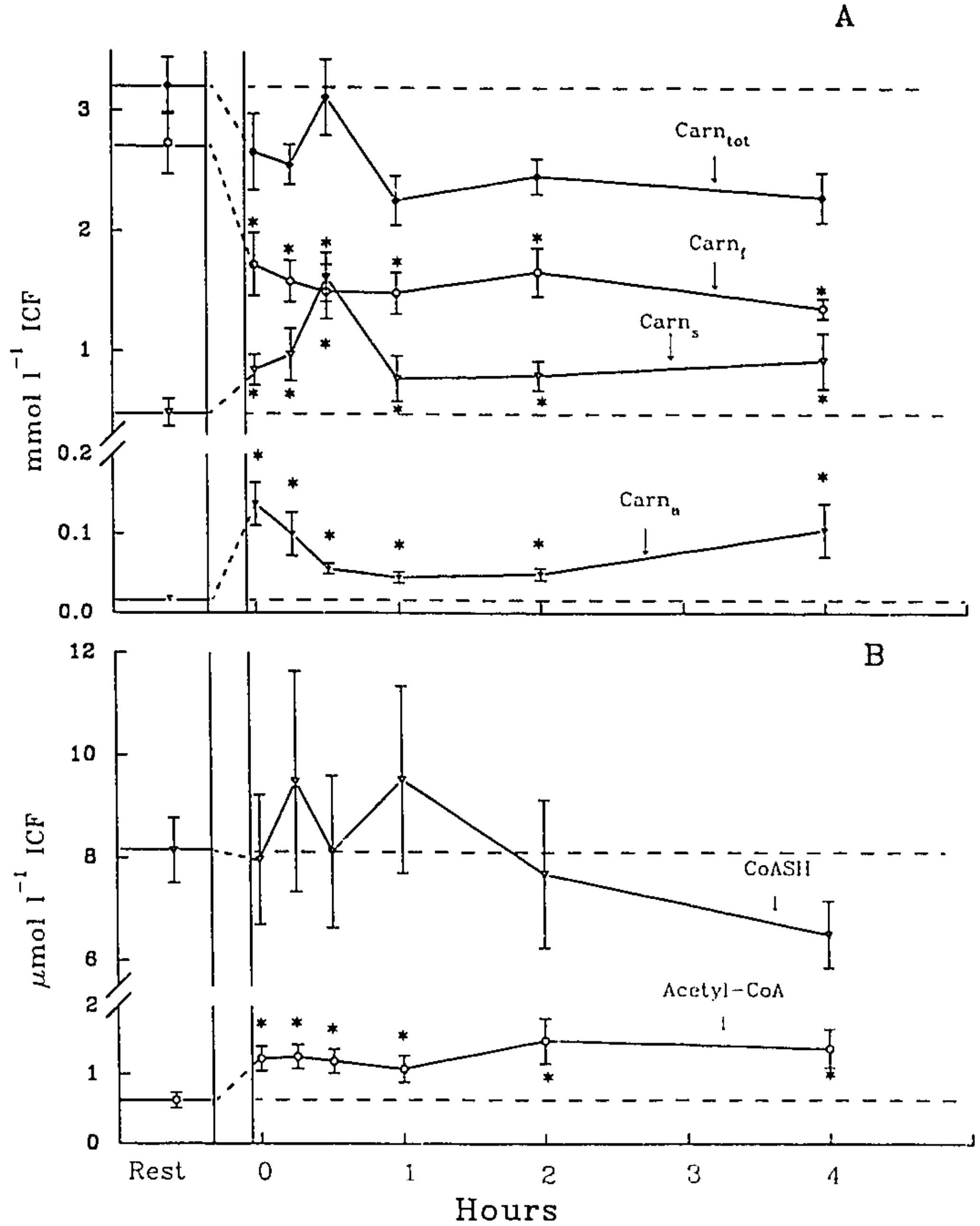
Changes in (A) white muscle intracellular inosine monophosphate (IMP) and adenosine triphosphate (ATP), and (B) intra- and extracellular ammonia (Amm). Other details as in the legend of Fig. 3-1.



A
mmol l-1 ICF
14
12
10
8
6
4
2
0
IMP
ATP
B
mmol l-1
6
4
2
0.3
0.2
0.1
0.0
AmmICF
AmmECF
Rest
0
1
2
3
4
Hours

Figure 3-7.

(A) Changes in white muscle intracellular total carnitine ($Carn_{tot}$), free carnitine ($Carn_f$), short chain acyl-carnitine ($Carn_s$) and acetyl-carnitine ($Carn_a$). (B) Changes in white muscle intracellular coenzyme A (CoASH) and acetyl coenzyme A (Acetyl-CoA). Other details as in the legend of Fig. 3-1.



A
3
2
1
0.2
0.1
0.0
mmol l^{-1} ICF
Carn$_{tot}$
Carn$_f$
Carn$_s$
Carn$_a$
B
12
10
8
6
2
1
0
µmol l^{-1} ICF
CoASH
Acetyl-CoA
Rest
0
1
2
3
4
Hours

Table 3-1.

Rainbow trout white muscle water content (C_W), intracellular fluid volume (ICFV), extracellular fluid volume (ECFV), and plasma protein concentration (C_{PP}) at rest and during 4 h of recovery from exhaustive exercise.

Treatment	C_W [a]	ICFV [a]	ECFV [a]	C_{PP} [b]
Resting	806 ± 4	738 ± 10	68 ± 11	27 ± 2
0 h	805 ± 5	741 ± 3	65 ± 6	28 ± 3
0.25 h	810 ± 6	734 ± 6	75 ± 6	30 ± 4
0.5 h	799 ± 7	737 ± 7	63 ± 6	28 ± 2
1 h	806 ± 4	740 ± 4	66 ± 4	30 ± 2
2 h	807 ± 3	737 ± 4	71 ± 5	31 ± 1
4 h	811 ± 6	750 ± 8	61 ± 7	27 ± 3

Values are means ± SEM (N = 8-13) in [a] ml kg^{-1}wet weight and [b] g l^{-1} plasma. There are no significant differences between resting and post-exercise fish in all four categories.

Table 3-2.

Electrolyte concentrations in intracellular fluid of rainbow trout white muscle at rest and during 4 h recovery from exhaustive exercise.

Treatment	K^+_{ICF}	Na^+_{ICF}	Ca^{2+}_{ICF}	Mg^{2+}_{ICF}	Cl^-_{ICF}	Lac^-_{ICF}
Rest	160.6 ± 5.8	11.6 ± 2.5	4.8 ± 1.4	30.3 ± 1.0	3.0 ± 2.7	2.4 ± 0.7
0h	168.1 ± 4.9	9.2 ± 1.1	7.9 ± 2.0*	32.0 ± 2.0	3.7 ± 1.1	36.4 ± 6.1*
0.25 h	173.0 ± 5.0*	11.6 ± 1.8	4.3 ± 0.6	35.3 ± 2.1*	4.1 ± 1.6	32.9 ± 3.9*
0.5 h	170.5 ± 5.6*	12.8 ± 1.9	3.8 ± 0.6	34.1 ± 3.0	5.8 ± 1.4	33.1 ± 3.7*
1 h	171.8 ± 5.7*	9.0 ± 2.4	3.8 ± 0.5	35.0 ± 1.4*	5.7 ± 1.0	24.6 ± 4.3*
2 h	171.4 ± 5.3*	10.2 ± 2.7	5.0 ± 0.4	35.5 ± 1.6*	7.1 ± 1.2*	16.1 ± 3.5*
4 h	162.2 ± 6.1	12.1 ± 2.9	5.0 ± 1.6	33.0 ± 0.9*	5.9 ± 2.0	18.5 ± 7.5*

Values are means ± SEM (N= 8-13) in mmol l^{-1} ICFV. * indicates significantly ($P<0.05$) from corresponding resting value.

Table 3-3.

Electrolyte concentrations in extracellular fluid of rainbow trout at rest and during 4 h recovery fromexhaustive exercise

Treatment	K^+_{ECF}	Na^+_{ECF}	Ca^{2+}_{ECF}	Mg^{2+}_{ECF}	Cl^-_{ECF}	Lac^-_{ECF}	Pi_{ECF}
Rest	1.9±0.2	155.8± 6.7	4.3±0.2	0.53±0.16	135.8±4.1	0.47±0.17	1.42±0.13
0 h	2.4±0.2*	160.4±6.5	4.8±0.3	0.74±0.12	143.6±2.1*	3.86±0.45*	2.47±0.29*
0.25 h	1.9±0.1	167.0±6.2*	5.5±0.5	0.92±0.09*	141.5±1.5*	5.11±0.66*	2.38±0.29*
0.5 h	2.1±0.2	167.7±6.8*	5.0±0.3	0.83±0.16*	138.9±1.8	9.75±1.39*	2.17±0.28*
1 h	2.5±0.3*	153.8±5.6	5.4±0.3	0.63±0.12	135.3±3.4	9.89±1.63*	2.28±0.26*
2 h	3.7±0.5*	165.9±4.7*	5.5±0.4	0.84±0.11*	132.4±2.2	9.13±1.25*	2.11±0.27*
4 h	3.2±0.5*	156.6±6.6	5.3±0.4	1.00±0.12*	123.4±4.9*	8.30±1.40*	1.79±0.33

Values are means ± SEM (N = 8-13) in mmol l^{-1} ECFV. * indicates significantly different ($P<0.05$) from corresponding resting value.

Table 3-4.

Measured and estimated Lac, Amm and H^+ distribution ratios across white muscle cell membrane according to transmembrane pH and voltage (E_m) gradients.

	Estimated E_m (mV)	Measured Lac ratio ICF/ECF	pH-Est. Lac ratio ICF/ECF	E_m-Est. Lac ratio ICF/ECF	Measured Amm ratio ICF/ECF	pH-Est. Amm ratio ICF/ECF	E_m-Est. Amm & H^+ ratio ICF/ECF	Measured H^+ ratio ICF/ECF
Rest	-95.2±7.3	2.89±0.89	0.184±0.023★	0.041±0.012★	9.54±2.62	6.05±0.97	36.5±9.1★	6.16±1.00†
0 h	-101.5±6.2	9.73±1.56*	0.324±0.129★	0.031±0.006★	23.51±4.07*	4.96±0.81★	45.9±12.7	4.98±0.82†
1/4 h	-97.9±6.4	7.23±0.83*	0.154±0.017★	0.037±0.006★	22.04±3.77*	7.22±0.78★	44.3±14.2	7.27±0.78†
1/2 h	-94.9±5.9	4.12±0.65	0.152±0.013★	0.040±0.007★	32.67±8.30*	7.00±0.70★	35.5±7.9	7.04±0.71†
1 h	-93.4±3.5	3.35±0.83	0.117±0.034★	0.038±0.004★	31.60±7.12*	8.49±1.51★	30.9±5.3	8.59±1.54†
2 h	-89.6±5.8	1.68±0.28	0.263±0.036★	0.050±0.008★	18.67±2.44*	4.57±0.57★	32.2±8.1	4.60±0.59†
4 h	-85.3±4.1	1.99±0.48	0.140±0.020★	0.051±0.007★	21.16±4.59*	8.05±1.24★	22.6±3.9*	8.17±1.24†

E_m values were estimated according to Goldman Hodgkin Katz equation (6) using values of $PK^+=0.8$, $PNa^+=0.008$, and $PCl^{-1}=1$ (Hodgkin and Horowicz, 1959). Values are mean ± S.E.M. (N = 8-13). * indicates significant ($P<0.05$) difference from corresponding resting values. ★ indicates significant ($P<0.05$) difference from corresponding measured ratios. † indicates significant ($P<0.05$) difference from corresponding estimated values.

Table 3-5. Measured intracellular Pyr/Lac ratio and estimated redox state ($NAD^+/NADH$) in trout white muscle.

	Pyr/Lac	Redox ($NAD^+/NADH$)
Rest	0.074 ± 0.011	2210.3 ± 331.2
0h	0.028 ± 0.006*	2275.9 ± 538.8
0.25h	0.031 ± 0.006*	2750.3 ± 557.5
0.5h	0.033 ± 0.009*	3227.7 ± 762.9
1h	0.034 ± 0.007*	2419.3 ± 672.4
2h	0.034 ± 0.006*	1530.5 ± 341.9
4h	0.086 ± 0.037	3148.8 ± 883.9

Redox values were estimated according to equation (9):

$$\frac{[NAD^+]}{[NADH]} = \frac{[Pyr]_{ICF}\,[H^+]_{ICF}}{[Lac]_{ICF}\,K}$$

Using $K = 2.106 \times 10^{-12}$ mol l^{-1}, based on correction of the value reported by Williamsen *et al.* (1967) to 15°C according to van' t Hoff equation (Williams & Williams, 1973) with a standard enthalpy $\Delta H° = 12550$ cal mol^{-1} from Somero & Siebenaller, 1975). Values are means ± S.E.M. (N = 8-13). * indicates significant ($P < 0.05$) difference from corresponding resting values.

CHAPTER 4

AMMONIA MOVEMENT AND DISTRIBUTION AFTER EXERCISE ACROSS WHITE MUSCLE CELL MEMBRANES IN RAINBOW TROUT: A PERFUSION STUDY

ABSTRACT

Manipulations of pH and electrical gradients in a perfused preparation were used to analyze the factors controlling ammonia distribution and flux in trout white muscle after exercise. Trout were exercised to exhaustion and then an isolated-perfused white muscle preparation with discrete arterial inflow and venous outflow was made from the posterior portion of the tail. The tail-trunks were perfused with low (7.4), medium (7.9), and high (8.4) pH saline, achieved by varying [HCO_3^-] at constant Pco_2. Intracellular and extracellular pH, ammonia, CO_2, K^+, Na^+, and Cl^- were measured. Muscle intracellular pH was not affected by changes in extracellular pH. Increasing extracellular pH caused a decrease in the transmembrane P_{NH3} gradient and a decrease in ammonia efflux. When extracellular [K^+] was increased from 3.5mM to 15mM in the medium pH group, a depolarization of the muscle cell membrane potential from -92 mV to -60mV and a 0.1 unit depression in intracellular pH occurred. Ammonia efflux increased despite a marked reduction in the P_{NH3} gradient. Amiloride (10^{-4} M) had no effect, indicating that Na^+/H^+, NH_4^+ exchange does not participate in ammonia transport in this system. A comparison of observed intracellular to extracellular

ammonia distribution ratios with those modelled according to either pH or Nernst potential distributions supports a model in which ammonia distribution across white muscle cell membranes is affected by both pH and electrical gradients, indicating that the membranes are permeable to both NH_3 and NH_4^+. Membrane potential, acting to retain high levels of NH_4^+ in the intracellular compartment, appears to be the dominant control during the post-exercise period. However, at rest, the pH gradient may be more important, resulting in much lower intracellular ammonia levels and distribution ratios. I speculate that the muscle cell membrane NH_3/NH_4^+ permeability ratio in trout may change between the rest and post-exercise condition.

INTRODUCTION

Ammonia is a respiratory gas which behaves as a weak electrolyte in solution (13,17). With a pK of 9.0 to 10 under physiological conditions, more than 95% exists as NH_4^+, and the remainder as NH_3 (4). While NH_3 is often considered to be highly lipophilic, available data indicate that this is not the case, and that the lipid *versus* water partition coefficient is less than 0.1 (7). Diffusion through water-filled channels is likely to be much faster than diffusion through lipoprotein bilayers. NH_4^+, is charged and larger in ionic radius, especially in the hydrated form, and likely diffuses more slowly across cell membranes, exclusively by water-filled channels. However, NH_4^+ is much more plentiful than NH_3 and may also move by various carrier-mediated mechanisms (*e.g.* substitution into Na^+/H^+ exchangers; see ref.,1).

Ammonia is the major end product of nitrogen metabolism (over 70%) in ammoniotelic animals such as teleost fish. At rest, most ammonia is produced by transamination and deamination of amino acids in the liver (see ref. 34, for review), but during exhaustive exercise and hypoxia, large additional amounts of ammonia are produced in white muscle through the deamination of adenylates as ATP stores are degraded (6,19,25,29). In mammals, ATP stores are defended and intracellular and extracellular ammonia levels remain relatively low (9,15,20), but in exercising fish muscle, ammonia production is sufficient to serve as an important intracellular buffer of the accompanying lactacidosis, as well as an important modulator of glycolytic flux at the level of phosphofructokinase (6). Furthermore, most of the ammonia produced appears to be retained in the muscle cells, together with IMP, so as to fuel later resynthesis of the adenylate pool in fish (19,29).

In mammals, ammonia movement and distribution across muscle cell membranes traditionally follow the theory of non-ionic diffusion (20) - *i.e.* ammonia moves as NH_3 according to P_{NH3} gradients and therefore distributes according to transmembrane pH gradients because NH_3 permeability is so much higher than NH_4^+ permeability (13,17). As illustrated in Fig. 4-1A, this will lead to distribution ratios (intracellular to extracellular) of approximately 4.0. However, several recent studies have questioned this theory in finding significant disagreement between the measured muscle to plasma distribution ratio for ammonia and that predicted by the pH gradient (9,10). In fish muscle, the situation is also unclear (24,34). Most experimental studies (27,29,38,39), but not all (19), have indicated that intracellular ammonia levels after exercise are far higher than explicable by a pH-driven distribution, but very close to those predicted by a Nernst distribution between intracellular (ICF) and extracellular fluid (ECF), assuming a muscle cell membrane potential (E_m) in the range of -80 to -100 mV. As illustrated in Fig. 4-1B, this will result in a distribution ratio of approximately 30.0. These studies suggest that the influence of E_m on NH_4^+ is the dominant factor governing transmembrane distribution, so that NH_4^+ permeability must be appreciable. However, this concept has been strongly criticized on theoretical grounds (11), most notably because an "NH_4^+ shuttle" could result in an inwardly directed flux of H^+ into the cells (38), creating an intolerable load for the pH_i regulatory mechanisms of the cell (Fig. 4-1B). The situation is further complicated by the finding of Tang *et al.* (27) on post-exercise trout *in vivo* that despite an ammonia distribution between ICF and ECF apparently in accord with E_m, experimentally increasing the pH_e - pH_i gradient by bicarbonate infusion resulted in a greater apparent retention of ammonia in white muscle. My own recent *in vivo* study on trout muscle (29) indicated that ammonia distribution might change from a distribution governed by the pH gradient at rest to a distribution governed by E_m after exercise, in contrast to an earlier study showing an E_m-driven distribution under both circumstances (39).

In light of these uncertainties, the present study concentrates on the post-exercise situation, and employs a perfused rainbow trout tail-trunk preparation. In the present report, the preparation is characterized, and then used to examine the factors governing transmembrane ammonia distribution and flux in rainbow trout white muscle. Parallel studies with this preparation have examined the factors governing transmembrane lactate, metabolic acid, bicarbonate, and CO_2 distribution and flux (Y. Wang, G.J.F. Heigenhauser and C.M. Wood, unpublished results). The approach eliminates many of the uncertainties associated with *in vivo* studies. In particular, the preparation allowed me to measure both the efflux to the perfusate, and the transmembrane distribution with respect to the venous side. Previous *in vivo* studies have not been able to monitor efflux, and may have overestimated ICF/ECF distribution ratios because only arterial ECF samples were obtained. Furthermore, the preparation allowed me to manipulate the pH and electrical gradients between ECF and ICF. Extracellular bicarbonate levels were varied to alter $pH_i - pH_e$, high ECF potassium was used to partially depolarize the muscle cell E_m, and amiloride (1) was employed to block the possible involvement of the Na^+/H^+, NH_4^+ carriers.

MATERIALS AND METHODS

Experimental Animals

Rainbow trout (500-600g) were obtained from Spring Valley Trout Farm (Petersburg, Ontario) and then raised for 2-6 months in a 800L fiberglass cylindrical tank with flowing dechlorinated Hamilton tapwater (Na^+, 0.6; Cl^-, 0.8; Ca^{2+}, 1.8; Mg^{2+}, 0.5; K^+, 0.04 mEq l^{-1}; pH = 8.0; temperature, 5-12°C) until the desired size (800-1000g) was reached. During this period the fish were fed with high protein trout grower floating pellet (Aquaculture Zeigler Bros.) three times a week. Prior to the experiment, fish were acclimated to 15 ± 1°C for 5-7 days without feeding to standardize metabolic status. Exercised fish were used in this perfusion study in order to elevate muscle ammonia (Amm) levels. Prior to the perfusion, the fish were manually chased for 6 min to exhaustion in a 150L cylindrical tank (29). Immediately upon the cessation of the exercise, the trout was transferred to a dark acrylic box containing 8L aerated water and anaesthetized with MS-222 (0.5g/L neutralized with NaOH). This resulted in loss of equilibrium and cessation of ventilation within 1 min without struggling. This method has been proven to cause minimum metabolic and acid-base disturbances (26,30). The tail was cut off at the level of the anus and weighed prior to perfusion.

Experimental Protocols

1. Experimental Design and Perfusate Preparation

This study was designed to investigate how changes in pH and electrical gradients between intracellular and extracellular fluids of muscle affect transmembrane Amm flux and distribution. Cortland salmonid saline with 3% bovine serum albumin (BSA fraction V, Sigma) was used as the basic perfusate. Ammonia already present in the distilled water and component salts was sufficient to provide normal resting arterial levels of T_{Amm} (approximately 50 μmol l^{-1}) in the perfusate (27,29,30,39). Pco_2 was kept constant at approximately 2 torr to represent the typical resting value of trout arterial blood *in vivo* (29). $NaHCO_3$ levels were adjusted accordingly to manipulate perfusate extracellular pH (pH_e) in different treatment groups (Table 4-1). Changes in the pH gradient were achieved by altering perfusate pH_e, while the electrical gradient was depressed by elevating perfusate potassium concentration from the normal level of ~3mM to 15 mM (high-K^+). The study comprises four treatment groups (6-11 fish per group): low, medium, high pH_e and high potassium (at medium pH_e) as shown in Table 4-1. The medium-pH_e group was used as the control group, with normal $pH_e \approx 7.9$, which mimics resting trout arterial plasma pH (29). The low-pH_e group ($pH \approx 7.4$) simulates the typical trout arterial plasma pH after exhaustive exercise (29). The high-pH_e group ($pH \approx 8.4$) was chosen to create an elevated transmembrane pH gradient, which could occur under some circumstances such as high environmental pH (31). The pH and [HCO_3^-] of the high-K^+ group ([K^+] $\approx$ 15mM) were kept at the control level. Changes in saline [HCO_3^-] were achieved by reciprocal changes in $NaHCO_3$ and NaCl so as to maintain [Na^+] constant in various treatment groups. However, in the high-K^+ group, because 12 mmol more KCl was added to elevate the perfusate K^+ level, the NaCl level was reduced correspondingly to avoid introducing excessive Cl^- into the saline.

Based on the results of these studies, it was apparent that one of the drug treatments used in our parallel study on lactate and metabolic acid transport in the perfused post-exercise trunk preparation (Y. Wang, G.J.F. Heigenhauser and C.M. Wood, unpublished results) could provide useful information on ammonia transport. Methods were identical to that of the low-pH_e treatment, except that 10^{-4} M amiloride HCl (Sigma) was present in the perfusate throughout the second 30 min of perfusion. Perfusate and tissue samples from these trunks ($N = 11$) were analyzed for all ammonia-relevant parameters; these data are presented in the current study.

2. Perfusion and Sampling

Immediately after the tail trunk was severed from the body and weighed (10-15s), catheters (Clay-Adams PE-90 tubing) were implanted into the caudal artery and caudal vein and secured by ligation around the vertebral column. Perfusion was started immediately at 2ml min^{-1} $100g^{-1}$ tail weight with Cortland saline plus BSA of the appropriate pH and K^+ concentration. The saline was heparinized with 50 iu ml^{-1} sodium heparin (Sigma) to prevent blood clotting. The tail trunk was submerged in a temperature-controlled saline bath (15 ± 0.5°C) during the entire perfusion period, while the perfusate was pumped through a heat-exchange coil in a 15°C water bath. Sampling ports were placed in arterial and venous catheters to allow subsequent collection of inflowing and outflowing perfusate samples, respectively, and the outflow of the venous catheter was set to the level of the saline bath - *i.e.* zero pressure.

The perfusion preparation setup is shown schematically in Fig. 4-2. The perfusate was gassed with 0.25% CO_2, balance O_2 , for at least 60 min in a plexiglass disc oxygenator to achieve the desired pH, Po_2 and Pco_2 levels. Precision gas mixtures were supplied by Wöstoff gas-mixing pumps (Bochum, Germany) and saturated with water vapor prior to contact with the perfusate. The oxygenated perfusate was then drawn through the heat exchanger by a peristaltic pump (Gilson Minipuls 3) and delivered to the tail trunk *via* a windkessel to dampen the pressure pulsatility. All tubing was stainless steel, to minimize loss of O_2 and CO_2. The perfusion rate was kept at 2 ml min^{-1} 100 g^{-1} tail weight throughout, approximately twice the blood perfusion rate in trout white muscle under exercise conditions *in vivo* (23,32). The perfusion pressure was monitored by a transducer (Narco Bio-System Inc. RP-1500) and registered on a Gilson ICT-5H polygraph chart recorder. This initial perfusion lasted for 30 min in order to ensure washout of red blood cells.

At 30 min, arterial and venous perfusate samples (2 ml) were collected *via* the sampling ports using gas-tight Hamilton syringes. A second (final) set of perfusate samples was taken after a subsequent 30 min of perfusion with non-heparinized saline of the same composition. Samples were analyzed for perfusate pH, Po_2, total CO_2 (Tco_2), protein content, total ammonia (T_{Amm}), lactate (Lac), Cl^-, Na^+, and K^+. Total CO_2, pH, Po_2, and protein content were measured immediately after sampling, while part of the perfusate sample (300 μl) was deproteinized with 8% PCA (600 μl), frozen in liquid N_2, and then stored at -70°C for later analysis of Lac. Perfusate (approx. 500 μl) was also frozen directly in liquid N_2 and stored at -70°C for analysis of T_{Amm} and electrolytes.

At the time when the tail was cut off, an initial muscle sample (3-5g) was taken from the area between the lateral line and the dorsal fin immediately *anterior* to the point of section. A final muscle sample (3-5g) was excised from the same position on the perfused tail-trunk after the second perfusate sample (*i.e* at 60 min). Upon excision, the muscle samples were freeze-clamped within 5 sec with aluminum tongs pre-cooled in liquid N_2, and then stored in liquid N_2. The muscle tissues were used to determine intracellular pH (pH_i), Tco_2, T_{Amm}, Lac, Cl^-, Na^+, K^+ and H_2O content. Lac and Tco_2 data in perfusate and tissue samples will be reported elsewhere (Y. Wang, G.J.F. Heigenhauser and C.M. Wood, unpublished results).

3. Analytical Techniques

Frozen muscle tissue was ground to a fine power in an insulated mortar and pestle cooled with liquid N_2. Muscle pH_i was measured by the homogenization technique (22). One part of frozen tissue powder (approx. 200 mg) was mixed with 5 parts of metabolic inhibitor (150 mmol l^{-1} potassium fluoride; 6 mmol l^{-1} sodium nitrilotriacetate, Sigma) in a 1.5ml sealed centrifuge tube. The homogenate was then centrifuged at 9,000 g for 30 sec and the supernatant was used to measure muscle pH_i at 15°C with a Radiometer microelectrode (E5021) and PHM84 acid-base analyzer; muscle Tco_2 was also measured on this supernatant (Y. Wang, G.J.F. Heigenhauser and C.M. Wood, unpublished results). Part of the powder (approx. 100 mg) was extracted with 1 ml 8% PCA, and the supernatant of this tissue extract was used to analyze tissue T_{Amm} by the glutamate dehydrogenase method as modified by Kun and Kearney (14). The rest of the frozen tissue powder was lyophilized for 64 h and then stored in a freezer at -70°C for later measurement of Lac.

For measurement of ions, freeze-dried tissue powder (20mg) was digested with 1N ultrapure HNO_3 (1 ml) at 40-50°C for 48h and then the supernatant was used for Na^+, K^+ and Cl^- analyses. White muscle water content ($[H_2O]_i$) was determined by drying fresh tissue (0.5-1g) to a constant weight at 80°C. Perfusate was also deproteinized with 1N HNO_3, and the supernatant used for Na^+ and K^+ analysis. For perfusate Cl^- measurements, undiluted perfusate was used. Both Na^+ and K^+ were determined with flame atomic absorption spectrometry (Varian AA-1275), while Cl^- was analyzed by coulometric titration (Radiometer CMT10).

Perfusate pH was determined with same acid-base apparatus as for muscle pH_i. Perfusate Po_2 was measured at 15°C with a Radiometer Po_2 electrode (E5046) connected to a

Cameron Instrument, OM-200 oxygen meter. Perfusate Tco_2 was determined on a Cameron Instrument Capni-Con total CO_2 analyzer (Model II). Pco_2 and $[HCO_3^-]$ were calculated by manipulation of the Henderson-Hasselbalch equation using appropriate constants (αCO_2 and pK') for rainbow trout true plasma at 15°C (3). Total protein and water content of saline was measured with an American Optical refractometer.

The perfusate T_{Amm} content was measured by the glutamate dehydrogenase assay using a Sigma kit. Along with tissue T_{Amm} analysis, these enzymatic measurements were conducted on an LKB UltraspecPlus 4053 spectrophotometer.

4. Perfusate Buffer Capacity

The non-bicarbonate buffer capacities (β) of each of the four perfusates were determined. In brief, perfusate samples (4ml) were placed in tonometer vessels (Instrumentation Laboratories 237) and equilibrated with 1, 2, 4, and 8 torr Pco_2, balance O_2, for 20 min at 15°C, then analyzed for pH and Tco_2 with aforementioned methods. HCO_3^- concentrations of the samples were calculated and β in various treatment groups was determined from the slope of the regression of HCO_3^- against pH:

$$\beta = \frac{\Delta\,[HCO_3^-]}{\Delta pH} \tag{1}$$

Calculations and Statistics

The perfusion pressure has been expressed as net inflow pressure exerted on the tail trunks only, calculated as the difference between the perfusion pressure with and without the tail trunks in the system (*i.e.* correcting for cannula resistance). Since the perfusion rate (2 ml 100 g^{-1} tail weight min^{-1}) was constant in each preparation, the perfusion pressure reflected the resistance of the tail trunk.

The extracellular fluid volume (ECFV, ml g^{-1}) and intracellular fluid volume (ICFV, ml g^{-1}) of white muscle in this perfusion preparation were estimated by muscle water content ($[H2O]_t$), and Na^+, K^+, and Cl^- concentrations of the muscle and extracellular fluid (ECF, venous perfusate water in this case) using the "Cl^--K^+ space" approach of Conway (5):

$$ICFV = [H_2O]_t - ECFV \qquad (2)$$

$$ECFV = \frac{[K^+]_t[Cl^-]_t - ([H_2O]_t)^2[Cl^-]_e[K^+]_e}{[K^+]_t[Cl^-]_e + [Cl^-]_t[K^+]_e - 2[H_2O]_t[Cl^-]_e[K^+]_e} \qquad (3)$$

where the subscripted *t* and *e* stand for the concentrations in whole muscle tissue (mmol/kg for ions, ml/g for H_2O) and in ECF (mmol/l perfusate H_2O) respectively. The estimate of ECFV was made based on the assumption that ions in extracellular fluid have been fully equilibrated with venous perfusate after 60 min of perfusion.

The ICF ions and T_{Amm} concentrations ($[S]_i$) were calculated as:

$$[S]_i = \frac{[S]_t - [S]_e \times ECFV}{ICFV} \qquad (4)$$

where subscripted *i* stands for the concentration in ICF, while S represents ions or T_{Amm}.

Oxygen consumption (Mo_2) and CO_2 efflux were calculated, using the Fick principle, from the perfusion rate and the differences of gas content between inflow and outflow perfusate - *e.g.*:

$$Mo_2\ (mmol\ kg^{-1}\ h^{-1}) = Perfusion\ rate \times \alpha O_2 \times \Delta Po_2 \qquad (5)$$

where ΔPo_2 is O_2 partial pressure difference between arterial and venous perfusate, and α_{O2} (1.77 μmol L^{-1} $torr^{-1}$) is the solubility coefficient of O_2 at 15°C (3). An analogous equation, with the corresponding ΔTco_2 values substituted for $\alpha O_2 \times \Delta Po_2$ was applied in the CO_2 efflux calculation.

Ammonia, as a metabolic substrate and product, exists as an anion, a respiratory gas, and a weak base (pK ≈ 9.7 at 15°C). Total ammonia (T_{Amm}) is the sum of the ionic and non-ionic forms of this substance:

$$T_{Amm} = [NH_4^+] + [NH_3] \tag{6}$$

$$P_{NH_3} = \frac{[NH_3]}{\alpha NH_3} \tag{7}$$

where αNH_3 is taken from Cameron and Heisler (4) and P_{NH3} is NH_3 partial pressure.

At physiological pH (6.0-8.0), according to the following equation:

$$pH = pK + log\frac{[NH_3]}{[NH_4^+]} \tag{8}$$

over 98% of ammonia exists as NH_4^+, with pK again from Cameron and Heisler (4). If the muscle cell membrane is permeable only to NH_3 and P_{NH3} is in equilibrium across the membrane, then the total ammonia distribution will be a function of the transmembrane pH gradient only:

$$\frac{[T_{Amm}]_{ICF}}{[T_{Amm}]_{ECF}} = \frac{1 + 10^{(pK-pH_i)}}{1 + 10^{(pK-pH_e)}} \tag{9}$$

Therefore, the relatively lower pH_i will trap more NH_4^+ in the ICF (Fig 1A).

However, if the cell membrane is permeable only to NH_4^+, ammonia will be distributed according to membrane Nernst potential (E_m) only:

$$E_m = -\frac{RT}{ZF}\ ln\frac{[NH_4^+]_i}{[NH_4^+]_e} = -\frac{RT}{ZF}\ ln\frac{[T_{Amm}]_i - [NH_3]_i}{[T_{Amm}]_e - [NH_3]_e} \quad . \qquad \textbf{(10)}$$

where R, T, Z and F are the gas constant, the absolute temperature, the valence, and Faraday's constant, respectively. Then under equilibrium conditions, the negatively charged ICF will trap a great deal more NH_4^+ (Fig. 4-1B) than if pH were governing the distribution. The E_m of the white muscle was estimated from the measured intra- and extracellular concentrations of K^+, Na^+, and Cl^- according to the Goldman-Hodgkin-Katz equation:

$$E_m = \frac{RT}{F} ln\frac{PK^+[K^+]_{ECF} + PNa^+[Na^+]_{ECF} + PCl^-[Cl^-]_{ICF}}{PK^+[K^+]_{ICF} + PNa^+[Na^+]_{ICF} + PCl^-[Cl^-]_{ECF}} \qquad \textbf{(11)}$$

where PK^+, PNa^+ and PCl^- are relative permeability coefficients, from Hodgkin and Horowicz (12).

Under the situation where the cell membrane has significant permeability to both ionic and non-ionic forms of ammonia, T_{Amm} distribution will be a function of both transmembrane pH and E_m gradients. According to Boron and Roos (2):

$$\frac{[T_{Amm}]_i}{[T_{Amm}]_e} = \frac{[H^+]_i + K}{[H^+]_e + K} \times \frac{(PNH_3\ /PNH_4^+) - F\times E_m/[RT(1-\gamma)]\times[H^+]_e/K}{(PNH_3\ /PNH_4^+) - F\times E_m\times\gamma/[RT(1-\gamma)]\times[H^+]_i/K} \qquad \textbf{(12)}$$

where K is the NH_3/NH_4^+ dissociation constant, PNH_3 and PNH_4^+ are the permeabilities to NH_3 and NH_4^+, respectively, and:

$$\gamma = exp(\frac{E_m F}{RT}) \qquad (13)$$

If the pH_e - pH_i gradient and the E_m are known, then the $[T_{Amm}]_i/[T_{Amm}]_e$ ratio will be a function of the permeability ratio PNH_3 /PNH_4^+ as outlined by Wright *et al.* (38) and Wood *et al.* (37).

All data are reported as means ± 1 S.E.M. (N). Significant differences between means in the four different perfusate groups were evaluated by one-way analysis of variance (ANOVA). If the ANOVA indicated significance ($P \leq 0.05$), then *post hoc* comparison by means of Duncan's multiple range and critical range test ($P \leq 0.05$) were performed, with reference to the medium-pH group as the control. Student's paired t-test was used to evaluate differences within treatment groups between 30 and 60 min values. Simple unpaired t-tests were used to evaluate differences between the low-pH_e treatment and the low-pH_e plus amiloride treatment.

RESULTS

Condition of the Perfused Tail-trunk Preparation.

The perfusion pressure started at about 15 cm H_2O, and thereafter slowly declined by about 5 cm H_2O as the perfusion proceeded for the first 30 min. However, this trend was reversed during the second 30 min, such that perfusion pressures at 60 min were 16 - 19 cm H_2O. There were no significant differences in pressure between the four groups at either 30 or 60 min (Table 4-2).

There was no visible red color in the outflow perfusate after 20 min of perfusion, indicating thorough washout of RBC's. There were also no detectable differences in protein concentration (approximately 3%) between inflowing and outflowing perfusate at any time. The $[H_2O]_t$ of each treatment group after 60 min perfusion (Table 4-3) was not significantly different from that of initial muscle samples (0.786 ± 0.016 ml g^{-1} wet tissue, N=32, pooled total of four groups), and was therefore not affected by the experimental treatments. However, the high-K^+ treatment caused a marked redistribution of internal fluid volumes, resulting in a 58% decrease in ECFV and a 5% increase in ICFV (Table 4-3). Similarly white muscle ICF Na^+, K^+ and Cl^- showed no significant changes as pH_e varied, but Na^+ and Cl^- altered markedly in response to high-K^+ perfusion (Table 4-4). ICF Na^+ and Cl^- increased by 35% and 280% respectively relative to the the control group, while K^+ remained unchanged despite the large increase of ECF K^+ (Table 4-4). It was not possible to compare these values with ICFV and ICF ion concentrations prior to perfusion because ECF measurements required for the intracellular calculations were not obtained when the initial muscle samples were collected. However the ICFV and ECFV values of the three pH treatment groups, as well as the intracellular ionic concentrations after 60 min perfusion, were comparable to those of our previous *in vivo* study on exercised trout (29).

There were no significant differences of $[Na^+]$ or $[Cl^-]$ between inflow and outflow perfusate (A-V difference) in any of the three pH treatment groups after 60 min of perfusion (Table 4-5). Despite the large increases in ICF Na^+ and Cl^- concentrations in the high-K^+ treatment, there were again no significant A-V differences in these two ions. However, $[K^+]_v$ increased by almost 20% relative to $[K^+]_a$ in both the low-pHe and the medium-pH_e (control) groups, indicating a net efflux of K^+ at 60 min (Table 4-5). This did not occur in the high-pHe treatment. In contrast the high-K^+ group exhibited a 10% decline in $[K^+]_v$ relative to $[K^+]_a$, indicating a net uptake of K^+ at 60 min (Table 4-5). The trend for lower absolute ECF $[Cl^-]$ with increasing pH in the three pH treatments, and the lower absolute ECF $[Na^+]$ in the high-K^+ treatment were due to the original make-up of the salines (see Materials and Methods).

E_m was not affected by perfusate acid-base status and averaged about -90 mV in the three pH treatments (Table 4-6). The increase in perfusate $[K^+]$ from 3 to 15 mM in the high-K^+ treatment caused the intended partial depolarization (35%), reducing white muscle E_m to about -59 mV (Table 4-6). In this group, slight muscle twitching was observed upon the start of perfusion with high-K^+ saline. In all treatments, E_m remained unchanged when calculated with respect to either the arterial or venous ECF perfusate compositions (Table 4-6).

Typically, there was a 250-350 torr decrease in Po_2 from the arterial to the venous perfusate, and O_2 uptake remained relatively stable over the course of perfusion in all four treatment groups (Table 4-7). The O_2 supply did not seem to be exhausted since venous Po_2

(over 75 torr) was far from being depleted. O_2 uptake did not vary significantly amongst the three pH treatments, though it tended to be lower at lower pH's. However, the high-K^+ group exhibited a 20% increase over the control group in O_2 consumption (Table 4-7).

CO_2 efflux was much greater than O_2 uptake, a difference which varied in accord with perfusate pH from 0.7-fold in the high-pH to 3-fold in the low-pH treatment (Table 4-7). Changes in pH altered CO_2 efflux significantly. While low-pHe treatment resulted in a 20% increase, the high-pH_e treatment led to a 70% depression in CO_2 efflux (Table 4-7). These data were in approximate inverse relation to the simultaneously measured metabolic acid flux data (Y. Wang, G.J.F. Heigenhauser and C.M. Wood, unpublished results), suggesting that most of the CO_2 efflux occurred in the form of HCO_3^-.

Acid-base Status

The intended differences in pH_a amongst treatments were achieved (Fig. 4-3, *cf.* Table 4-1), and non-bicarbonate buffer capacities (β) of perfusate used in the four treatment groups were not significantly different. The overall mean value was -5.54 ± 0.15 mM $[HCO_3^-]$. pH unit $^{-1}$. As pH_a increased from 7.4 to 8.4, the arterial-venous difference (ΔpH_{a-v}) also increased from 0.13 to 0.34 units (Fig. 4-3). However, the substantial differences in pH_a amongst treatments (approx. 1.0 unit between high- and low-pH perfusates) had no effect on muscle pH_i, which remained at about 6.6 in this post-exercise preparation. Therefore, the overall transmembrane pH gradient (ΔpH_{v-i}) varied linearly with pH_a, from 0.70 at low pH_a to 1.50 at high pH_a (Fig. 4-3). It should be noted that pH_i measured in initial muscle samples taken immediately prior to the start of perfusion was 6.596 ± 0.026 (N = 30) , and therefore identical to the ~6.6 measured after 60 min in the three pH treatments.

In contrast to the pH treatments, the 35% reduction of the muscle cell E_m induced by higher extracellular [K^+] resulted a significant depression in pH_i to about 6.4 (Fig. 4-3). However, the overall transmembrane pH gradient ($\Delta pH_{v\text{-}i}$ = 1.20) did not change in comparison to the control group ($\Delta pH_{v\text{-}i}$ = 1.16). Venous pH also fell significantly, so $\Delta pH_{a\text{-}v}$ almost doubled relative to the control group.

pH Effects on Ammonia Distribution and Efflux

Intracellular ammonia concentration ($T_{Amm}i$) was about 9,000 μM in this post-exercise preparation, and there were no significant differences amongst the three pH treatment groups after 60 min of perfusion (Fig. 4-4). This may be compared with an initial value of 7712 ± 355 μM (N = 30) measured in initial muscle samples taken prior to the start of perfusion. Thus there was a small but significant increase in $T_{Amm}i$ over the course of the 60 min perfusion.

Compared to the control group, there was a 47% decrease in venous ammonia ($T_{Amm}v$) in the high-pH_e group, but no significant change in $T_{Amm}v$ in the low-pH_e group (Fig. 4-4). In accord with the unchanged $T_{Amm}i$ and pH_i, there were no significant differences in intracellular P_{NH3} amongst the three pH treatment groups; intracellular P_{NH3} averaged about 150 μTorr (Fig. 4-5). Nevertheless, as extracellular pH increased, transmembrane P_{NH3} gradients (intracellular to venous) dropped from 134.5 to 92.5 μTorr (Fig. 4-5). This depressed transmembrane P_{NH3} gradient was mainly due to the elevation of venous P_{NH3} induced by higher extracellular pH.

In parallel to the depressed transmembrane P_{NH3} gradient as extracellular pH increased, T_{Amm} efflux tended to decrease (Fig. 4-6). The high-pH_e treatment caused a significant 47% decrease in T_{Amm} efflux relative to the control group, while the 12% increase at low-pHe was not significant (Fig. 4-6).

The measured transmembrane T_{Amm} ratio ($T_{Amm}i/T_{Amm}e$, calculated relative to the venous end) increased markedly as extracellular pH increased (Fig. 4-7). The lower venous T_{Amm} (Fig. 4-4) contributed primarily to the higher ratio. For comparison, the transmembrane pH gradients at the venous end were used to estimate the T_{Amm} distribution ratios (Equation 9) assuming that the white muscle membrane is effectively permeable only to NH_3. It is clear that T_{Amm} ratios calculated in this manner from the pH gradients were far from matching the measured ratios and indeed greatly underestimated them in all three pH treatment groups (Fig. 4-7). Nonetheless, as transmembrane pH gradient increased, the pH-estimated ratio increased in parallel with the elevation in the measured T_{Amm} ratio (Fig. 4-7). The absolute differences between the measured and the pH-estimated ratios remained almost unchanged as extracellular pH increased.

E_m Effects on Ammonia Distribution and Efflux

The membrane depolarization caused by the increase in extracellular K^+ did not alter $T_{Amm}i$ significantly (Fig. 4-4), but caused substantial elevations in both $T_{Amm}v$ (Fig. 4-4) and T_{Amm} efflux (Fig. 4-6) relative to the control group. This 77% increase in T_{Amm} efflux occurred despite a substantial fall in intracellular P_{NH3} from about 170 to 100 μTorr and an associated 64% decrease in transmembrane P_{NH3} gradient (Fig. 4-5). The net driving force on NH_4^+, calculated relative to the venous perfusate as the difference between the measured E_m and the Nernst E_m for NH_4^+ (from Equation 10), increased by two-fold from 6.6 ± 2.6 mV (N = 6) to 20.1 ± 4.7 mV (N = 11).

For comparison, the values of E_m tabulated in Table 4-6 were used to estimate the T_{Amm} distribution ratios (Equation 10) assuming that the white muscle cell membrane is effectively permeable only to NH_4^+. As E_m did not vary significantly among the three pH treatments (Table 4-6), the E_m-estimated $T_{Amm}i/T_{Amm}e$ ratios did not change significantly (Fig. 4-7). In the low-pH_e and medium-pH_e (control) treatments, these E_m-estimated ratios were not significantly different from the measured ratios. They therefore agreed much more closely with the measured ratios than did the pH-estimated ratios (Fig. 4-7). However, in the high-pHe treatment, the E_m-estimated ratio and the pH-estimated ratio were similar, and both were significantly lower than the measured ratio. Relative to the control group, the high-K^+ treatment caused a significant decrease in the measured T_{Amm} distribution ratio, which was paralleled by a significant decline in the E_m-estimated ratio. In contrast, the pH-estimated ratio increased slightly. In this case, the two estimated ratios were similar, and both significantly lower than the measured ratio, similar to the situation at high-pH (Fig. 4-7).

To look at the matter from another perspective, Nernst potentials were calculated from the measured intra- and extracellular $[NH_4^+]$ concentrations, assuming that the muscle cell membranes are permeable only to NH_4^+ (Equation 10). The NH_4^+-estimated Nernst potentials agreed closely with recorded E_m values in the low-pH_e and medium-pHe treatments, but were significantly more negative than E_m in the high-pH_e and high-K^+ treatments (Fig. 4-8). In general, these findings agree with the analysis based on distribution ratios (Fig. 4-7).

Amiloride Effects on Ammonia Distribution and Efflux

In general, the T_{Amm} distribution ratios predicted from E_m by Equation 10, assuming that the white muscle cell membrane was effectively permeable only to NH_4^+, agreed with the observed distribution ratios much better than did those predicted from pH gradients by

Equation 9 assuming effective permeability to NH_3 only (Fig. 4-7). This agreement was strongest in the low-pH_e treatment. We therefore evaluated whether amiloride (10^{-4} M), an inhibitor of both Na^+/H^+, NH_4^+ exchange and Na^+ channels at this concentration (1), influenced the distribution and efflux of ammonia in the low-pH_e treatment.

Amiloride had no significant effect on pH gradients or E_m in the preparation (data not shown). Amiloride also had no significant effect on either the measured T_{Amm} distribution ratio or the measured T_{Amm} efflux rate (Table 4-8).

DISCUSSION

Evaluation of the Tail-trunk Perfusion Preparation: Comparison with in vivo Studies

The present perfused trout trunk preparation is similar to that of Moen and Klungsoyr (18). In contrast to whole trunk preparations (16,28,33) only the post-anus region of the tail was perfused, thereby avoiding involvement of the kidney and allowing discrete collection of venous outflow from the caudal vein. In this region, white muscle makes up approximately 90% of the total soft tissue volume (8), the balance being mainly red muscle in a discrete band under the lateral line. The preparation therefore facilitates the measurement of metabolite fluxes between white muscle and extracellular fluid, but cannot preclude small contributions from red muscle. The perfusion flow rate of 2 ml min^{-1} 100 g^{-1} tail weight was chosen as a compromise between O_2 delivery requirements and measurement accuracy for ΔT_{Amm} and other metabolites between inflow and outflow. This flow rate is about twice the blood flow rate to white muscle recorded during aerobic exercise in trout, or about 3-4 fold resting flow rates (23,32). The only estimates of white muscle blood flow in trout after exhaustive exercise of the type used here are those of Neumann *et al.* (21), who reported a 1.5 fold increase from resting levels.

In preliminary tests, we found that the use of 3% bovine serum albumin to provide colloid osmotic pressure was a major improvement relative to previous preparations employing polyvinylpyrrolidone or dextran as oncotic agents (16,18,28,33). Vascular reactivity was sustained, edema did not occur, and the preparation exhibited fluid volumes and intracellular ion, pH and T_{Amm} levels all comparable with those of our *in vivo* study on exhaustively exercised trout (29). The rate of O_2 consumption (Table 4-7) was about 25% of that measured *in vivo* on whole trout at rest or about 10% of that seen after exhaustive exercise (36). In view of the low metabolic rate of white muscle relative to aerobic tissues such as liver and gills, these figures seem quite reasonable. Certainly, the relatively high venous Po_2 (70 - 120 torr; Table 4-7) indicated that O_2 supply was not compromised. In our preliminary tests, when the perfusion rate was reduced to one third of the above rate or the arterial Po_2 was decreased to about 200 torr, venous Po_2 could be depressed to as low as 10-20 torr. This also suggests that the O_2 delivery to the preparation was far from insufficient.

Acid-base Status

Intracellular pH_i in the perfused trunk preparation (about 6.6; Fig. 4-3) was very similar to that measured in the white muscle of exhaustively exercised trout *in vivo*, and may be compared with resting values of about 7.2 both *in vivo* (25,27,29,36) and *in vitro* (16). As extracellular "respiratory" acid-base status was maintained constant at a resting level of Pa_{CO_2} in the post-exercise tail-trunks, the acidotic pH_i was due to intracellular "metabolic acidosis" (low HCO_3^-, confirmed by Y. Wang, G.J.F. Heigenhauser and C.M. Wood,unpublished results). This was undoubtedly due to H^+ generation from lactate production and ATP breakdown during exercise as documented in many studies, such as those cited above. The significantly lower pH_i in the high-K^+ group was likely caused by additional muscle twitching induced by membrane depolarization; these trunks had higher intracellular lactate levels.

In the present experimental design, changes in extracellular acid-base status were achieved by purely "metabolic" adjustments (*i.e.* changes in HCO_3^-) at constant Pco_2 (Table 4-1), whereas *in vivo* after exhaustive exercise, both factors change - increased Pco_2 and decreased HCO_3^-. White muscle pH_i remained constant at the post-exercise level independent of extracellular HCO_3^- and pH_e in both this *in vitro* study (Fig. 4-3) and in trout *in vivo* infused with a large dose of HCO_3^- after exhaustive exercise (27). These results suggest that metabolic acid-base disturbance in the ECF has minimal influence on intracellular acid-base status in white muscle, in contrast to the well-documented influence of ECF respiratory acid-base disturbance on muscle pH_i both *in vitro* (16) and *in vivo* (35).

Ammonia Distribution and Efflux

Assuming that passive movements of NH_3 and NH_4^+ are the only routes by which ammonia can be released from muscle cells, then changes in P_{NH3} gradients should dictate movements of the former, and changes in the net electrochemical gradients for NH_4^+ should dictate the latter. Our experimental design manipulated the former by changing pH_e (Fig. 4-3) and the latter by changing E_m (Table 4-6). The results show clearly that both factors had a significant influence on T_{Amm} efflux from the preparation. Thus, in the absence of changes in E_m (Table 4-6), T_{Amm} efflux decreased as pH_e increased (Fig. 4-6) and therefore the P_{NH3} gradient decreased (Fig. 4-5). Conversely T_{Amm} efflux increased with a partial depolarization by high-K^+ (Fig. 4-6; Table 4-6), *despite a marked reduction in the* P_{NH3} *gradient.* Specifically, a 47% decrease in efflux at high-pHe relative to med-pH_e was associated with an apparent 27% decrease in the P_{NH3} gradient from ICF to venous perfusate. Conversely, a 77% increase in efflux in the high-K^+ treatment was associated with a two-fold increase in the electrochemical gradient for NH_4^+ yet a 67% decrease in the P_{NH3} gradient. While both NH_3 and NH_4^+ movements are clearly important, it is impossible to calculate the exact contribution of each component without knowledge of exactly how the gradients are distributed along the arterial to venous pathway in muscle capillaries, and whether the absolute permeabilities change.

An alternative approach to the same general question is to examine the intracellular to extracellular distribution ratio of T_{Amm} (34,37,38), following the basic principles elaborated by Boron and Roos (2). By Equation 10, if permeability to NH_4^+ (PNH_4^+) predominates, then a relatively high $[T_{Amm}]i/[T_{Amm}]e$ dictated by E_m will result (*e.g.* Fig. 4-1B), whereas if permeability to NH_3 (PNH_3) predominates, by Equation 9 a relatively low $[T_{Amm}]i/[T_{Amm}]e$ dictated by the pH_e - pH_i gradient will result (*eg.* Fig. 4-1A). These ratios have fixed values for any fixed values of pH_i - pH_e and E_m, but will change when pH_i - pH_e and E_m vary. In the situation where both permeabilities are significant, the distribution ratio will be intermediate between the asymptotes set by pH_i - pH_e (low value of the ratio) and by E_m (high value of ratio) - see Fig. 4-1 of ref. 34 for a graphical representation of the relationship. The exact value will be dictated by the exact value of PNH_3 / PNH_4^+ as outlined in Equation 12. In absolute terms, PNH_4^+ does not have to quantitatively exceed PNH_3 for the distribution ratio to be dictated by E_m. Because there is so much more NH_4^+ than NH_3 in solution at physiological pH, even if PNH_4^+ is only 10% of PNH_3 (*i.e.* $PNH_3 / PNH_4^+ \approx 10$), then the ratio will approach the asymptote dictated by E_m. It is important to note that this analysis assumes steady-state conditions and that all ammonia is freely diffusible.

Application of this approach to the present data set revealed several interesting features (Fig. 4-7). Firstly, it reinforces the conclusion that both the pH gradient, acting on NH_3 distribution, and E_m, acting on NH_4^+ distribution, are important in setting the distribution of T_{Amm}. Thus, as pH_e increased at constant E_m, the measured distribution ratio increased in parallel to the pH-predicted distribution ratio. Conversely, as E_m was lowered by partial depolarization by high-K^+, the measured distribution ratio decreased in parallel to the E_m - predicted ratio. These changes occurred even though the ratios predicted by the reciprocal controlling factors did not change significantly. Secondly, at least at low-pH_e and medium-pH_e, the observed ratios were not significantly different from the E_m- predicted values, but remained far above the pH-predicted values. Thirdly, under all conditions, the measured distribution ratios were greater than the maximum ratios predicted by E_m, although these differences were only significant in the high-pH_e and high-K^+ treatments (Fig. 4-7). This situation precluded calculation of PNH_3 / PNH_4^+ from Equation 12.

This latter situation can only occur if steady-state conditions were not achieved, and/or if some of the intracellular ammonia was not freely diffusible. Both explanations are possible. Although we perfused for 1 h before making measurements, and calculated the distribution ratios with respect to the venous outflow, it remains possible that disequilibrium persisted, especially if intracellular ammonia production was continuing. This is suggested by the fact that intracellular T_{Amm} actually increased over the 60 min of perfusion. Recent data (summarized in Fig. 4-9) from our study (R. Henry, Y. Wang, and C.M. Wood, unpublished results) on perfused tail-trunks from resting trout casts some light on the situation. In that investigation, ammonia released from resting trunks was negligible, suggesting that production did not occur, and the measured distribution ratio was far below the maximum value predicted by E_m. Alternately or additionally, there is considerable evidence from mammalian studies that a portion of intracellular ammonia may be protein-bound or otherwise compartmentalized (see ref. 38 for a detailed discussion), and therefore not available for free diffusive exchange.

Amiloride was tested in the low-pH_e treatment where the observed distribution ratio most closely matched that expected from the effect of E_m on NH_4^+. The complete lack of effect of this drug (Table 4-8) at a concentration (10^{-4} M) which should block both Na^+/H^+, NH_4^+ exchangers and Na^+ channels (1) suggests that carrier mediation of this type is not involved in the movement of NH_4^+ across the white muscle cell membranes.

Comparison with In vivo Studies

Overall, the present results indicating an ammonia distribution after exhaustive exercise close to that predicted by E_m, at least under the physiologically realistic condition of low-pH_e, agree well with a number of investigations on exhaustively exercised teleost fish *in vivo* (27,29,38,39). All of these studies have indicated that ammonia is distributed between white muscle ICF and ECF approximately according to E_m, and not according to pH_e - pH_i after exercise, *i.e.* that NH_4^+ permeability effectively predominates. In the present study, artificially raising pH_e by HCO_3^- infusion after exhaustive exercise *in vivo* markedly increased the distribution ratio as more T_{Amm} accumulated in the ICF, and less appeared in the blood plasma (27). Therefore, both *in vivo* and *in vitro*, PNH_4^+ is sufficiently large relative to PNH_3 after exhaustive exercise that ammonia distribution is largely governed by E_m acting on NH_4^+. Nevertheless, PNH_3 is substantial, so ammonia efflux responds sensitively to the pH_e-pH_i gradient. These statements are not contradictory for flux and distribution ratio are not the same quantity. Thus, in the dynamic post-exercise situation at low-pH_e, the Nernst potential for NH_4^+ is almost identical to E_m (Fig. 4-8) so there is no *net* driving force for NH_4^+ to leave the cells, but there is a large P_{NH3} gradient for NH_3 to leave the cells (Fig. 4-5).

Under resting conditions, intracellular production of ammonia is presumably very small due to an absence of adenylate breakdown. Nevertheless, most *in vivo* studies on resting teleost fish have indicated that ammonia is again distributed according to E_m (27,31, 38,39). Under these conditions, the electrochemical gradient for inward movement of NH_4^+ would be balanced by the P_{NH3} gradient for outward movement of NH_3 (Fig. 4-1B). Heisler (11) has objected to this conclusion on theoretical grounds, specifically that the resulting inward "H^+ shuttle" would place an intolerable load on the pH_i regulatory mechanisms of the cells.

Fig. 4-9 illustrates that in the perfused trout trunk under resting conditions (R. Henry, Y. Wang, and C.M. Wood, unpublished results) and in our recent *in vivo* study on trout (29), the $[T_{Amm}]i/[T_{Amm}]e$ distribution ratio was much lower than in all previous studies, far lower than that predicted by E_m, and in fact approximated that predicted by pH_e-pH_i. The reason for the discrepancies between these resting data and those of many previous studies is unclear, but it should be noted that resting ratios are subject to the greatest error because of the low plasma T_{Amm} concentrations, the greatest bias due to arterial *vs.* venous sampling, and the difficulty of obtaining muscle samples from resting fish without ammonia production from adenylate breakdown (30). These problems do not apply in the resting perfused trunk preparation.

The *in vitro* and *in vivo* patterns of Fig. 4-9 show remarkable agreement, and suggest that the situation may change from a pH-dictated distribution at rest to a E_m-dictated distribution during recovery from exhaustive exercise. As pointed out earlier (29), the advantages of this switchover could be considerable. At rest, the costly H^+ shuttle would be avoided, whereas after exercise, the E_m-dictated distribution would help retain much higher levels of ammonia in the muscle for greater intracellular buffering and ATP resynthesis. If this is the case, then the PNH_3 /PNH_4^+ ratio of the white muscle cell membranes must decrease between rest and post-exercise; acidosis itself might be a controlling factor.

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Figure 4-1.

Schematic models of ammonia transport and distribution across muscle cell membrane. Model A shows the scenario when the membrane is only permeable to NH_3 and Model B shows the scenario when the membrane also has a significant permeability to NH_4^+. Note the very different distribution ratios which result.

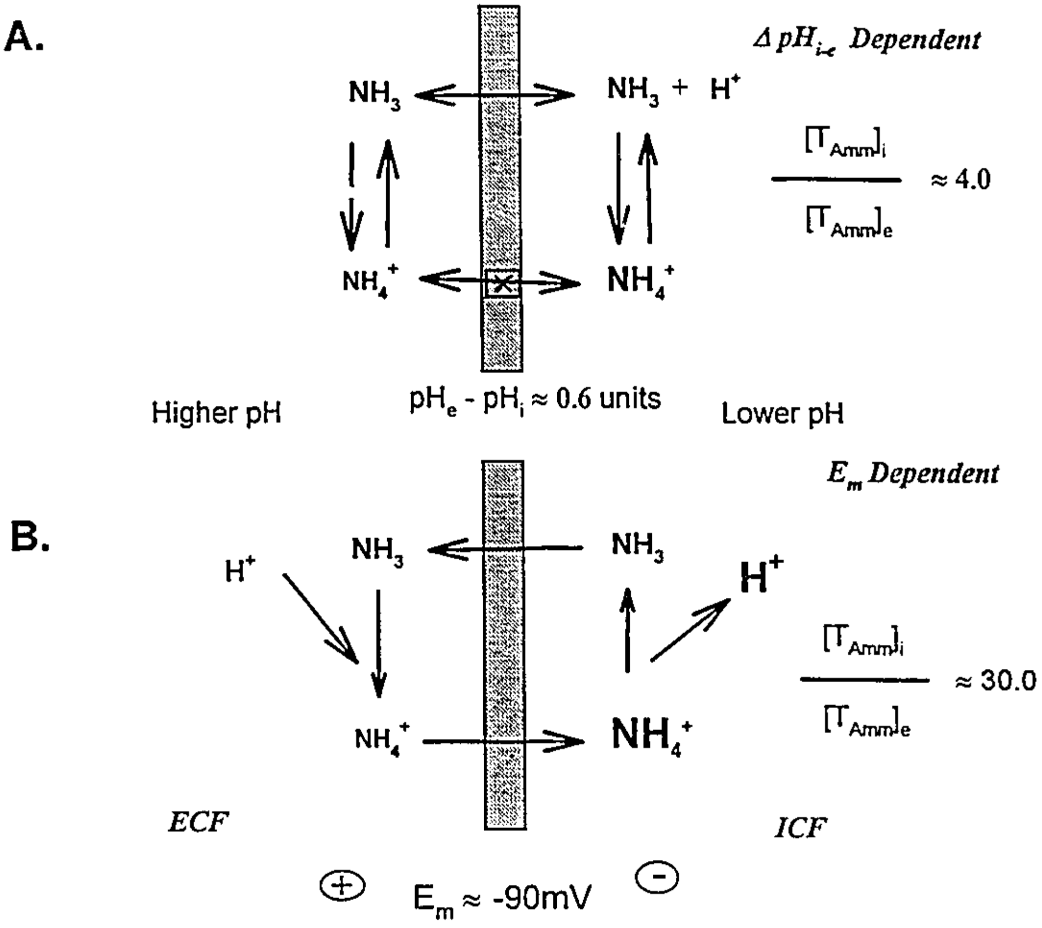
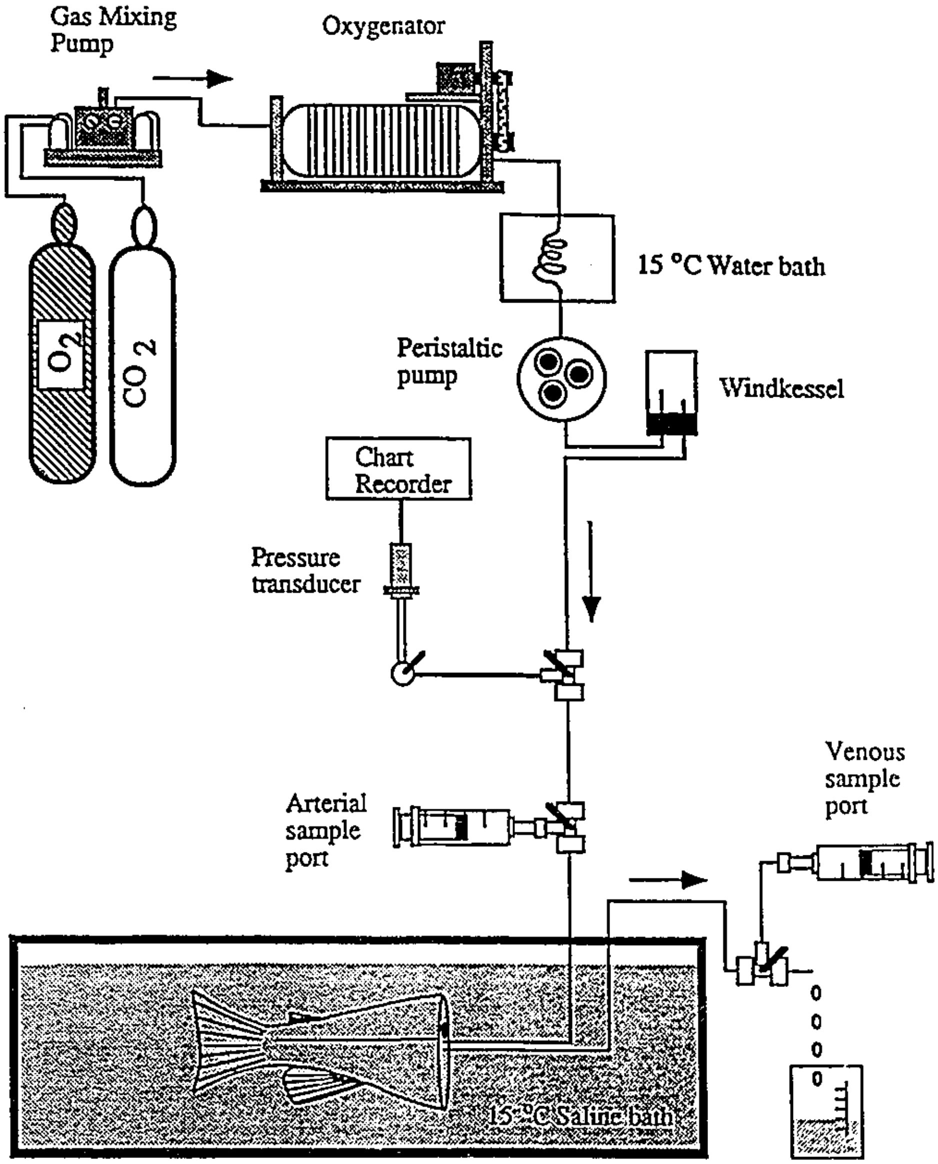

A.
ΔpH_{i-e} Dependent
NH₃
NH₃ + H⁺
NH₄⁺
NH₄⁺
[T_Amm]_i / [T_Amm]_e ≈ 4.0
Higher pH
pH_e - pH_i ≈ 0.6 units
Lower pH
E_m Dependent
B.
H⁺
NH₃
NH₃
H⁺
NH₄⁺
NH₄⁺
[T_Amm]_i / [T_Amm]_e ≈ 30.0
ECF
ICF
+
E_m ≈ -90mV
-

Figure 4-2.

Schematic diagram of the isolated-perfused tail-trunk preparation of the rainbow trout.


Gas Mixing Pump
Oxygenator
O2
CO 2
15 °C Water bath
Peristaltic pump
Windkessel
Chart Recorder
Pressure transducer
Arterial sample port
Venous sample port
15 °C Saline bath

SDR Production

Figure 4-3.

pH in the inflowing saline (arterial, pH_a, filled bars), outflowing saline (venous, pH_v, open bars), and intracellular fluid (pH_i, stippled bars) of white muscle in the four treatment groups of isolated-perfused tail-trunk preparations. Trunks were taken from trout exercised to exhaustion, and values recorded after 60 min perfusion. Values are means ± 1 S.E.M., N = 7, 11, 8, 6 for the low-pH, medium-pH (= control), high-pH, and high-K^+ groups, respectively. * indicates significant difference ($P \leq 0.05$) from the corresponding control value.

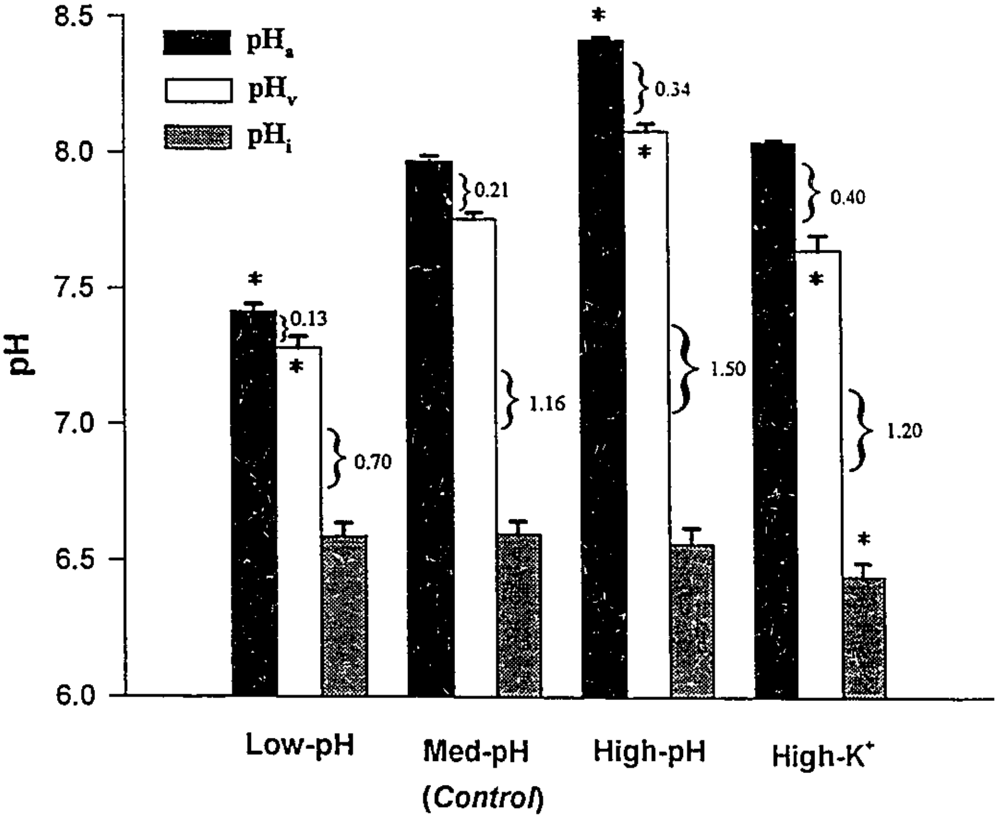

8.5
8.0
7.5
7.0
6.5
6.0
pH
pH_a
pH_v
pH_i
*
0.13
*
0.70
0.21
1.16
*
0.34
*
1.50
0.40
*
1.20
*
Low-pH
Med-pH
(*Control*)
High-pH
High-K$^+$

Figure 4-4.

Total ammonia concentrations in inflowing saline (arterial, $T_{Amm}a$, filled bars), outflowing saline (venous, $T_{Amm}v$, open bars), and intracellular fluid ($T_{Amm}i$, stippled bars) of white muscle in the four treatment groups of isolated-perfused tail-trunk preparations. Trunks were taken from trout exercised to exhaustion, and values recorded after 60 min perfusion. Other details as in legend of Figure 4- 3.

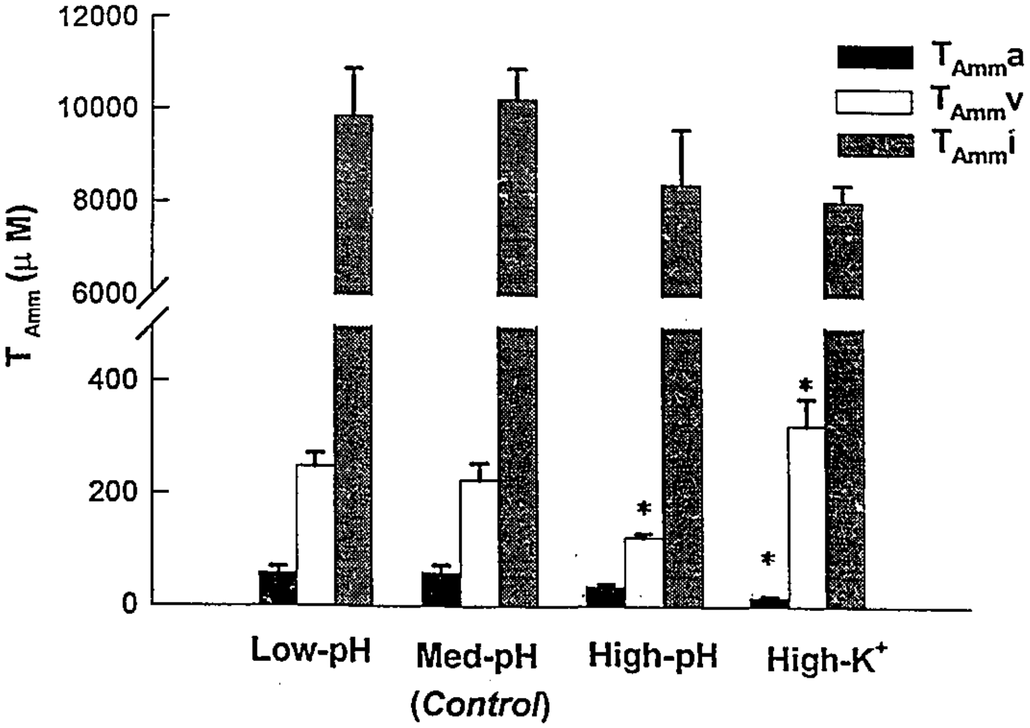
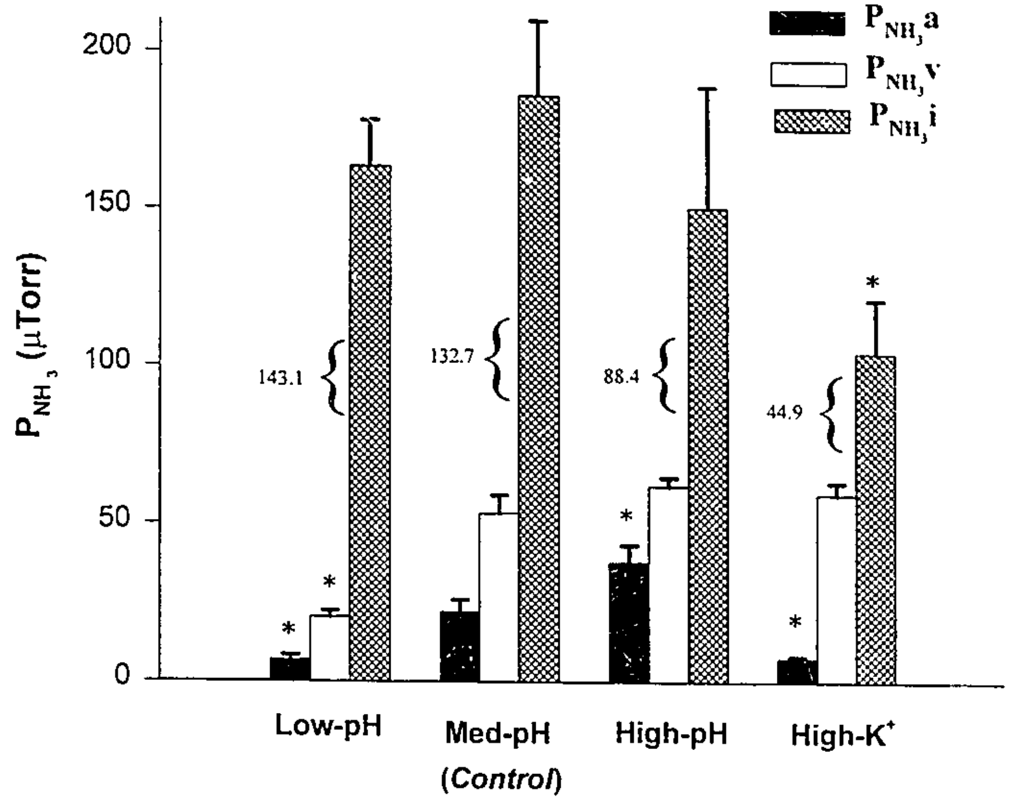

T_{Amm} (μM)
12000
10000
8000
6000
400
200
0
$T_{Amm}a$
$T_{Amm}v$
$T_{Amm}i$
*
*
*
Low-pH
Med-pH
(*Control*)
High-pH
High-K$^+$

Figure 4-5.

Partial pressures of NH_3 in inflowing perfusate (arterial, $P_{NH3}a$, filled bars), outflowing perfusate (venous, $P_{NH3}v$, open bars) and intracellular fluid ($P_{NH3}i$, stippled bars) of white muscle in the four treatment groups of isolated-perfused tail-trunk preparations. Brackets indicate the sizes of the mean $P_{NH3}i$ -$P_{NH3}v$ gradients. Trunks were taken from trout exercised to exhaustion, and values recorded after 60 min perfusion. Other details as in legend of Figure 4-3. (70 μTorr = 10 mPa).



$P_{NH_3}a$
$P_{NH_3}v$
$P_{NH_3}i$
P_{NH_3} (μTorr)
0
50
100
150
200
143.1
132.7
88.4
44.9
*
Low-pH
Med-pH
(*Control*)
High-pH
High-K$^+$

Figure 4-6.

Total efflux rates of ammonia (T_{Amm}) from the post-exercise trout tail-trunk preparations after 60 min perfusion in the four treatment groups. Efflux rates were expressed as μmol h^{-1} kg^{-1} wet weight of tail-trunk. Other details as in legend of Figure 4-3.

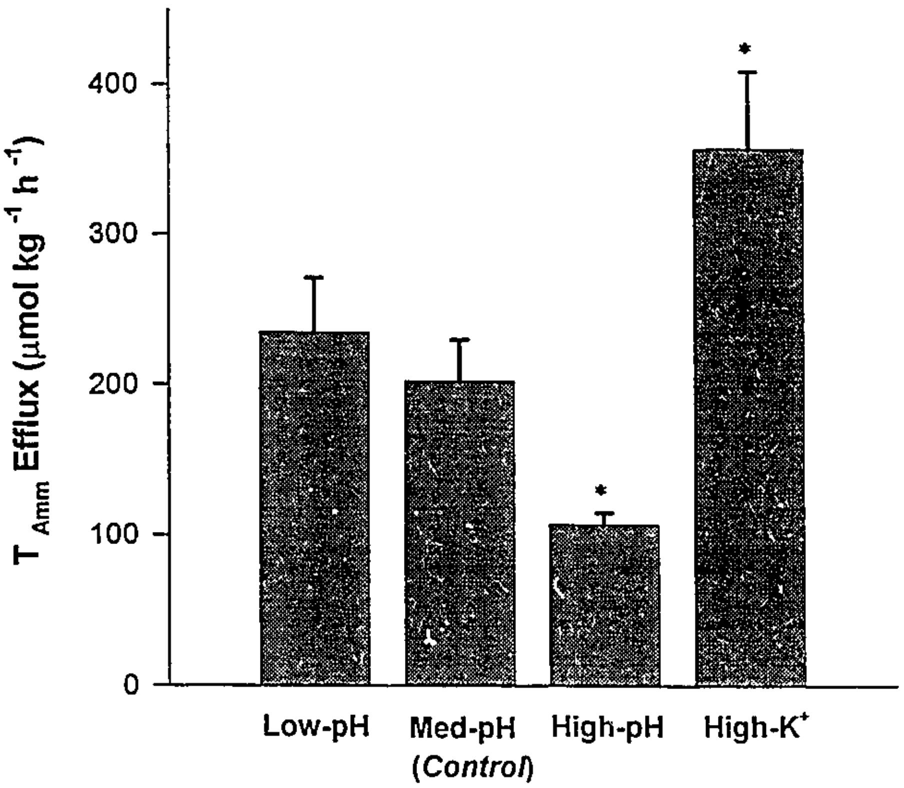

T Amm Efflux (μmol kg -1 h -1)
0
100
200
300
400
*
*
Low-pH
Med-pH (Control)
High-pH
High-K+

Figure 4-7.

Transmembrane distribution ratios of total ammonia ($T_{Amm}i/T_{Amm}e$) from intra- to extra-cellular fluid (venous outflowing perfusate) of white muscle in the four treatment groups of isolated-perfused tail-trunk preparations. Measured values (open bars) are compared with values (solid bars) predicted from the measured transmembrane pH gradients (ΔpH_{i-v}) by equation 9, assuming permeability only to NH_3, and with values (stippled) predicted from the membrane potential E_m by equation 10, assuming permeability only to NH_4^+. # indicates significant difference ($P \leq 0.05$) from the corresponding measured ratio. * indicates significant difference ($P \leq 0.05$) from the corresponding ratios in the medium pH (= control) group. Other details as in legend of Figure 4-3.

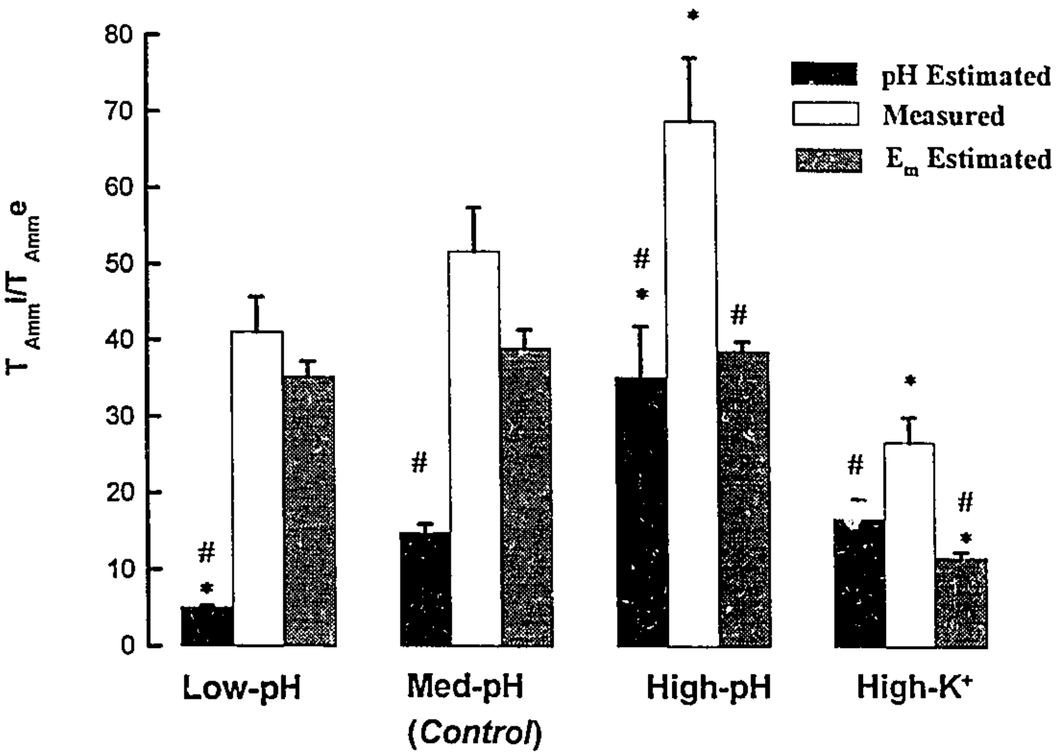

80
70
60
50
40
30
20
10
0
$T_{Amm}{}^{i}/T_{Amm}{}^{e}$
pH Estimated
Measured
E_m Estimated
#
*
#
#
*
*
#
#
*
#
#
*
Low-pH
Med-pH
(*Control*)
High-pH
High-K$^+$

Figure 4-8.

Comparison between the membrane potentials (E_m) estimated from measured intra- to extracellular K^+, Na^+ and Cl^- distributions by equation 11 (stippled bars), and Nernst potentials calculated from measured NH_4^+ distribution ratios by equation 10 (open bars) in the four treatment groups of isolated-perfused tail-trunk preparations. * indicates significant difference ($P \leq 0.05$) from the corresponding value in the medium-pH (= control) group. # indicates significant difference ($P \leq 0.05$) from the corresponding value estimated by equation 11. Other details as in legend of Figure 4-3.

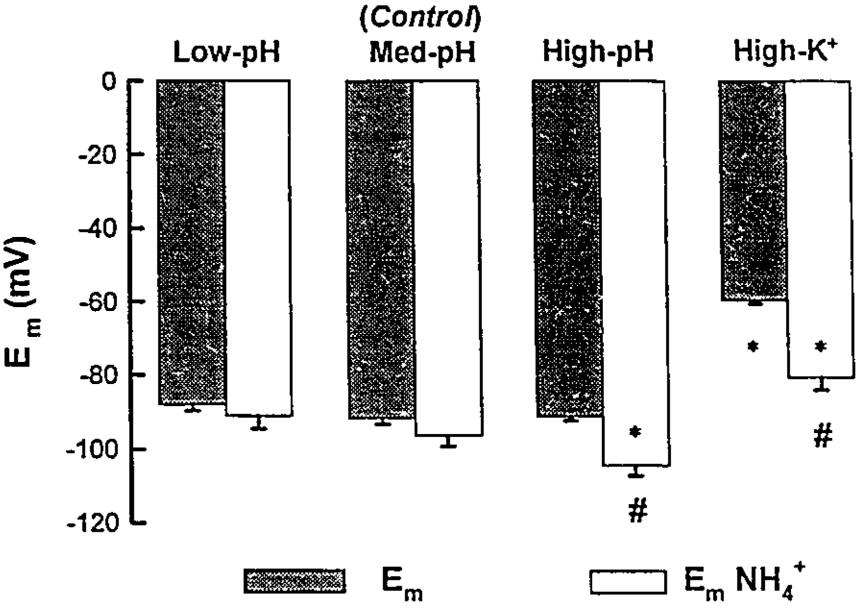

(Control)
Low-pH
Med-pH
High-pH
High-K+
0
-20
-40
-60
-80
-100
-120
E m (mV)
*
*
*
#
#
E m
E m NH4+

Figure 4-9.

A comparison of total ammonia distribution ratios ($T_{Amm}i/T_{Amm}e$) across white muscle cell membranes *in vivo* (at rest and at 60 min post-exercise) and *in vitro* in an isolated tail-trunk perfusion preparation (at rest and low-pH_e 60 min post-exercise). The measured values (open bars) are compared with values (solid bars) predicted from the measured transmembrane pH gradients by equation 9 and values (stippled bars) predicted from the membrane potential E_m (Equation 10). * indicates significant difference ($P \leq 0.05$) from corresponding measured ratios. The resting *in vitro* data were obtained from a separate study (R. Henry, Y. Wang, and C.M. Wood, unpublished results). The *in vivo* post-exercise data were obtained from Wang *et al.* (29).

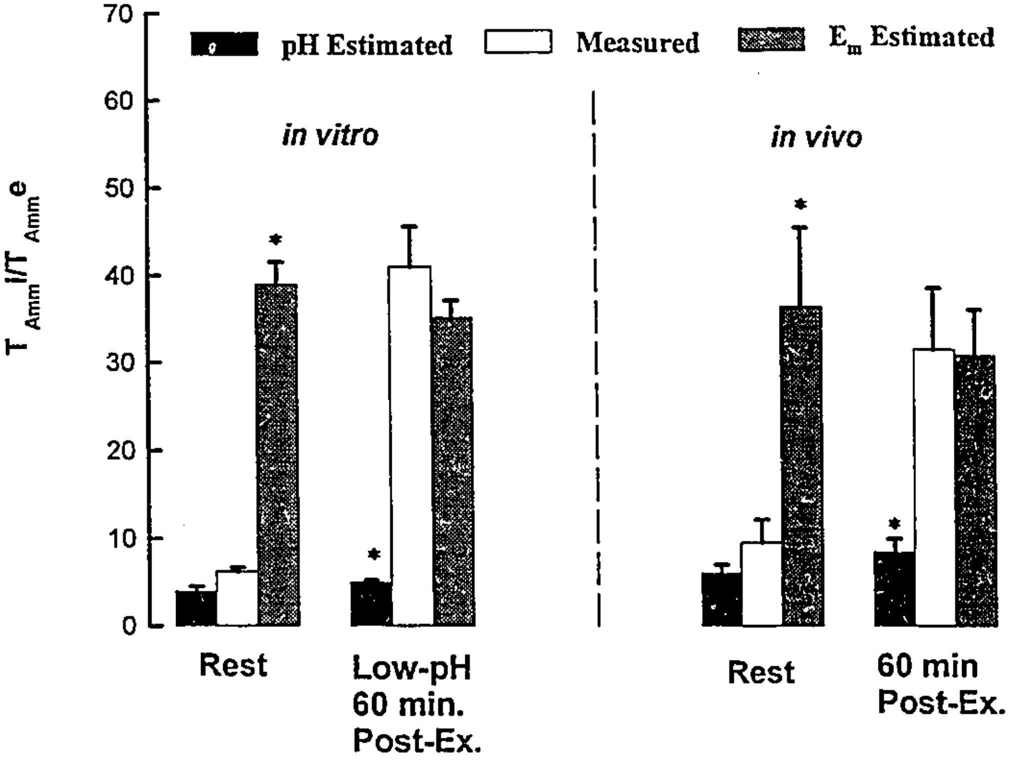

pH Estimated
Measured
E_m Estimated
$T_{Amm}i/T_{Amm}e$
0
10
20
30
40
50
60
70
in vitro
in vivo
*
*
*
*
Rest
Low-pH 60 min. Post-Ex.
Rest
60 min Post-Ex.

Table 4-1. Expected inflow saline pH, Pco_2, $[HCO_3^-]$, and $[K^+]$ in the four experimental treatment groups.

Treatment Groups	**pH**	**Pco_2 (Torr)**	**$[HCO_3^-]$ (mM)**	**$[K^+]$ (mM)**
Low-pH	7.4	2	2	3
Medium-pH (Control)	7.9	2	7	3
High-pH	8.4	2	18	3
High-K^+ (Depolarized)	7.9	2	7	15

Table 4-2. Net perfusion pressure after 30 and 60 min perfusion in the four experimental treatment groups.

	Low-pH	**Medium-pH (Control)**	**High-pH**	**High-K$^+$ (Depolarized)**
30min	10.3±1.1	11.6±1.0	11.1±1.4	8.7±0.9
60min	17.7±2.7*	18.6±2.2*	16.1±3.1*	16.7±2.7*
	N = 7	N = 11	N = 8	N = 6

Values are means ± SEM for each treatment group (cm H_2O).

* Significantly different from the corresponding 30 min values.

Table 4-3. The white muscle tissue water content ($[H_2O]_t$), intracellular fluid volume (ICFV), and extracellular fluid volume (ECFV) after 60 min of perfusion in the four experimental treatment groups.

	Low-pH	**Medium-pH (Control)**	**High-pH**	**High-K^+ (Depolarized)**
$[H_2O]_t$ ($ml\ g^{-1}$)	0.795 ± 0.005	0.784 ± 0.001	0.779 ± 0.002	0.769 ± 0.009
ICFV ($ml\ g^{-1}$)	0.719 ± 0.005	0.703 ± 0.008	0.698 ± 0.008	0.735 ± 0.012*
ECFV ($ml\ g^{-1}$)	0.076 ±0.005	0.081 ± 0.008	0.080 ± 0.008	0.034 ± 0.016*
	N = 7	N = 11	N = 8	N = 6

Values are means ± SEM for each treatment group (ml g^{-1} wet tissue).

* Significantly different from the corresponding control values.

Table 4-4. The intracellular Na^+, K^+ and Cl^- concentrations in trout white muscle after 60 min of perfusion in the four experimental treatment groups.

	Low-pH	Medium-pH (Control)	High-pH	High-K^+
$[Na^+]_i$	10.8±0.7	15.6±2.2	10.4±2.0	21.0±0.9*
$[K^+]_i$	150.0±3.6	160.8±5.1	156.9±3.7	150.5±7.4
$[Cl^-]_i$	3.6±0.2	3.3±0.2	2.7±0.1	12.5±0.9*
	N = 7	N = 11	N = 8	N = 6

Values are means ± SEM for each treatment group (mmol l^{-1} ICF water).

* Significantly different from the corresponding control values.

Table 4-5. Ionic concentrations (Na^+, K^+, and Cl^-) in arterial and venous perfusate water after 60 min perfusion in the four experimental treatment groups.

	Low-pH	**Medium-pH (Control)**	**High-pH**	**High-K^+ (Depolarized)**
$[Na^+]_a$	154.7± 8.8	165.8 ±5.2	163.8 ±5.9	142.8 ±1.7
$[Na^+]_v$	154.5 ±4.3	167.3 ±4.2	163.1± 7.6	144.6 ±0.8
$[K^+]_a$	3.08±0.12	2.96±0.16	2.94±0.12	14.05±0.71
$[K^+]_v$	3.65±0.22*	3.52±0.19*	3.29±0.10	12.45±0.76*
$[Cl^-]_a$	153.4±2.4	151.0±3.3	128.7±1.9	161.8±2.3
$[Cl^-]_v$	148.4±2.3	146.8±1.6	130.5±2.2	157.0±1.4
	N = 7	N = 11	N = 8	N = 6

Values are mean ± SEM for each treatment group (mmol l^{-1} perfusate water).
* Significantly different from the corresponding arterial values.

Table 4-6. Transmembrane Nernst potentials (E_m) in the four experimental treatment groups after 60 min perfusion, as estimated from the measured intra- and extracellular [Na^+], [K^+], and [Cl^-], according to the Goldman-Hodgkin-Katz equation (Eq. 11). E_m values calculated with respect to arterial inflow and venous outflow perfusate compositions are compared.

	Low-pH	**Medium-pH (Control)**	**High-pH**	**High-K^+ (Depolarized)**
Arterial	-89.9±1.1	-93.7±1.6	-91.5±1.4	-58.8±2.0*
Venous	-88.0±1.6	-91.8±1.5	-91.4±1.0	-59.7±1.0*
	N = 7	N = 11	N = 8	N = 6

Values are means ± SEM for each treatment group.

* significantly different from corresponding control value.

Table 4-7. In- and outflowing perfusate Po_2, O_2 consumption, and CO_2 efflux rate of the tail-trunk preparation after 60 min perfusion.

	Low-pH	**Med-pH (Control)**	**High-pH**	**High-K$^+$ (Depolarized)**
Po_2a (torr)	321.4±24.0*	399.0±16.3	413.8±14.0	417±5.95
Po_2v (torr)	85.0±10.4	115.6±22.8	105.5±12.1	74.0±7.4*
Mo_2 (mmol kg^{-1} h^{-1})	0.48±0.03*	0.60±0.03	0.66±0.03	0.73±0.02*
CO_2 efflux (mmol kg^{-1} h^{-1})	1.45±0.02*	1.20±0.15	0.35±0.23*	1.47±0.14
	N = 7	N = 11	N = 8	N = 6

Values are means ± SEM for each treatment group.

* Significantly different from the corresponding control values.

Table 4-8. A comparison of total ammonia distribution ratio across the white muscle cell membrane (intracellular to venous) and related parameters between the low-pH and low-pH plus amiloride (10^{-4} M) treatment groups.

Treatment Group	Amm Flux (μmol kg^{-1} h^{-1})	Measured Amm Ratio	E_m-est Amm ratio	pH-est Amm ratio	$E_{NH_4^+}$ (mV)
Low-pH (N=7)	228.37±34.40	40.99±4.64	35.12±2.03	4.93±0.26	-91.14±3.37
Low-pH plus Amiloride (N=11)	251.26±22.95	35.59±3.16	40.12±0.95	5.33±0.28	-88.66±4.15

Values are means ± SEM for each treatment group.

There were no significant differences ($P > 0.05$) in any parameters between the two groups.

CHAPTER 5

LACTATE AND METABOLIC H^+ TRANSPORT AND DISTRIBUTION AFTER EXERCISE ACROSS WHITE MUSCLE CELL MEMBRANES IN RAINBOW TROUT: A PERFUSION STUDY

ABSTRACT

An isolated-perfused tail-trunk preparation was employed to study the influence of transmembrane pH gradient and membrane potential (E_m) on the transport and distribution of L(+)-lactate (Lac), metabolic H^+ (ΔH_m^+), and related parameters in post-exercise rainbow trout white muscle. One resting (arterial pH, $pH_a \approx 7.9$) and four post-exercise treatments (low-$pH_a \approx 7.4$, medium-$pH_a \approx 7.9$, high-$pH_a \approx 8.4$, and high-K^+, $pH_a \approx 7.9$, partially depolarized by 15 mM K^+), were examined. The desired arterial extracellular pH (pH_a) was achieved by varying HCO_3^- concentration (2-18 mM) in a lactate-free saline while maintaining a constant P_aco_2 (≈ 2 torr). Fish were manually chased for 6 min to exhaustion to establish a typical post-exercise lactacidosis in white muscle. The elevated intracellular Lac (≈ 50 mM) remained unchanged after 60 min of perfusion due to very low rates of lactate efflux and oxidation, the latter indicated by the lack of increase in O_2 consumption. H^+, HCO_3^-, and Lac^- distributions were all well out of electrochemical equilibrium. Relatively high transmembrane Pco_2 gradients were attributed to a CO_2/HCO_3^- disequilibrium induced by the

lack of carbonic anhydrase in the perfusate. Total CO_2 efflux was reduced at high extracellular pH (high-pH_e); alterations in the net driving force (NDF) on HCO_3^- may have overshadowed the influence of Pco_2 gradients in driving total CO_2 efflux. Despite a well matched intracellular loading of Lac and ΔH_m^+, Lac efflux and ΔH_m^+ flux were completely uncoupled. ΔH_m^+ flux reacted to both acid-base and electrochemical gradients as ΔH_m^+ efflux dropped and even reversed since pH_e decreased, while partial depolarization in conjunction with depressed intracellular pH (pH_i) resulted in elevated ΔH_m^+ efflux. Lac efflux did not respond to changes in pH_e. Changes in Lac efflux corresponded more closely to changes in the Lac^- concentration gradient than to alterations in the HLac gradient. This study provides circumstantial evidence for the involvement of electroneutral mechanisms (*i.e.* Lac^-/H^+ co-transport and/or Lac^-/anion exchange) in Lac efflux, but does not eliminate the possibility of an active transport mechanism contributing to the retention of Lac against substantial passive gradients in trout white muscle.

INTRODUCTION

In fish as well as in many other animals, intensive exercise induces an accelerated lactate and metabolic H^+ production in skeletal muscle, especially in fast twitch glycolytic muscle (or type IIa, white), *via* "anaerobic" glycolysis. Intramuscular lactic acidosis occurs when ATP breakdown exceeds ATP supply through oxidative metabolism. The fate of this lactate has drawn tremendous attention in the last few decades due to its important roles in both metabolic and acid-base regulation; in mammals, the current picture, while somewhat controversial, suggests multiple fates. Owing to the paucity of mitochondria in white muscle, the traditional view has been that efflux of lactate *via* the bloodstream to the liver for both oxidation and glucose/glycogen resynthesis (*i.e.* the Cori cycle) is the major fate. More recent studies suggest that oxidation in cardiac and slow oxidative skeletal muscle may also occur and even that *in situ* oxidation and glycogenesis within the white muscle itself are possible. Nevertheless, the release of lactate from muscle to blood has been demonstrated to account for a large portion of its total disappearance (5,9,10,28,29).

In fish, the situation appears to be rather different. A large number of studies have now described the phenomenon of post-exercise lactacidosis using the rainbow trout as a model system (see 27,45,47 for reviews). There is general accord that net lactate release to the bloodstream is very low, that the Cori cycle does not occur (indeed, liver ablation does not slow lactate clearance - 21), and that the vast majority of the lactate and metabolic H^+ ions stay in the muscle to be slowly oxidized or resynthesized into glycogen *in situ*. The details of this metabolism remain poorly understood; nevertheless, the white muscle appears to function almost as a closed system in this regard.

Despite the limited knowledge on the mechanisms responsible for this large "retention of lactic acid" in white muscle of fish, or on the mechanisms which permit the movements of only small amounts of lactate and metabolic H^+ into the bloodstream, some evidence suggests that lactate and metabolic H^+ effluxes occur at differential rates, but since most of this evidence is based on measurements of blood concentrations rather than actual flux rates, the conclusion is uncertain (47). Other evidence suggests that lactate may be retained in white muscle by an inwardly directed active transport mechanism, though the data are at best circumstantial (2,21,40). Recently, we carried out a detailed analysis of the pH and electrical gradients in the white muscle of rainbow trout *in vivo* after exhaustive exercise (41). This indicated that neither lactate nor H^+ were distributed according to the membrane potential E_m. Indeed, both were held well out of electrochemical equilibrium, with deviations in opposite directions - *i.e.* lactate was retained against the driving gradient, whereas H^+ was extruded against the driving gradient. The alternate possibility, that the distribution of both was driven by the partitioning of the free acid "HLac" according to the transmembrane pH gradient, was also not supported by the data. This is in contrast to the findings in higher vetebrates, where the changes in extracellular pH resulted in corresponding intracellular pH and transmembrane lactate relocation (17,33,44).

The objective of the present study was to extend this analysis using an *in vitro* preparation of the rainbow trout in which the both the pH gradients and electrical gradients could be independently manipulated, and the resulting distributions and effluxes of lactate and metabolic H^+ recorded. Recently, I have developed a perfused tail-trunk preparation for these purposes and used it to characterize the distribution and transport of ammonia in trout white muscle after exercise (42). In the present study, I have used the preparation to study the behavior of lactate, metabolic H^+ ions, and related metabolic parameters - O_2, CO_2, and HCO_3^- - in post-exercise trout muscle. Some of the original evidence for the differential movement of lactate and metabolic H^+ out of trout white muscle, and for the active retention of lactate, was obtained using a perfused tail-trunk preparation (40).

MATERIALS AND METHODS

This study is based on the same set of experiments with the isolated-perfused tail-trunk preparations of the rainbow trout reported by Wang *et al.* (42). In the previous study, the experiments characterized post-exercise transport and distribution of ammonia across white muscle cell membranes. The present study presents the simultaneous characterization of post-exercise lactate, metabolic acid, and CO_2 transport and distribution. An additional resting series was also performed. Except for differences in some analytical procedures and calculations which are described here, the reader is referred to Wang *et al.* (42) for detailed methods.

Experimental Animals

Exhaustive exercised protocol was used to elevate white muscle lactate, CO_2 and metabolic H^+ levels in fish. Rainbow trout (800 - 1000 g), acclimated to 15 ± 1°C, were manually chased for 6 min to induce exhaustive exercise, a treatment which has been shown to produce very uniform changes in white muscle metabolite and acid-base status (39,41,43, 47). Immediately after exercise, the fish were sampled. A resting series was performed as a comparative benchmark for the post-exercise series. Resting fish were held in dark acrylic boxes served with aerated flowing water at 15 ± 1°C for about 48 h prior to the experiment. All trout were sacrificed without disturbance by exposure to a high concentration of neutralized MS-222 (0.5 g l^{-1}). The portion of the fish posterior to the anus was severed for the isolated-perfused tail-trunk preparation.

Experimental Design and Perfusion Protocols

This study was designed to examine the effect of changes in transmembrane pH and electrical gradients on the flux and distribution of lactate, CO_2, HCO_3^- and metabolic H^+.

Lactate-free Cortland salmonid saline with 3% bovine serum albumin (Sigma, Fraction V) was used as the perfusate. Changes in the transmembrane pH gradient were accomplished by varying inflow (arterial) saline pH (pH_a = pH_e), and changes in transmembrane electrical gradient, by partial depolarization using elevated perfusate [K^+]. This study consisted of one resting ($pH_e \approx 7.9$) and four post-exercise treatment groups as shown in Table 5-1: low-$pH_e \approx 7.4$, medium-$pH_e \approx 7.9$, high-$pH_e \approx 8.4$, and high-K^+ ($pH_e \approx 7.9$, partially depolarized). The medium-pH_e treatment was designated as the control for the exercise groups. The desired pH_e in each treatment was manipulated by altering inflow (arterial) [HCO_3^-] from 2 to 18mM (Fig. 5-2) with reciprocal changes in [Cl^-] (*i.e.* [Na^+] unchanged) at constant $Pco_2 \approx 2$ torr (Fig. 5-3a). Partial depolarization of the transmembrane electrical gradient (E_m) was achieved by elevating perfusate [K^+] from ~3mM to ~15mM (high-K^+) with KCl, while pH and [HCO_3^-] levels were kept at the control level. Unlike the pH treatment groups, where [Na^+] was kept intact, saline [NaCl] was reduced in accordance with elevated [KCl] to avoid excessive [Cl^-] in the saline.

The tail-trunk preparation was set up and perfused as described previously - see Fig. 5-2 of Wang *et al.* (42). Perfusion saline was equilibrated with ~0.25% CO_2, balance O_2, the perfusion rate was 2 ml min^{-1} $100g^{-1}$ tail weight, and the experimental temperature throughout the perfusion was 15±0.5°C. Sampling ports allowed the collection of discrete arterial inflow (caudal artery) and venous outflow (caudal vein) samples. The first 30 min of perfusion served to stabilize the preparation and purge red blood cells. At 30 and 60 min, arterial and venous perfusate samples (2 ml) were collected into gas-tight Hamilton syringes. The samples were analyzed immediately for perfusate pH, Po_2, total CO_2 (Tco_2), and protein content, while a portion (300 μl) was deproteinized with 600 μl of 8% perchloric acid (PCA) and stored at -

70°C for later analysis of Lac. The remainder of the sample (500 μl) was also frozen for later electrolyte measurements (Na^+, K^+, Cl^-).

Muscle tissue was sampled to determine intracellular pH (pH_i), Tco_2, Lac, Cl^-, Na^+, K^+ and water content. At the time the tail was cut off, an initial white muscle sample (3-5g) from the area between the dorsal fin and the lateral line immediately *anterior* to the point of section was preserved by freeze-clamping with pre-cooled aluminum tongs in liquid N_2. After 60 min of perfusion, a final muscle sample was similarly preserved from the same position, but on the perfused tail-trunk. The muscle samples were stored at -70°C prior to analysis.

Analytical Techniques

Muscle tissue was ground to a fine powder using an insulated mortar and pestle cooled with liquid N_2; the frozen powder was then used directly for various analyses or lyophilized for 64 h and then stored again at -70°C. Methods for measurement of muscle Na^+, K^+, Cl^-, and total water content have been described previously (42). Muscle pH_i and Tco_2 were both measured according to the homogenization technique (31). In brief, approximately 200 mg of frozen muscle powder was transferred to 800 μl of ice-cold metabolic inhibitor (150 mmol l^{-1} potassium fluoride; 6 mmol l^{-1} sodium nitrilotriacetate, Sigma) in a 1.5 ml sealed centrifuge tube, mixed thoroughly, and weighed exactly. The homogenate was then centrifuged at 9,000g for a few seconds. One portion of the supernatant was used for pH_i measurement (42), and another portion (0.2 ml) was injected into a 5 ml gas-tight Hamilton syringe filled with 2.5 ml of helium and 0.5 ml of 0.1N HCl. The resulting gas phase sample was analyzed on a gas chromatograph analyzer (Shimadzu GC-8A with sample and reference columns packed with porapack Q 80/120, column/injection temperature 80/100°C). Helium (28 ml min^{-1}, 2 kg cm^{-2}) was used as carrier gas.

For the measurement of muscle Lac, lyophilized tissue powder (~20mg) was weighed, extracted with 1ml of ice-cold 8% PCA for 1 h, then centrifuged at 9,000g for 2 min. The supernatant was neutralized with Tris buffer and analyzed for Lac by the lactate dehydrogenase, NAD/NADH assay (3).

Parallel analyses of extracellular pH, Tco_2, Po_2, electrolytes and Lac were carried out on both arterial and venous perfusate by the same methods as for muscle tissue and plasma described above and/or as in our earlier studies (41,42). Total protein and water content of saline were measured with an American Optical refractometer to monitor possible fluid shifts between compartments.

Calculations and Statistics

Both intra- and extracellular Pco_2 (P_ico_2, P_aco_2, and P_vco_2) and HCO_3^- ($[HCO_3^-]_i$, $[HCO_3^-]_a$, and $[HCO_3^-]_v$) were calculated by manipulation of the Henderson-Hasselbalch equation using appropriate constants (αCO_2 and pK') for rainbow trout plasma and white muscle at 15°C (4,12,30).

The 'metabolic acid' (ΔH_m^+) released from the perfused muscle ICF to the perfusate was calculated according to the following equation (26):

$$\Delta H_m^+ = [HCO_3^-]_1 - [HCO_3^-]_2 - \beta(pH_1 - pH_2) \tag{1}$$

where subscripts *1* and *2* refer to measured values in arterial inflow and venous outflow perfusate, respectively. The non-bicarbonate buffer capacity ($\beta = -5.54 \pm 0.15$ mM $[HCO_3^-]$ pH $unit^{-1}$) of the perfusion saline was determined previously (42) and did not differ amongst treatments. The ΔH_m^+ concentration in post-exercise muscle ICFV could also be calculated according to Eq. 1. where subscripts *1* and *2* stand for resting and post-exercise values, respectively. The β value for rainbow trout white muscle ICF (-73.59 ± 4.87 mM [HCO_3^-] pH unit^{-1}) was taken from Milligan and Wood (25).

Flux rates of metabolic substrates/products - *i.e.* O_2, CO_2, HCO_3^-, metabolic acid , Lac, and ions were calculated using the Fick principle, based on the constant perfusion rate (2 ml 100 g^{-1} tail weight min^{-1}) and the measured differences in concentration between arterial and venous perfusate (42).

The extracellular (ECFV, ml g^{-1}) and intracellular fluid volumes (ICFV, ml g-1) of white muscle in this preparation were estimated by the "Cl^--K^+ space " equation of Conway (for detailed formula see 6,42), based on measured total water, Na^+, K^+, and Cl^- concentrations in muscle and extracellular fluid (venous perfusate water). Intracellular (ICF) concentrations of all substances in the muscle tissue were calculated by correcting total tissue measurements for the amount of the substance present in trapped ECF (see Eq. 4 of 42).

Lactate, with a $pK \approx 3.7$, exists in both dissociated (Lac^-) and non-dissociated (HLac) forms. Total lactate (Lac) is the sum:

$$Lac = [HLac] + [Lac^-] \tag{2}$$

At physiological pH (6.0-8.0), according to the following equation:

$$pH = pK + \log\frac{[Lac^-]}{[HLac]} \tag{3}$$

[Lac^-] is 3 to 4 orders of magnitude greater than [HLac].

According to Equations 2 and 3, [HLac] is given by:

$$[HLac] = \frac{[Lac]}{1+10^{pH-pK}} \tag{4}$$

Assuming the membrane is permeable solely to non-ionic HLac, such that $[HLac]_i \approx [HLac]_e$, then the Lac distribution at equilibrium will be a function of the transmembrane pH gradient only:

$$\frac{[Lac]_i}{[Lac]_e} = \frac{1+10^{(pH_i-pK)}}{1+10^{(pH_e-pK)}} \tag{5}$$

Therefore, the lower pH_i of white muscle after strenuous exercise should create a relatively steeper outward HLac gradient to facilitate lactate release, despite a very low absolute HLac concentration. In parallel, it is conceivable that an increase in pH_e would also promote lactate release (Fig.1A).

On the other hand, if the cell membrane is permeable only to Lac^-, the Lac distribution at equilibrium will be governed by the transmembrane Nernst potential (E_m):

$$E_m = -\frac{RT}{ZF} ln\frac{[Lac^-]_e}{[Lac^-]_i} = -\frac{RT}{ZF} ln\frac{[Lac]_e-[HLac]_e}{[Lac]_i-[HLac]_i} \tag{6}$$

where R, T, Z and F have their usual values. In other words, intracellular Lac^- should be driven outwards by the negative charge in the ICF (Fig. 5-1B). The E_m of the white muscle was estimated from measured intra- and extracellular $[K^+]$, $[Na^+]$ and $[Cl^-]$ according to the Goldman-Hodgkin-Katz equation (for detailed calculation see 13,42). The net driving force (NDF) on Lac^- was then estimated as:

$$NDF = E_m + \frac{RT}{ZF} ln\frac{[Lac^-]_e}{[Lac^-]_i} \tag{7}$$

Equations analogous to Eq. 7 were used to calculate NDF for HCO_3^- and H^+ across muscle cell membranes.

All data are expressed as means ± 1 S.E.M. (N). Significant differences among means in the five treatment groups were tested by one-way ANOVA, followed by *post hoc* comparison using Duncan's multiple range and critical range test ($P \leq 0.05$). Within the four post-exercise treatments, the medium-pH group served as the control. Student's paired t-test was used to examine the differences between 30 and 60 min values within each treatment group.

RESULTS

Intra-and Extracellular pH.

In the resting perfused trunk preparation, white muscle pH_i remained stable at the initial *in vivo* value of about 7.25. The 6 min exercise regime induced intracellular acidosis, as pH_i dropped more than 0.6 unit in the exercise control group compared with the resting group (Table 5-1). Again, this pH_i remained stable over the the 60 min perfusion period. In parallel, the arterial-venous pH difference (ΔpH_{a-v}) in the control post-exercise group almost doubled in comparison with the corresponding value in the resting group (from 0.11 to 0.21, Table 5-1). Similarly, the transmembrane pH difference (ΔpH_{v-i}) almost doubled (from 0.64 to 1.16) as a result of exercise. Relative to the medium-pH_a treament, the low-pH_a and high-pH_a treatments under post-exercise conditions resulted in lower and higher values of pH_v, as well as correspondingly lower and higher values of the arterial-venous pH difference (ΔpH_{a-v}), respectively. However, there were no significant differences in pH_i among the three pH treatment groups. Therefore, the transmembrane pH gradient (ΔpH_{v-i}) more than doubled from the low-pH to high-pH groups (from 0.69 to 1.53; Table 5-1).

E_m was maintained at about -90 mV independent of rest or exercise, but underwent about a 35 % depolarization in response to high perfusate [K^+] (Table 5-1). This partial depolarization resulted in a significant pH_i depression and a two-fold increase in ΔpH_{a-v} over the control group (Table 5-1), although ΔpH_{v-i} in the depolarized group remained unchanged. It is worth mentioning that high ECF potassium (15mM) did cause some degree of muscle twitching at the beginning of each perfusion.

Respiratory Gas Exchange

In all treatment groups, P_aco_2 was maintained at about 2 torr (Fig. 5-2A) while HCO_3^-

in the inflowing saline was manipulated from 2 to 18 mM in order to adjust pH_a to the desired levels (Table 5-1).

Compared with the resting group, P_vco_2 (measured at 60 min), as expected, increased markedly in all four post-exercise groups (Fig. 5-2). There were no differences in P_vco_2 among the three pH treatments under post-exercise conditions, but high-K^+ treatment resulted in a further significant increase in P_vco_2 (~20%, Fig. 5-2). In all post-exercise preparations, P_vco_2 declined steadily over the 60 min perfusion period (data not shown) which more or less reflects the *in vivo* post-exercise situation reported elsewhere (24). These declines in P_vco_2 were most prominent in the high-pH (1 torr) and high-K^+ treatments (1.2 torr) and least in the low-pH treatment (0.2 torr).

All post-exercise groups except the high-pH treatment exhibited significantly higher P_ico_2 (2-3 fold; Fig. 5-2), and all except the high-K^+ treatment exhibited significantly lower T_ico_2 (Table 5-2) relative to the resting group. Furthermore, in the high-pH group, T_ico_2 was about 60% lower than in the medium-pH and low-pH groups, which correlated with the lack of P_ico_2 elevation in this treatment. As a result, the transmembrane Pco_2 gradient ($P_{i-v}co_2$) in the high-pH group was depressed to about 50% of the resting level and was less than one fifth of the value in the low-and medium-pH groups. Figure 5-2 clearly demonstrates that the depressed transmembrane Pco_2 gradient in the high-pH group was due to lower P_ico_2 rather than higher P_vco_2 in outflowing saline. However, the question remains as to whether the lower T_ico_2 and P_ico_2 in the high pH treatment were due to greater CO_2 washout prior to this sampling point *i.e.*, during the 60 min of high-pH perfusion, or whether they were a consequence of reduced CO_2 production. On one hand, Figure 5-3A demonstrates clearly that Tco_2 efflux in the high-pH treatment at 60 min was lower than in the other two post-exercise groups, and lower even than under resting conditions. On the other hand, there was no indication that aerobic metabolic rate, as quantified from measurements of O_2 uptake (Fig. 5-3B), was depressed in the high-pH treatment (see below).

The high-K^+ treatment exhibited significant $P_i\mathrm{co}_2$ elevation relative to the post-exercise control group (medium-pH), and the transmembrane $P\mathrm{co}_2$ gradient was raised by almost two-fold (Fig. 5-2, Table 5-2). $P_v\mathrm{co}_2$ was also significantly increased (Fig. 5-2). In spite of the much increased transmembrane $P\mathrm{co}_2$ gradient, this partial depolarization treatment did not significantly elevate $T\mathrm{co}_2$ efflux (Fig. 5-3A).

With a relatively constant $P_a\mathrm{o}_2$ (approximately 350-400 torr) in inflowing saline in both the resting and post-exercised groups, $P_v\mathrm{o}_2$ was not depleted, remaining above 70 torr in all treatments (data not shown). Compared with the resting group, the three post-exercise pH treatments did not exhibit higher $M\mathrm{o}_2$ values (Fig. 5-3B). However, the high-K^+ group did post a modest but significant elevation in $M\mathrm{o}_2$ relative to the post-exercise control group (medium-pH). Within the pH treatments, $M\mathrm{o}_2$ was lowest in the low-pH group (a significant difference) and highest in the high pH-group (not significant; Fig. 5-3B). This pattern of $M\mathrm{o}_2$ against pH was exactly opposite to that of $T\mathrm{co}_2$ efflux against pH (Fig. 5-3A). These data allow estimation of the gas exchange ratio (RE) of the preparation - approximately 1.8 at rest, 3.0 at low-pH, 2.0 at medium-pH and high-K^+, and 0.7 at high-pH. Note that RE does not represent true respiratory quotient (RQ) because it includes both CO_2 efflux due to titration of HCO_3^- stores by metabolic acid and transmembrane HCO_3^- fluxes in addition to aerobic CO_2 production.

HCO_3^- Distribution

Venous ($[HCO_3^-]_v$) was significantly higher than arterial ($[HCO_3^-]_a$) in all groups, but as pH_e increased due to experimental $[HCO_3^-]_a$ manipulation (from 2 to 18 mM) in the three pH treatments, the A-V difference of HCO_3^- declined (Fig. 5-4). Nonetheless, the groups with same pH_a and $[HCO_3^-]_a$, namely resting, medium-pH and high-K^+, all shared very similar A-V differences of HCO_3^- after 60 min of perfusion (Fig. 5-4). Relative to resting levels, intracellular levels ($[HCO_3^-]_i$) were more or less reduced in all post-exercise groups (Fig. 5-4). Trends were similar to those in T_iCO_2 levels, such that $[HCO_3^-]_i$ was lowest in the high-pH treatment (Table 5-2). As the pH_e and $[HCO_3^-]_e$ increased, the inwardly directed transmembrane HCO_3^- concentration gradient from venous saline to ICF also increased (Fig. 5-4). In consequence, the outwardly directed NDF on HCO_3^- dropped significantly from about -85 mV at low-pH to almost zero at high-pH (Table 5-2). Partial depolarization by high-K also significantly reduced the NDF on HCO_3^-. The NDF in resting preparations was highly negative (-85 mV; Table 5-2).

The Effect of pH Gradient on Lactate and H^+ Distribution

In the resting preparation, intracellular lactate ($[Lac]_i$) of white muscle remained low and stable at about 3mM throughout the 60 min perfusion period (Table 5-3; Fig. 5-5A). Exhaustive exercise elevated $[Lac]_i$ to about 50 mM, a level which remained unchanged throughout perfusion in the three pH treatment groups. $[Lac]_v$ was low but measurable (about 0.32 mM) in the resting preparation (Table 5-3; Fig. 5-5A) and stayed unchanged between 30 and 60 min. $[Lac]_v$ in all post-exercise groups were elevated more than 3-fold relative to resting levels, and again stayed constant from 30 to 60 min. There was no effect of extracellular (perfusate) pH on $[Lac]_v$ (Table 5-3; Fig. 5-5A).

Since experimental manipulation of pH_e affected neither $[Lac]_i$ nor $[Lac]_v$ (Fig. 5-5A), the transmembrane concentration differences remained the same at low-, medium-, and high-pH. The transmembrane HLac gradients were also unaffected, despite the decreasing $[HLac]_v$ caused by rising pH_e (Fig. 5-5B). However, in contrast to the Lac concentration gradients in the post-exercise treatments, which were 15 - 20 times of the resting value, the HLac gradients were elevated approximately 100-fold. Moreover, the Lac concentration gradients were about three orders of magnitude greater than the HLac ones (Eq. 2). The NDF on Lac^- also stayed fairly constant at -180 to -190mV in the three pH treatments, and significantly greater than the resting value (Table 5-3). Given the fact that all these possible driving gradients remained unchanged at different pH_e levels, the very similar Lac efflux rates in these three groups were not surprising (Fig. 5-6).

Fig. 5-7 models the $[Lac]_i/[Lac]_e$ distributions that would have occurred at equilibrium under different scenarios. Firstly, note that distribution ratios far less than 1.0 would have occurred had Lac been equilibrated either according to the pH gradient (*i.e.* only HLac permeant), or according to the membrane potential (E_m; *i.e.* only Lac^- permeant), or according to both, in contrast to the observed ratios of 35 to 55. Furthermore, had the pH gradient been the dominant factor, then an increase in the distribution ratio should have occurred as pH_e declined. The measured Lac distribution ratio at rest (approximately 7) was much lower than after exercise (35-55), but still significantly above that predicted by either the pH gradient or E_m (Fig. 5-7).

Intracellular ΔH_m^+ loading was very much correlated with ICF Lac accumulation in the three pH treatment groups (Table 5-3). Similar to the Lac in ICF, the ΔH_m^+ level also remained constant after 60 min of perfusion (Table 5-3). Metabolic acid (ΔH_m^+ flux) rates appeared to be completely uncoupled from Lac movements in the resting, low- and medium-pH groups (Fig. 5-6). Fig. 5-6 also shows that ΔH_m^+ efflux increased from a negative value (net uptake) at low-pH to a value not significantly different from net Lac efflux at high-pH. Thus, the ΔH_m^+ efflux increased as the transmembrane pH_{v-i} gradient increased (Table 5-1). Interestingly, the ΔH_m^+ flux exhibited a negative value (net uptake) in the resting group, as in the low-pH group, despite the fact that Lac efflux was clearly visible. Notably, the pH_{v-i} gradients were identical in these two treatments, despite the very different absolute pH values in ICF and ECF in them (Table 5-1). In general, the NDF on H^+calculated with respect to the arterial perfusate was substantially higher than with respect to the venous perfusate (Table 5-3). The overall negative NDF on H^+ in the resting and low-pH groups would favor proton influx and support the ΔH_m^+ influx data, while the positive NDF on H^+ at high-pH would favor ΔH_m^+ efflux (Table 5-3, Fig. 5-6).

The Effect of E_m on Lactate and H^+ Distribution

The 35% depolarization in the high-K^+ treatment resulted in a significant increase in $[Lac]_i$ and ΔH_m^+ over the 60 min perfusion period (Table 5-3), which was correlated with the greater ICF acidosis reported earlier (Table 5-1). Notably, this treatment started the 60 min of perfusion with post-exercise $[Lac]_i$ and ΔH_m^+ identical to those of the other exercised groups, showing that the high-K^+ perfusion was the sole cause of $[Lac]_i$ and ΔH_m^+ build-up in the intracellular compartment. In parallel with the almost 40% $[Lac]_i$ elevation was a doubling of $[Lac]_v$ (Table 5-3) accompanied by a 40% increase of the Lac concentration difference (Fig. 5-5A). Due to the ICF and ECF acidosis, [HLac] also increased dramatically in both compartments, thereby creating a steeper HLac gradient (Fig. 5-5B). If the muscle cell membranes were permeable to Lac^-, then one would expect reduced E_m and NDF (Table 5-3) to help retain Lac within the ICF. On the contrary, Lac efflux was significantly enhanced (Fig. 5-6), and this was consistent with the elevated outward HLac gradient. To look at this matter from a different aspect, E_m estimated Lac distribution ratio in the depolarized group was still far below the measured one, but was very close to the pH estimated ratio (Fig. 5-7).

As with Lac efflux, ΔH_m^+ efflux was also increased significantly over the control value by partial depolarization (Fig. 5-6). Although ΔH_m^+ efflux did not quite match the Lac efflux in this treatment (but was not significantly different either), the E_m influence seemed to be evident. Again, the positive NDF of H^+ and the much elevated ICF ΔH_m^+ loading were all in favor of this increasing ΔH_m^+ unloading from muscle ICF (Table 5-3, Fig. 5-6).

Fig. 5-8 compares the actual transmembrane $[H^+]$ distribution ratio (directly determined from pH_i and pH_v measurements) with that predicted assuming passive equilibration with E_m. Only at high-pH and in the partially depolarized state was H^+ distribution in equilibrium with E_m. The resting and the low-pH groups shared very similar $[H^+]$ ratios due to similar pH gradients. In the post-exercise preparations, the measured ratio increased as pH_e increased (Fig. 5-8), whereas the distribution predicted by E_m remained unchanged.

DISCUSSION

Comparison with in vivo Studies

The isolated-perfused tail-trunk preparation provides a useful tool to study the metabolic and acid-base state of fish white muscle, by allowing the manipulation of some intra- and extracellular variables with a flexibility which would be impossible *in vivo*. A detailed assessment of the physiological conditions in the preparation was provided in our earlier paper (42).

Six min of strenuous exercise induced a substantial metabolic acidosis in white muscle ICF; pH_i levels of about 7.26 and 6.60 (Table 5-1) in the resting and post-exercise preparations, respectively, were comparable to those observed *in vivo* (38,39,41). Intracellular $[Lac]_i$ and ΔH_m^+ build-ups of about 50 mM in white muscle (Table 5-3) were slightly greater than in our previous *in vivo* study (41), but well within the ranges reported in other investigations on exhaustively exercised trout (25,36,38,48). All of these *in vivo* studies reported little net change in pH_i, ΔH_m^+, or $[Lac]_i$ over the first 60 min of post-exercise recovery, again in accord with the stability of these values over the 1 h perfusion period in the present preparation (Tables 1, 3). One potential difference from the *in vivo* situation was the fact that in order to set perfusate pH, we elected to hold inflowing perfusate Pco_2 (P_aco_2) constant at resting levels, whereas *in vivo*, P_aco_2 rises after exercise and then declines close to resting levels by 1 h. However, respiratory acidosis appears to be largely of endogenous origin in the muscle, as indicated by the persistent elevation of P_vco_2 *in vivo* long after P_aco_2 has declined (25). Intracellular Pco_2 certainly increased in the perfused preparation despite the constancy of P_aco_2 (Fig. 5-2), and indeed these elevations may have been larger than *in vivo*, for reasons discussed below. Regardless, because white muscle is so well buffered,

Pco_2 elevation in this range is a relatively minor component of the total intracellular acidosis (23,39). Variations in this factor should not materially affect the present conclusions about lactate and metabolic H^+ distribution.

The lack of change in $[Lac]_i$ and $[Lac]_v$ over the 60 min perfusion suggests that the preparation was in a relatively stable, aerobic state. Given that Mo_2 was similar to that of resting preparations, the elimination of Lac in muscle ICF *via* oxidation did not, as yet, play a prominent role, nor did the low Lac efflux from muscle, which would have lowered $[Lac]_i$ by less than 5% over the perfusion period. Furthermore, according to our *in vivo* work (41), glycogen restoration was also negligible at this time.

The significantly lower pH_i and additional accumulation of intracellular ΔH_m^+ and $[Lac]_i$ over the 60 min perfusion period in the high-K^+ treatment (Tables 1, 3) was likely due to additional muscle twitching induced by membrane depolarization. This activity probably was fueled by additional glycolytic production of Lac and metabolic acid as well as by Mo_2 (Fig. 5-3B). The significantly increased P_ico_2 in the high-K^+ treatment shown in Fig. 5-2 was probably a result of the combination of ICF metabolic acidosis (*i.e.*, higher $[Lac]_i$ and ICF ΔH_m^+ load, Table 5-1, 3) and higher Tco_2 (Table 5-2) induced by muscle twitching.

Respiratory Gas Exchange

The lack of increase in the Mo_2 of white muscle (Fig. 5-3B) and the stability of $[Lac]_i$ for the one hour period after exercise supports the view that aerobic metabolism of lactate in white muscle is not an important contributor to the EPOC ("excess post-exercise oxygen consumption") seen *in vivo* at this time (35). Tco_2 efflux was not elevated, either, but tended to decrease as pH increased in the three pH treatment groups, while Mo_2 exhibited the opposite trend (Fig. 5-3A, B). Tco_2 efflux consists of three components: the true respiratory

CO_2 production, the portion of CO_2 generated from the acid titration of "on board" HCO_3^-, and transmembrane HCO_3^- movements. The tendency of Mo_2 to increase with pH strongly indicates that respiratory CO_2 production did not fall, and indeed may have risen slightly at higher pH, reflecting the well known effect of acidosis on metabolic rate (7,16,37). Inasmuch as intracellular $[Lac]_i$ and metabolic H^+ loads were the same amongst the pH treatments, it is unlikely that acid titration of HCO_3^- stores varied greatly with the change in pH_e. However, the NDF on HCO_3^- was outwardly directed, but declined significantly as the pH gradient and $[HCO_3^-]_e$ increased (Table 5-2). At high-pH, the NDF was zero. The concomitant fall in Tco_2 efflux suggests that transmembrane HCO_3^- movement may play an important role in Tco_2 efflux. Indeed, the virtual elimination of HCO_3^- efflux in the high-pH treatment may explain why only in this group did the RE value (approx. 0.7) approach a true RQ. To further illustrate this point, the sharing, between the resting and low-pH groups, of similar NDF on HCO_3^- (but very different Pco_2 gradients) resulted in an almost identical Tco_2 efflux rate (Fig. 5-3, Table 5-2). In spite of the soaring P_ico_2 in the high-K^+ group, Tco_2 efflux remained unchanged compared with the control or even the resting groups (Fig. 5-3A). The lower NDF on HCO_3^- as the result of partial depolarization may have reduced HCO_3^- efflux and offset the elevated Pco_2-driven efflux of Tco_2 (Table 5-2).

The contribution of the transmembrane Pco_2 gradient in driving Tco_2 efflux in the preparation is unclear. Certainly the Tco_2 efflux (Fig. 5-3) did not parallel the gradient overall, though it did within the three post-exercise pH treatments. Interestingly, despite the differences in pH_e and transmembrane Pco_2 gradient, the elevated post-exercise P_vco_2 remained uniform among the three groups (Fig. 5-2). CO_2 is generally thought to be very diffusible through cell membranes, so the absolute levels of P_ico_2 (7 - 17 torr depending on

pH_e, Fig. 5-2) and the size of the transmembrane Pco_2 gradients were surprisingly high. *In vivo* measurements in postexercise trout muscle indicate P_ico_2 values approximately 60% lower at comparable pH_e (8,39). A possible explanation is the absence of red blood cells in the perfused preparation. The presence of carbonic anhydrase (CA) in the red blood cells (RBC) *in vivo* catalyzes the CO_2 hydration reaction, thereby helping to load CO_2 into the blood in the white muscle while maintaining relatively small transmembrane Pco_2 gradients (7). Despite the existence of sarcolemmal-bound CA, the absence of blood CA in the perfused preparation may have elevated P_ico_2 and/or resulted in disequilibrium conditions in the venous capillary perfusate. In other words, the elevated P_ico_2 reflected the true perfusate Pco_2 in the capillary due to the very high permeability of membranes to CO_2, whereas the "transmembrane Pco_2 gradients" shown in Figure 2 only exist when this disequilibrium conditions is slowly dissipated with time in a closed system (*i.e.* in outflowing venous saline).

Lactate and ΔH_m^+ Transport and Distribution

The lactate efflux rates from the present perfused white muscle preparation after exercise (Fig. 5-6) were very similar to those estimated by a ^{14}C-lactate turnover study *in vivo* (22), as well as in a previous perfused whole trunk study (40). Lactate clearance from white muscle by efflux alone would take more than 20 h, whereas it is normally complete within 8-10 h in salmonids *in vivo* (21,22,36,41). These data reinforce the view (27,45,47) that the major portion of lactate produced during exhaustive exercise never leaves white muscle, but rather is metabolized *in situ*. Unlike the trout, the distribution ratio of Lac in higher vertebrate muscles is much lower (≤ 10 *vs.* ≥ 30 in fish), while the Lac flux and pH_i are very much affected by pH_e, suggesting that the permeabilities of muscle cell membranes to Lac and H^+ are very different (17,19,38,40,41,44).

Multiple questions remain. Is ΔH_m^+ movement coupled with Lac movement? How is the transmembrane Lac gradient maintained in fish white muscle? Is there any active retention of Lac involved in counter-balancing the small Lac "leakage" (*cf.* 2,21,40) or is the slow release of Lac merely the consequence of very low permeability alone? Are free diffusion of HLac and Lac^- involved in the Lac release from muscle, or is a carrier mediated mechanism involved? In higher vertebrates, there now exists evidence for both free diffusion of $HLac/Lac^-$ (*cf.* 17,20,33,44) plus several carrier mediated mechanisms by which Lac may move across cell membranes (*cf.* 5,10,11,15,18,19,29,33,44). The present data cast some light on these issues.

Firstly, the present data prove unequivocally that Lac efflux and ΔH_m^+ efflux are completely dissociated from one another quantitatively (Fig. 5-6), as was long suspected from blood measurements *in vivo* (reviewed by Wood, 47). Indeed, in the low-pH treatment we see the extreme situation where there is net ΔH_m^+ uptake, but net Lac efflux (Fig. 5-6). ΔH_m^+ movement appeared to vary as a function of the transmembrane pH gradient (Table 5-1) or NDF (Table 5-3), whereas Lac efflux was much more constant and independent of these parameters. However, it must be appreciated that ΔH_m^+ movement is a composite acid-base measure which cannot in itself distinguish between base efflux and acid uptake, or *vice versa*. Earlier we argued that there were substantial differences in base efflux (*i.e.* HCO_3^- movements) dependent upon pH_e, so it would be naive to attribute the ΔH_m^+ movements to simple H^+ movements alone. Furthermore, since both H^+ and HCO_3^- are clearly held well out of electrochemical equilibrium across the cell membrane (Table 5-2, 3; Fig. 5-8), the observed movements may well not be passive. Nevertheless, it does appear that the ΔH_m^+ flux responds to acid-base and electrochemical gradients (*i.e.* NDF on H^+ and HCO_3^-) in the expected fashion. Overall, these results support the "equilibrium limitation" model of Holeton and Heisler (14) such that ΔH_m^+ efflux will fall as pH_e declines, eventually reaching a point of reversal.

Lac was retained in the intracellular compartment of white muscle well out of equilibrium with either the electrical or pH gradients under all conditions, including rest (Fig. 5-7). The small outward movement of Lac which did occur was not influenced by the extracellular pH treatment (Fig. 5-6), was elevated rather than reduced by partial depolarization (Fig. 5-6) in contrast to earlier studies on frog muscle (17), and was increased only 3-fold (between rest and exercise) in the face of a 100-fold increase in the HLac concentration gradient (Fig. 5-5B). Overall, the best correlation, though by no means perfect (see below), was with the transmembrane concentration gradient for total Lac (Fig. 5-5A). The simplest explanation for these observations (though by no means the only possible one) is that Lac^- efflux occurs by an *electrically neutral* carrier mechanism, such that only the chemical concentration gradient is important. At least two such mechanisms have been identified in higher vertebrates, an electroneutral antiporter which exchanges Lac^- against Cl^- or HCO_3^- (29,32,44) and an electroneutral co-transporter which moves equimolar amounts of Lac^- and H^+ (1,29,32,34,44,46). It should be noted that HLac diffusion, Lac^-/HCO_3^- exchange, and H^+-Lac^- co-transport would all result in equimolar effluxes of ΔH_m^+ and Lac^-; the observed lack of agreement between ΔH_m^+ and Lac^- movements discussed above could be considered an argument against each of these mechanisms, leaving only Lac^-/Cl^- exchange. However, also as pointed out earlier, the potential for independent movements of H^+ and HCO_3^- (*e.g.* by diffusion, by Na^+/H^+ and Cl^-/HCO_3^- exchange) renders this argument overly simplistic.

One weakness of the idea of an electroneutral carrier(s) driven by the simple Lac$^-$ concentration gradient is the fact that Lac efflux increased only about 3-fold from rest to exercise (Fig. 5-6), while the concentration gradient increased about 15 to 20-fold (Fig. 5-5A). However, the availability and activity of such carriers may be pH-dependent (15,17,20, 33), and of course their rates will depend not only on Lac$^-$ gradients, but also on gradients for the other transported ions (H^+, HCO_3^-, Cl^-), all of which change between rest and exercise. Furthermore, there is no evidence in the current data to exclude the idea of an active Lac uptake carrier which opposes efflux. The present experiments measured the net Lac flux, but not unidirectional flux. It is quite possible that an active retention mechanism becomes more effective in the post-exercise situation simply because of the greater availability of Lac in the extracellular fluid for the proposed uptake carrier. This, in itself, would attenuate the increase in net Lac efflux relative to the apparent driving concentration gradient.

The present study has served to define the actual net fluxes of Lac and ΔH_m^+, as well as the relevant pH, electrochemical, and concentration gradients, and to demonstrate how the fluxes respond to experimental manipulations of these gradients. Clearly, the next step is to adopt a pharmacological approach to factor out the possible carrier mechanisms involved in both the efflux and influx directions.

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Figure 5-1.

Schematic models of transmembrane lactate transport and distribution in trout white muscle cell after exercise. (A) shows the situation when only HLac is diffusible across the cell membrane and the pH gradient is governing Lac distribution and transport. (B) demonstrates the scenario where the muscle cell membrane is permeable only to Lac^- and the electrochemical gradient is the driving force for Lac distribution. Note the substantially greater Lac^- concentration within each compartment and much greater outward Lac^- concentration gradient compared with that of HLac.

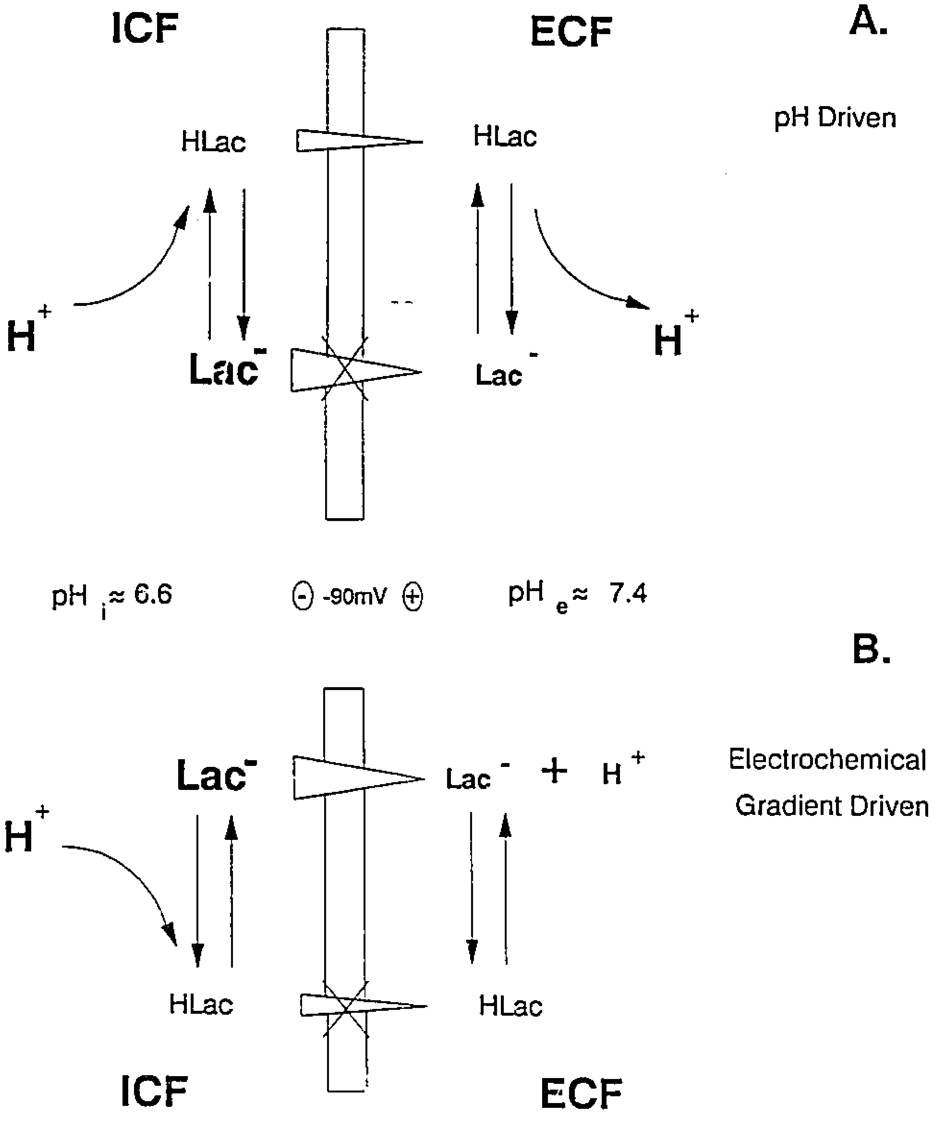

ICF
ECF
A.
pH Driven
HLac
HLac
H^+
H^+
Lac^-
Lac^-
$pH_i \approx 6.6$
⊖ -90mV ⊕
$pH_e \approx 7.4$
B.
Lac^-
$Lac^- + H^+$
Electrochemical
Gradient Driven
H^+
HLac
HLac
ICF
ECF

Figure 5-2.

CO_2 partial pressure (Pco_2) in inflowing saline (arterial, P_aco_2, filled bars), outflowing saline (venous, P_vco_2, open bars), and intracellular fluid (P_ico_2, stippled bars) of white muscle in the five treatment groups of isolated tail-trunk perfusion preparations after 60 min of perfusion. The numbers beside the brackets indicate the mean P_ico_2-P_vco_2 gradients (7.5 torr = 1kPa). Values were recorded after 60 min of perfusion and are means ± S.E.M., N = 5, 7, 11, 8, 6 for resting, low-pH, medium-pH, high-pH, and high-K^+ groups, respectively. * indicates significant difference ($P \leq 0.05$) from the corresponding control values.

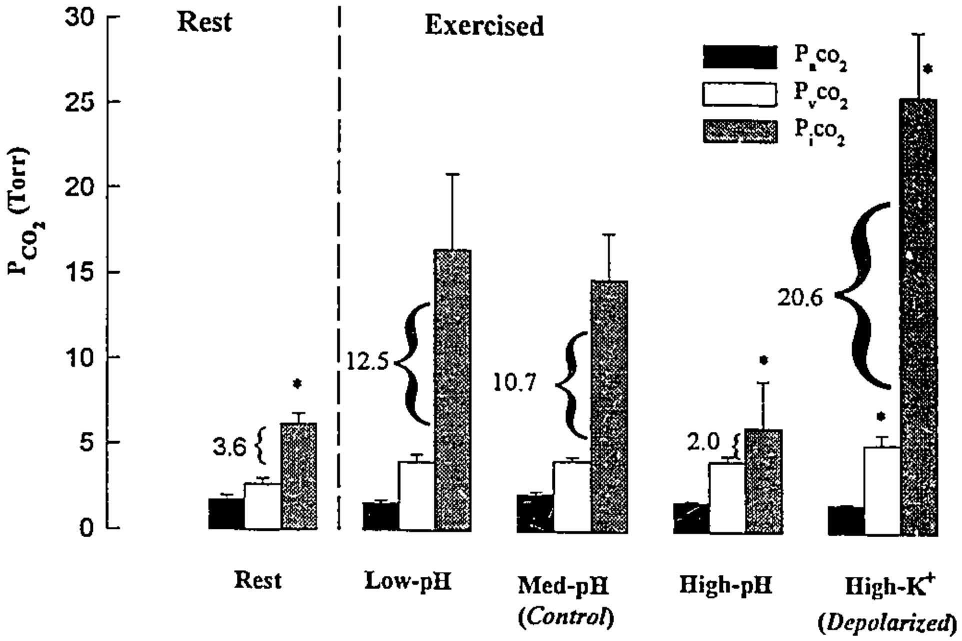

Rest
Exercised
P_aCO_2
P_vCO_2
P_iCO_2
P_{CO_2} (Torr)
30
25
20
15
10
5
0
3.6
12.5
10.7
2.0
20.6
*
Rest
Low-pH
Med-pH (Control)
High-pH
High-K$^+$ (Depolarized)

Figure 5-3.

(A) Net efflux rates of total CO_2 (Tco_2) and (B) uptake rates of O_2 by the perfused trout tail-trunk preparation after 60 min of perfusion. The exercised trunks were taken from trout exercised to exhaustion. Flux rates are expressed as mmol h^{-1} kg^{-1} wet weight of tail-trunk. Other details as in legend of Figure 5-2.

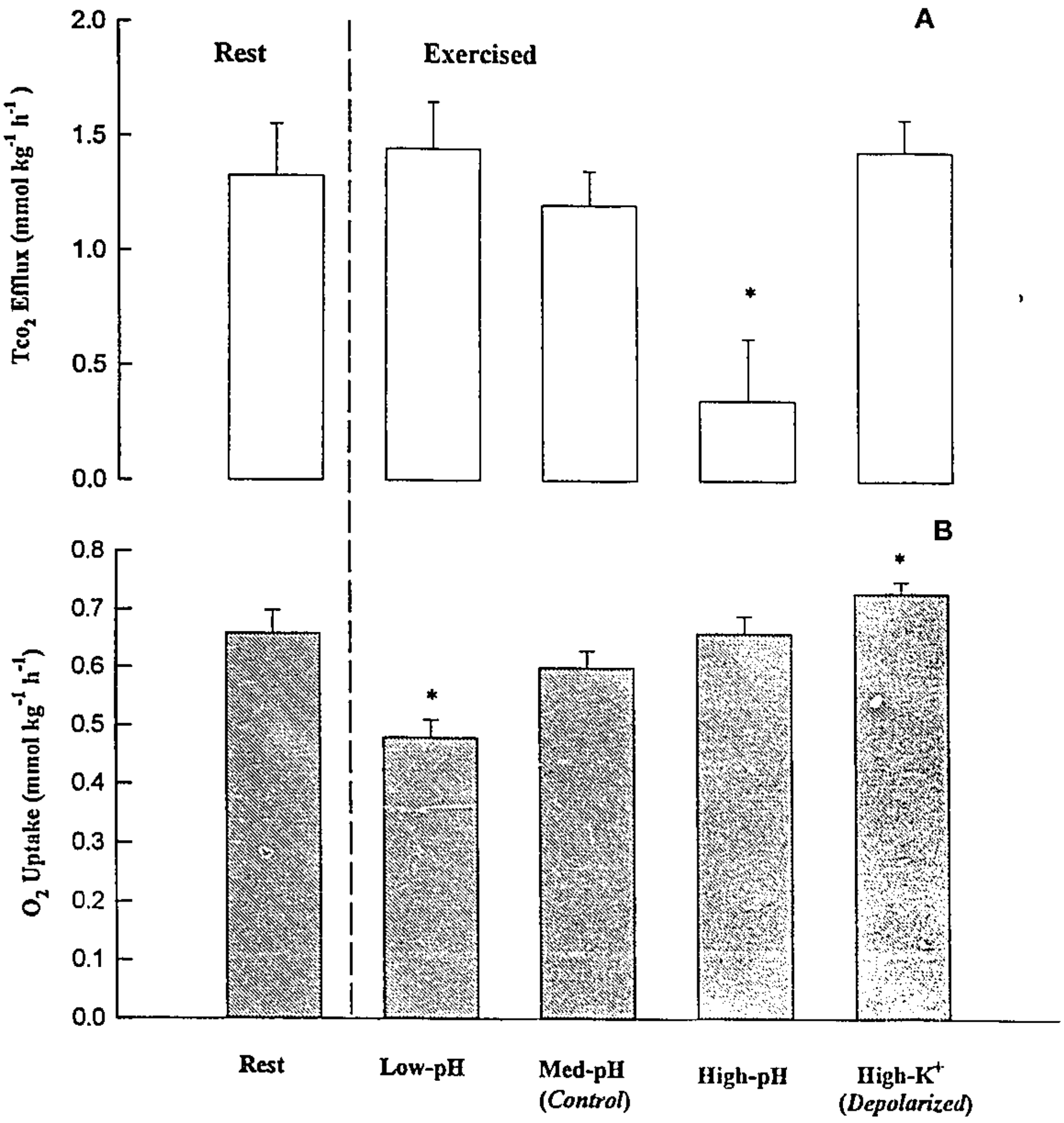
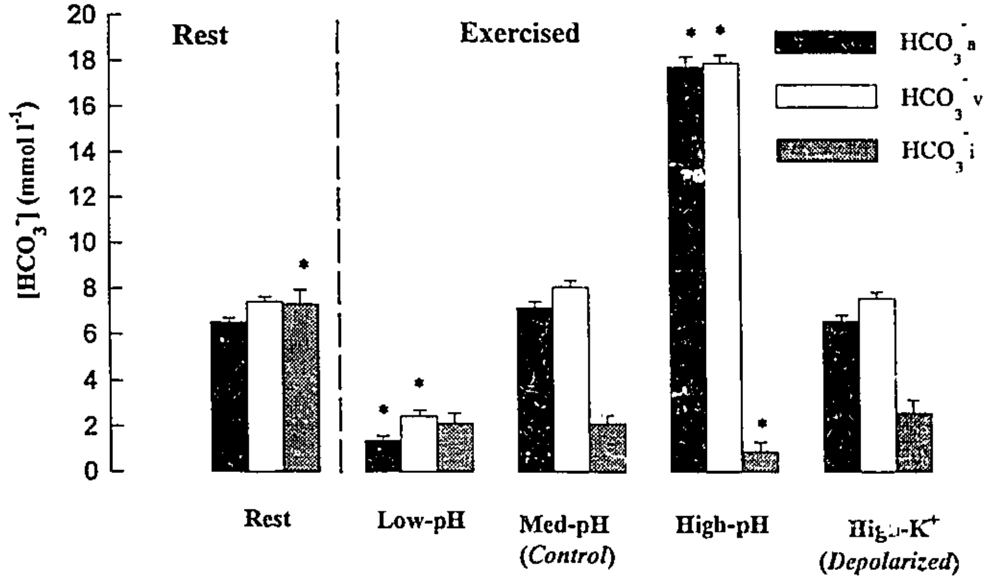

A
B
Rest
Exercised
Tco_2 Efflux (mmol kg^{-1} h^{-1})
O_2 Uptake (mmol kg^{-1} h^{-1})
2.0
1.5
1.0
0.5
0.0
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
*
*
Rest
Low-pH
Med-pH
(Control)
High-pH
High-K$^+$
(Depolarized)

Figure 5-4.

HCO_3^- concentrations in the inflowing saline (arterial, $[HCO_3^-]_a$, filled bars), outflowing saline (venous, $[HCO_3^-]_v$, open bars), and intracellular fluid ($[HCO_3^-]_i$, stippled bars) of white muscle in the resting and four exercised treatment groups of isolated tail-trunk perfusion preparations. Exercised trunks were taken from trout exercised to exhaustion. Other details as in legend of Figure 5-2.



$[HCO_3^-]$ (mmol l^{-1})
20
18
16
14
12
10
8
6
4
2
0
Rest
Exercised
*
*
*
*
*
*
HCO_3^- a
HCO_3^- v
HCO_3^- i
Rest
Low-pH
Med-pH
(*Control*)
High-pH
High-K^+
(*Depolarized*)

Figure 5-5.

(A) Total lactate concentrations in outflowing saline (venous, $[Lac]_v$, open bars) and intracellular fluid ($[Lac]_i$, stippled bars) of white muscle in the five treatment groups of isolated-perfused tail-trunk preparations. Brackets indicate the size of the mean $[Lac]_i$ - $[Lac]_v$ gradients (mmol l^{-1}). Note that inflowing saline Lac concentrations ($[Lac]_a$) are zero. (B) Non-dissociated form of lactate concentration in outflowing perfusate (venous, $[HLac]_v$, open bars) and intracellular fluid ($[HLac]_i$, stippled bars) of white muscle in the five treatment groups (Eq. 4). Brackets indicate the size of the mean $[HLac]_i$ - $[HLac]_v$ gradients (μmol l^{-1}). Other details as in legend of Figure 5-2.

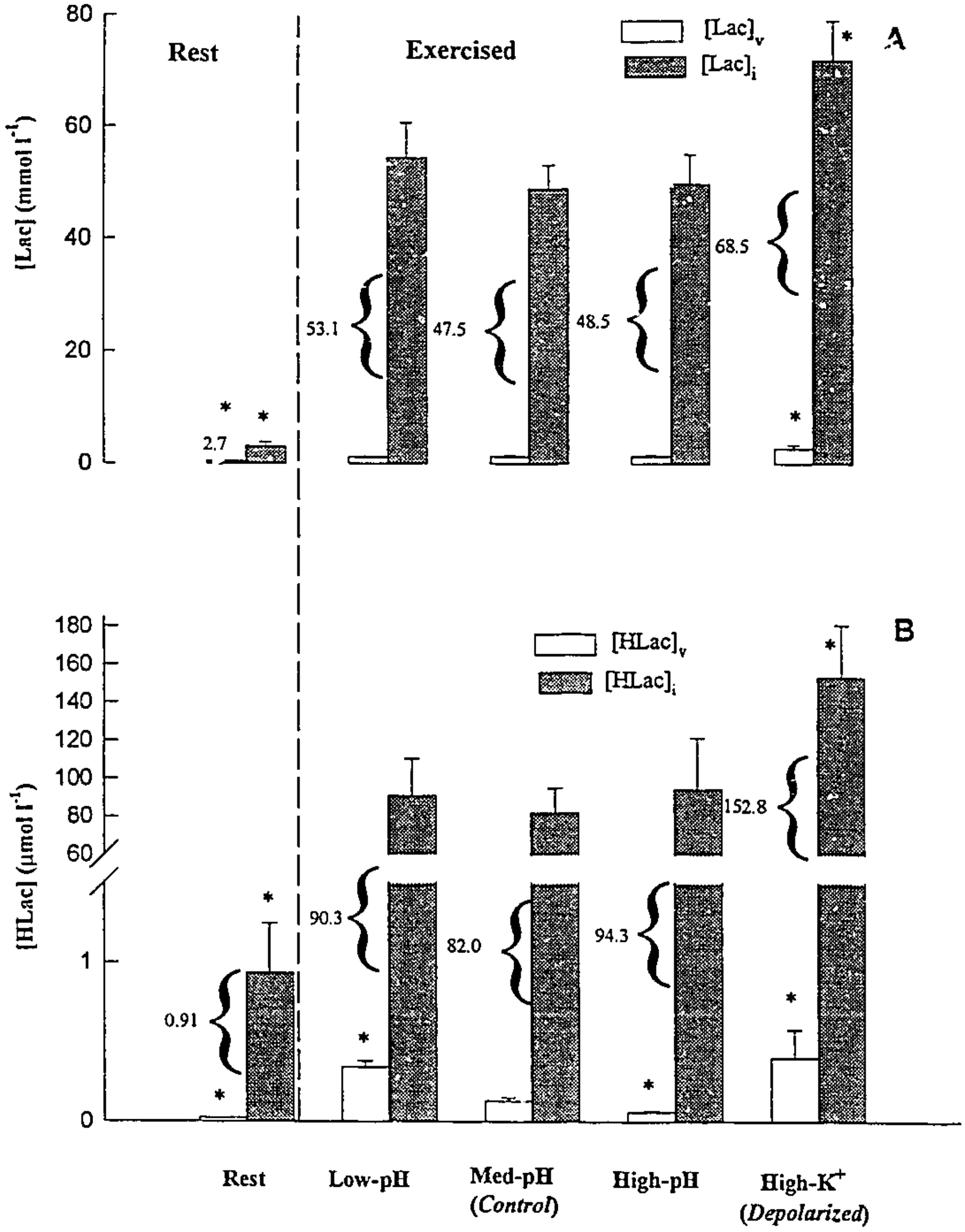

A
[Lac] (mmol l^{-1})
80
60
40
20
0
Rest
Exercised
[Lac]$_v$
[Lac]$_i$
2.7
53.1
47.5
48.5
68.5
*
B
[HLac] (μmol l^{-1})
180
160
140
120
100
80
60
1
0
[HLac]$_v$
[HLac]$_i$
0.91
90.3
82.0
94.3
152.8
Rest
Low-pH
Med-pH
(*Control*)
High-pH
High-K$^+$
(*Depolarized*)

Figure 5-6.

Net flux rates of total lactate (Lac flux) and metabolic acid (ΔH_m^+ flux) by the trout isolated-perfused tail-trunk preparations after 60 min of perfusion in the five treatment groups. Flux rates are expressed as mmol h^{-1} kg^{-1} wet weight of tail-trunk. Positive and negative values indicate net efflux from and influx to muscle, respectively. ‡ indicates significant difference ($P \leq 0.05$) from the corresponding Lac flux values. Other details as in the legend of Figure 5-2.

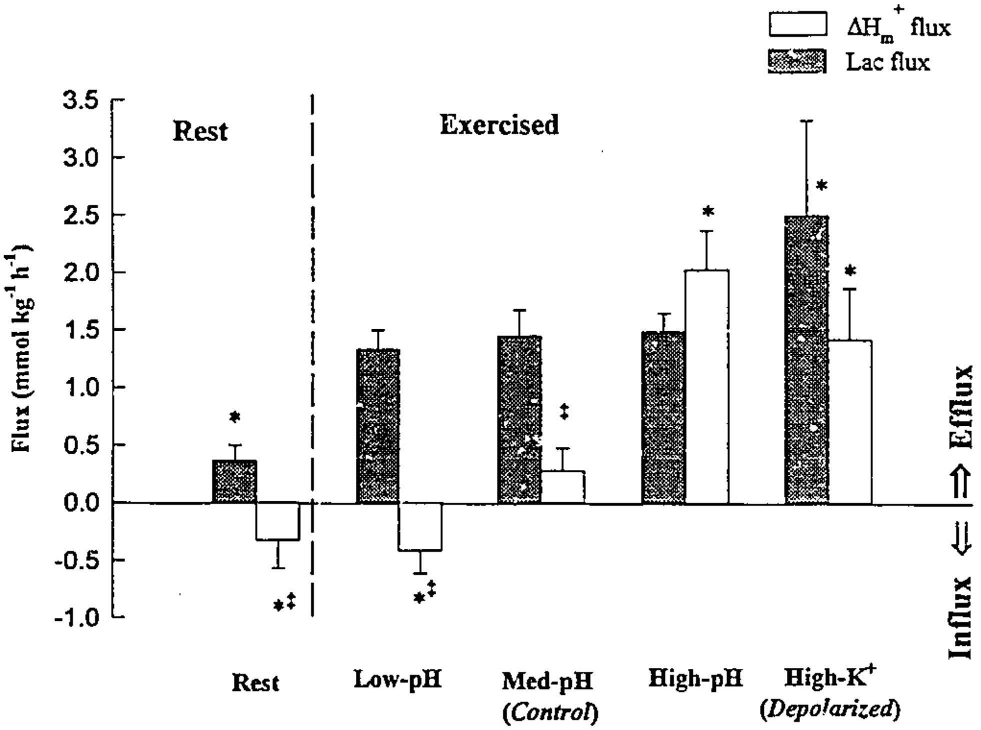

ΔH$_m^+$ flux
Lac flux
Flux (mmol kg^{-1} h^{-1})
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
Rest
Exercised
*
*‡
*‡
‡
*
*
*
Rest
Low-pH
Med-pH
(Control)
High-pH
High-K$^+$
(Depolarized)
Efflux ⇧
⇩ Influx

Figure 5-7.

Transmembrane distribution ratios of total lactate ($[Lac]_i/[Lac]_e$) from intra- to extra-cellular fluid (venous outflowing perfusate) of white muscle in the five treatment groups of isolated-perfused tail-trunk preparations. Measured ratio values (stippled bars) are compared with ratio values (filled bars) predicted by distribution according to the transmembrane pH gradient (ΔpH_{i-v}) by equation 5, assuming permeability only to HLac, and with ratio values (open bars) predicted by distribution according to the transmembrane potential E_m by equation 6, assuming permeability only to Lac^-. * indicates a significant difference ($P \leq 0.05$) from the corresponding control ratio values in the medium-pH group. ‡ indicates a significant difference ($P \leq 0.05$) from the measured ratio values. Other details as in the legend of Figure 5-2.

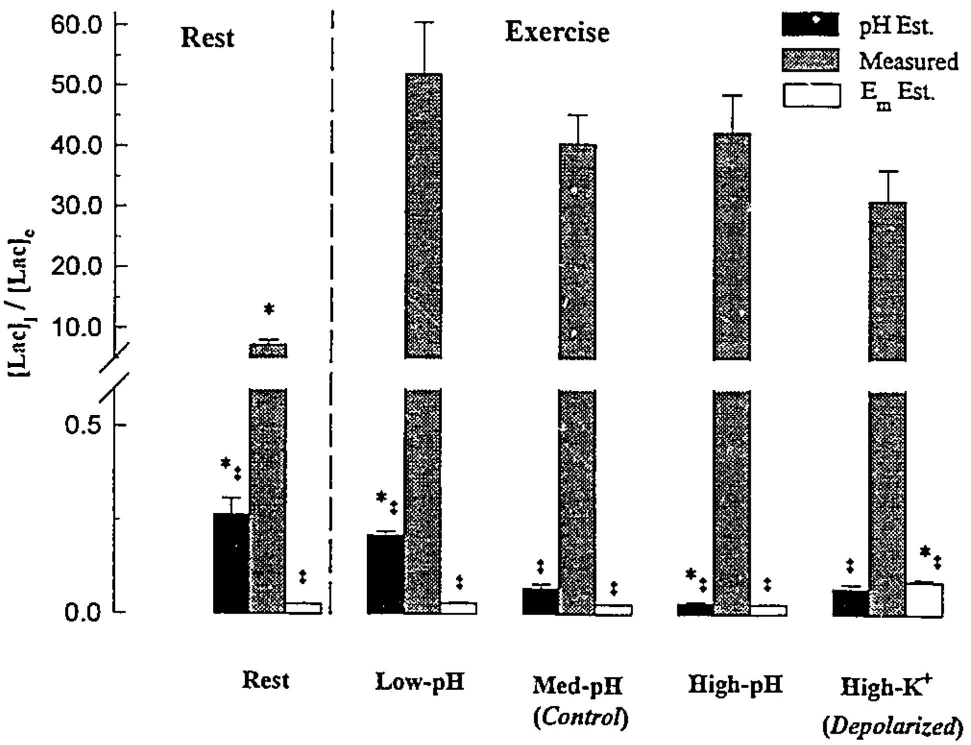

60.0
50.0
40.0
30.0
20.0
10.0
0.5
0.0
[Lac]i / [Lac]e
Rest
Exercise
pH Est.
Measured
Em Est.
Rest
Low-pH
Med-pH
(Control)
High-pH
High-K+
(Depolarized)

Figure 5- 8.

Transmembrane distribution ratios of H^+ ($[H^+]_i/[H^+]_e$) from intra- to extra-cellular fluid (venous outflowing perfusate) of white muscle in the five treatment groups of isolated-perfused tail-trunk preparations. Measured ratio values (stippled bars) are compared with ratio values (open bars) predicted by distribution according to the transmembrane potential (E_m) by an equation analogous to equation 6, assuming cell membranes are permeable to H^+. * indicates a significant difference ($P \leq 0.05$) from the corresponding control ratios. ‡ indicates a significant difference ($P \leq 0.05$) from the corresponding E_m estimated ratios.

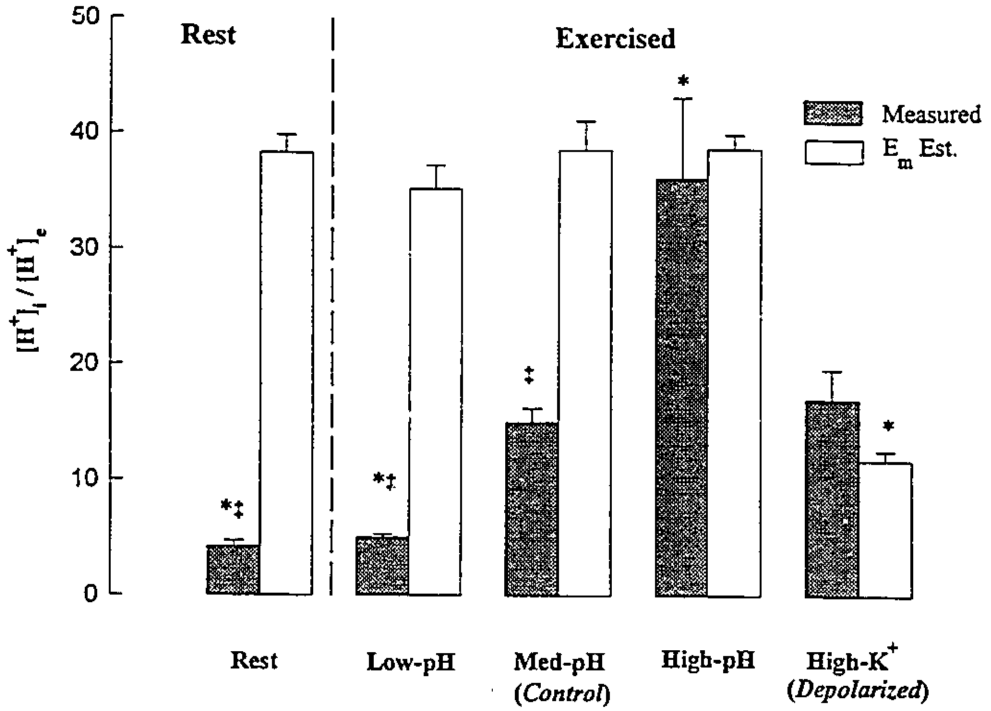

Rest
Exercised
Measured
E_m Est.
$[H^+]_i / [H^+]_e$
50
40
30
20
10
0
*‡
*‡
‡
*
*
Rest
Low-pH
Med-pH
(Control)
High-pH
High-K+
(Depolarized)

Table 5-1. The intra- and extracellular pH, arterial to venous pH difference ($\Delta pH_{a\text{-}v}$), transmembrane pH gradient at the venous end ($\Delta pH_{v\text{-}i}$), and membrane potential (E_m) in the resting and the four post-exercise treatment groups (low-pH, medium-pH, high-pH and high-K$^+$) after 60 min of perfusion. E_m was estimated according to the Goldman-Hodgkin-Katz equation.

	Resting	Post-Exercise			
		Low-pH	Medium-pH *(Control)*	High-pH	High-K$^+$ *(Depolarized)*
pH_a	8.014 ± 0.038	7.411 ± 0.030	7.966 ± 0.020	8.416 ± 0.011	8.040 ± 0.010
pH_v	7.901 ± 0.034‡	7.279 ± 0.042	7.753 ± 0.023	8.081 ± 0.029	7.643 ± 0.057‡
pH_i	7.291 ± 0.025‡	6.587 ± 0.048	6.595 ± 0.048	6.561 ± 0.061	6.441 ± 0.052‡
$\Delta pH_{a\text{-}v}$	0.114 ± 0.012‡	0.132 ± 0.021‡	0.213 ± 0.015	0.335 ± 0.037‡	0.397 ± 0.054‡
$\Delta pH_{v\text{-}i}$	0.609 ± 0.065‡	0.692 ± 0.022‡	1.158 ± 0.040	1.528 ± 0.068‡	1.202 ± 0.075
E_m (mV)	-91.34 ± 0.97	-88.02 ± 1.56	-91.80 ± 1.50	-91.49 ± 1.00	-59.64 ± 1.00‡
	(N=5)	(N=7)	(N=11)	(N=8)	(N=6)

Values are Mean ± 1 S.E.M. (N).

‡ significantly different from the corresponding post-exercise control values.

Table 5-2. Post-exercise white muscle intracellular Tco_2, Pco_2, $[HCO_3^-]$ and NDF on HCO_3^- (Eq. 7) in the five treatment groups after 60 min of perfusion.

	Resting	Post-Exercise			
		Low-pH	Medium-pH (*Control*)	High-pH	High-K^+ (*Depolarized*)
Tco_2	6.21 ± 0.61*	3.21 ± 0.73	3.21 ± 0.57	1.30 ± 0.64*	4.29 ± 0.79
Pco_2 (mmHg)	7.31 ± 0.63*	16.48 ± 4.46	14.80 ± 2.71	6.12 ± 2.79*	25.72 ± 3.80*
$[HCO_3^-]_i$	5.37 ± 0.58*	2.13 ± 0.45	2.13 ± 0.38	0.89 ± 0.41*	2.59 ± 0.57
NDF (mV)	-84.57 ± 2.56*	-82.65 ± 7.53*	-53.75 ± 6.04	-2.33 ± 14.71*	-31.47 ± 5.41*

Values are Mean ± 1 S.E.M. (See Table 5-1 for N in each group). Tco_2 and $[HCO_3^-]_i$ are expressed in mmol l^{-1} ICF.

* significantly different ($P \leq 0.05$) from the corresponding control values.

Table 5-3. The extra- and intracellular concentrations of total lactate (Lac), intracellular metabolic H^+ (ΔH_m^+, Eq. 1), and the NDF on Lac^- and H^+ over the course of a 60 min perfusion (Eq. 7).

	Resting	Post-Exercise			
		Low-pH	Medium-pH (*Control*)	High-pH	High-K^+ (*Depolarized*)
$[Lac]_v$ (60min)	0.32 ± 0.08‡	1.14 ± 0.15	1.22 ± 0.19	1.24 ± 0.17	2.66 ± 0.67‡
$[Lac]_i$ (0min)	3.45 ± 0.96‡	48.38 ± 3.11	51.46 ± 4.24	49.98 ± 3.71	57.27 ± 4.68
$[Lac]_i$ (60min)	3.04 ± 0.79‡	57.19 ± 6.69	51.56 ± 4.39	52.60 ± 5.29	70.45 ± 6.49*‡
ΔH_m^+ (ICF, 0 min)	--------	50.37 ± 2.99	51.51 ± 4.16	52.88 ± 4.38	57.94 ± 6.01
ΔH_m^+ (ICF, 60 min)	--------	54.83 ± 3.46	54.71 ± 3.75	58.46 ± 4.58	65.04 ± 4.30‡
Lac^- NDF (mV)	-144.64±2.77‡	-185.26±5.92	-190.25±8.97	-183.25±3.76	-143.70±5.41‡
H^+ $NDF_{i\text{-}a}$ (mV)	-30.77±2.17‡	-42.89±1.52‡	-13.54±3.63	14.52 ± 4.20‡	31.62 ± 3.94‡
H^+ $NDF_{i\text{-}v}$ (mV)	-56.55±3.70‡	-45.38±2.44‡	-19.92±3.40	3.48 ± 3.46‡	17.27 ± 4.17‡

Values are Mean ± 1 S.E.M. (See Table 5-1 for N in each group).

* significantly different ($P \leq 0.05$) from the corresponding 0 min values.

‡ significantly different ($p \leq 0.05$) from the corresponding control values.

Note: The NDF on Lac^- was calculated based on the intracellular to venous gradient values at 60 min. The NDF on H^+ was calculated based on the intracellular to arterial and venous gradient values at 60 min, respectively. Lac and ΔH_m^+ were expressed as mmol l^{-1} ICF or ECF, respectively.

CHAPTER 6

LACTATE TRANSPORT BY PERFUSED RAINBOW TROUT WHITE MUSCLE: KINETIC CHARACTERISTICS AND SENSITIVITY TO INHIBITORS

ABSTRACT

This study examined the uptake and release of lactate and metabolic protons (ΔH_m^+) in resting and exercised fish white muscle, using an isolated-perfused tail-trunk preparation of rainbow trout. In exercised muscle, L(+)-lactate (L-Lac) efflux was partially inhibited (~40%) by 5 mM α-cyano-4-hydroxycinnamate (CIN), but not by 0.5 mM 4-acetamido-4'-isothiocyano-stilbene-2,2'-disulphonate (SITS) or by 0.1 mM amiloride. These results suggest that lactate release occurs through a lactate ion (Lac^-)/H^+ cotransporter (symport) and the free diffusion of HLac or Lac^-, but not *via* the band 3 anion exchanger (Lac^- *versus* HCO_3^- or Cl^- antiporter). Lactate efflux was accompanied by ΔH_m^+ influx in all treatments, and increased ΔH_m^+ influx occurred following treatment with SITS. In resting muscle, Lac uptake rates were greater than Lac efflux rates in the post-exercise preparation. L-Lac influx exhibited partial saturation kinetics, while D(-)-lactate (D-Lac) influx was linearly related to its extracellular concentration (range: 0 to 32 mM). At an extracellular L-Lac concentration of 16 mM, in conjunction with a negligible transmembrane L-lactic acid (HLac) gradient and an

outwardly directed net driving force on L-Lac$^-$, CIN reduced net L-Lac uptake by 75%, while SITS caused a 45% inhibition. At the same extracellular concentration (16 mM), D-Lac influx was 64% of the net L-Lac influx. These results suggest that at 16 mM extracellular L-Lac, the Lac$^-$/H$^+$ cotransporter accounts for 30-36%, the Lac$^-$ /HCO$_3^-$- Cl$^-$ anion exchanger for 39-45%, and diffusion for 19-25% of uptake, though the latter is probably overestimated and the former underestimated for methodological reasons. Net L-Lac efflux was not affected by extracellular D-Lac concentration and/or D-Lac influx, implying the existence of a concurrent lactate efflux during lactate uptake. The D-lactate influx kinetics data indicated that the Lac$^-$ /HCO$_3^-$ exchanger was not saturable in the extracellular D-Lac concentration range of 0 to 32 mM. This study clearly demonstrates the involvement of carrier-mediated transport in transmembrane lactate movement in fish muscle, and supports the "lactate retention" mechanism proposed by Turner and Wood (54).

INTRODUCTION

In higher vertebrates, the exchange of lactate across skeletal muscle cell membranes is regarded as an important process in the regulation of its production, redistribution, and utilization. Lactate is now considered an important metabolic intermediate between carbohydrate energy reserves (glycogen and glucose) and the end products (CO_2 and H_2O). Thus, instead of simply being treated as a terminal waste product of "anaerobic glycolysis", causing fatigue and acid-base disturbance, and eventually being eliminated from the synthesis sites (*e.g.* skeletal muscles), the released lactate may be transferred, as a precursor, to red muscle, cardiac muscle, or even other less activated white muscles, for oxidation. Hepatic gluconeogenesis (*i.e.* the Cori cycle), and *in situ* glyconeogenesis in white and red muscle may also occur, using lactate as a substrate (1,3,6,17,41), although the latter remains controversial (46). The glycolytic fibers (white muscle), once thought to be the massive production site of lactate, are also involved in the removal of extracellular lactate from other tissues or exogenous sources (for reviews see 5,12,44). In mammals, work has been carried out to identify the mechanisms for lactate transport and to characterize the kinetics of lactate exchanges amongst various tissue compartments (16,23,29,31,32,48,60). Transmembrane lactate (Lac, refers to total lactate) movement has been found to occur *via* passive diffusion of undissociated lactic acid (HLac) or ionic Lac^-, as well as through carrier-facilitated transport, namely Lac^-/H^+ symports and Lac^-/HCO_3^--Cl^- antiports. The contribution of each route, however, varies depending on species, pH gradient, lactate concentration gradient, age, training, metabolic state, and hormonal conditions (5,16,19,23-25,27,31,33,48,59).

In fish, in contrast to higher vertebrates, our understanding of lactate transport mechanisms remains fragmentary (19,57,59,61). Recent studies on fish have suggested that the major portion of the post-exercise lactate load is retained in the white muscle by active or passive mechanisms, and is metabolically removed *in situ* by oxidation and/or glycogen resynthesis (26,35-38,51,54,55,57,59,61). This clearance of the post-exercise lactate load from fish white muscle can take more than 12 h in contrast to less than 1 h in higher vertebrates. The non-release or slow release of lactate from white muscle is thought to be advantageous for fish for several reasons (61): 1) The fish body is composed of as much as 70% white muscle, and extracellular fluid (ECF) is relatively poorly buffered and small in volume compared to muscle intracellular fluid (ICF) (20,37,38,52). 2) During exhaustive exercise in fish, the white muscle fibers are uniformly activated and loaded with lactate; consequently the removal of lactate to less exercised white muscle for metabolic processing, as occurs in mammals, may not be an option. 3) Lactate oxidation in cardiac muscle, and hepatic gluconeogenesis play a very minor part in total lactate clearance (13,34,35,40,50,61). However, the pertinent question remains: do lactate and H^+ efflux occur at rates well below the membrane specific transferring capacity due to the "equilibrium limitation" (20), or is there an active regulating mechanism involved in the process, as originally proposed by Turner and Wood (54).

Recently, I have developed an isolated-perfused tail-trunk preparation of the rainbow trout for the study of metabolite fluxes in white muscle (56,57). The objectives of the present study were to utilize this preparation to characterize the transmembrane movement of lactate (Lac) and metabolic H^+ (ΔH_m^+) in post-exercised and resting white muscle. Specific inhibitors of carrier-mediated transport, and manipulations of perfusate concentrations of L(+)-lactate and its isomer D(-)-lactate, were employed to identify the roles of carrier-mediated transporters and passive diffusion in lactate release and uptake in white muscle.

MATERIALS AND METHODS

Experimental Animals

Rainbow trout (*Oncorhynchus mykiss*) of either sex (400-600 g) were obtained from a local trout hatchery and held as described previously (56). During the holding period of 2-4 months, trout were fed approximately 2% (wet body weight per day) of trout grower floating pellets (Aquaculture Zeigler Bros., 50% protein, 15% lipid) until the desired size (800-1000 g) was reached. Fish were acclimated to the experimental temperature of 15 ± 1°C for 5-7 days without feeding prior to experimentation. Fish were placed for 48 h in darkened acrylic flux chambers supplied with flowing dechlorinated water to establish "resting conditions". "Exercised" fish were then manually chased in a 150 L tank for 6 min to exhaustion. The resting or exercised fish was then anaesthetized with a high dose of MS-222 (0.5 g L^{-1}) neutralized with NaOH, and sacrificed within 1 min without struggling. The portion of the fish posterior to the anus was cut off for the tail-trunk perfusion study. At this time, an initial white muscle sample (3-5 g) from the area between the dorsal fin and the lateral line immediately *anterior* to the point of section was preserved by freeze-clamping (see below) to establish the metabolic condition of the preparation prior to the start of perfusion.

Perfused Tail-Trunk Preparation and Experimental Design

The isolated-perfused tail-trunk preparation used in this study has been described previously (see Wang *et al.*, 56,57, for detailed set-up and evaluation). The study consisted of two series: a post-exercise series, which was designed to examine lactate transport mechanisms during post-exercise lactate release, and a resting series, which was designed to characterize lactate uptake mechanisms and their kinetics (Table 6-1).

Cortland salmonid saline, supplemented with 3% bovine serum albumin (Sigma, Fraction V), was used as the perfusate. The saline was equilibrated with 0.25% CO_2/balance O_2, and the desired pH (resting plasma pH ≈ 8.0; exercised pH ≈ 7.5) achieved by varying the saline [$NaHCO_3$] from 7 to 2 mmol l^{-1} under a constant partial pressure of CO_2 (Pco_2 ≈ 2 torr). Inflowing Po_2 was kept in the range of 480 - 530 torr. Arterial and venous sampling ports were implanted into the caudal artery and caudal vein, respectively, to allow the collection of inflowing and outflowing perfusate samples into gas-tight Hamilton syringes at 30 and 60 min after the perfusion was started (see Fig. 6-2 of Wang *et al.*; 56, for a schematic diagram of the set-up). The perfused trunk preparation was placed in a 15 ± 0.5°C water bath, and the perfusion rate of 2 ml min^{-1} 100 g^{-1} tail weight was delivered by a peristaltic pump. In all treatments, the perfusate, for the first 30 min, consisted of heparinized lactate-free saline, to facilitate blood purging, and to secure a steady state and a red blood cell-free preparation. Experimental treatments, as summarized in Table 6-1, were introduced in the second 30 min. A pressure transducer, attached to the constant flow perfusion line, monitored variations in the vascular resistance of the preparation. In our previous studies, the acid-base and metabolic conditions of the perfusion preparation were proven to be satisfactory over the experimental period (56,57) and the exercise protocol was shown to be sufficient to elevate intracellular [Lac] to a level comparable to that seen *in vivo* after exhaustive exercise (37,38,53,55,58).

At 30 and 60 min into the perfusion, arterial and venous samples (2 ml each) of perfusate were collected, and pH, total CO_2 (Tco_2), Po_2 and protein content were analyzed immediately. A portion (300 μl) of the saline was deproteinized with two portions of 8% perchloric acid (PCA). The supernatant of this PCA extract was then stored at -70°C for later analysis of [Lac]. The remainder of the perfusate sample (500 μl) was used to measure $[Na^+]$, $[K^+]$, and $[Cl^-]$.

Immediately after the termination of the 60 min perfusion, a white muscle tissue sample (3-5 g) from the area above the lateral line of the tail-trunk was preserved by freeze-clamping in liquid N_2 with a pair of pre-cooled aluminum tongs. The muscle samples were stored at -70°C prior to further analysis. Intracellular [Lac], pH (pH_i), $[Cl^-]$, $[Na^+]$, $[K^+]$ and water content were measured on white muscle tissue.

(1) The Post-exercise Series (Efflux Experiments)

This series continued the approach of our previous work, in which the effects of transmembrane potential and pH gradients on Lac efflux were studied (57). In the present study, various pharmacological inhibitors (Table 6-1) were applied to post-exercise trunks in order to identify the transport processes involved in Lac efflux (*cf.* Fig. 6-1A). In each experiment, Lac-free saline, containing one of the following inhibitors, was used: 5 mM α-cyano-4-hydroxycinnamic acid (CIN, Sigma), 0.5 mM 4-acetamido-4'-isothiocyano-stilbene-2,2'-disulphonate (SITS, Sigma), or 0.1 mM amiloride (Sigma). An extra 5 mM of $NaHCO_3$ was added to the saline containing CIN to neutralize the acidification introduced by this acid. CIN is considered a specific competitive blocker of the Lac^-/H^+ co-transporter (Fig. 6-1A, type A) and a non-competitive blocker of the Lac^-/HCO_3^--Cl^- antiporter (band-3, Fig. 6-1A, type C). SITS, at 0.5 mM, blocks only the anion exchanger (Fig. 6-1A, type C; 44). Amiloride blocks the Na^+/H^+ exchanger (Fig. 6-1A, type D). This exchanger may influence the acid-base status across the muscle cell membrane and thereby indirectly affect the pattern of Lac flux (43,44).

As the amiloride tests were performed on a different batch of trout with quantitatively different post-exercise responses, a separate control series was performed for comparison to the amiloride treatment. As DMSO (0.5% v/v) was required to dissolve the pharmacological agents in the saline, control experiments, with or without DMSO (0.5% v/v) in the perfusate, were also conducted on the post-exercise series to identify any potential effects introduced by this solvent.

(2) The Resting Series (Influx Experiments)

The resting series, consisting of five experiments (Table 6-1), was designed to study mechanisms of transmembrane Lac uptake in white muscle (Fig. 6-1B).

Two parallel sets of experiments, with various extracellular concentrations of L(+)- or D(-)-Lac (0, 1, 2, 4, 8, 16, and 32 mmol l^{-1}), were performed to characterize the kinetics of Lac uptake *via* carrier-mediated transporters and free diffusion in the resting muscle (Fig. 6-1B). L(+)- and D(+)-Lac (Sigma) were added as sodium salts. The Lac^-/H^+ symport is considered to be stereo-specific for the L-Lac isomer, whereas the anionic antiport (Lac^- *vs.* HCO_3^- or Cl^-) does not discriminate between L- and D-Lac (44).

The roles of carrier-mediated monocarboxylate transporters and free diffusion in Lac uptake in white muscle were also assessed by applying pharmacological blockers (5 mM CIN or 0.5 mM SITS) at the 16 mmol l^{-1} extracellular L(+)-Lac level (Table 6-1).

In addition, a low extracellular pH treatment ($pH_e \approx 7.6$) at 16 mM L(+)-Lac was conducted to study the effect of extracellular pH depression on Lac uptake (Table 6-1). The rationale behind the low pH treatment was to evaluate whether HLac diffusion is involved in Lac uptake by increasing the extracellular HLac concentration.

Analytical Protocols

Extracellular pH and Po_2 were measured in arterial and venous saline at 15°C, using, respectively, a Radiometer microelectrode (E5021) and PHM-84 acid-base analyzer and a Radiometer Po_2 electrode (E5046) connected to a OM-200 O_2 meter (Cameron Instrument). Measurement of perfusate Tco_2 was carried out using a Corning total CO_2 analyzer (Model 965, Corning Canada Inc.). Pco_2 and $[HCO_3^-]$ were calculated using the Henderson-Hasselbalch equation with appropriate constants (αCO_2 and pK') for trout true plasma at 15°C (4). The perfusate electrolytes (Na^+, K^+, and Cl^-) were measured by means of an AVL specific electrode (Model 983-S electrolyte Analyzer). The specific L-lactate dehydrogenase (Sigma) and D-lactate hydrogenase (Sigma), NAD/NADH assays described by Bergmeyer (2) were used to analyze the perfusate L- and D-Lac concentrations, respectively. Perfusate total protein and water content were measured with a refractometer (American Optical). The non-bicarbonate buffer capacities (β) of salines containing DMSO, CIN, SITS, or amiloride were determined according to the methods described previously (56); L-Lac and D-Lac had no measurable effect on β.

Freeze-clamped muscle samples were pulverized in liquid N_2 with a mortar and pestle. A portion of the frozen muscle tissue (200-300 mg), without pulverization, was used to determine the tissue water content (C_w) by drying in an oven at 70°C for 48 h to constant weight. The muscle tissue powder was then used for pH_i measurements or lyophilized for 64 h to a dry powder for analysis of Lac and ion concentrations. Muscle pH_i was measured by the homogenization technique described elsewhere (45,56). The freeze-dried muscle powder (approx. 20 mg) was weighed, then extracted with 1 ml of 8% perchloric acid (PCA). The supernatant of the PCA extracted tissue was used to determine tissue L- or D-Lac levels enzymatically (2). L(+)-Lac measurements on saline and muscle tissue were also carried out in the D-Lac series to monitor the potential effects of D-Lac treatment on L-Lac movement. For tissue ion measurements, the freeze-dried tissue powder (approx. 20 mg) was weighed, then extracted in 1 N HNO_3 (1 ml) at 50°C for 48 h. Flame atomic absorption spectrometry (Varian AA-1275) was used to determine $[Na^+]$ and $[K^+]$ in the diluted supernatant, while $[Cl^-]$ was analyzed by coulometric titration. Common standards were used to calibrate all the instruments involved in saline and tissue ion analyses.

Calculations.

Flux rates for Lac, ΔH_m^+, Tco_2, HCO_3^-, O_2, and ions were calculated according to the Fick principle, using the perfusion rate (2 ml 100 g^{-1} min^{-1}) and the measured concentration differences between the arterial and venous perfusate sample.

The muscle extracellular space (ECFV, ml g^{-1}) was estimated by the "Cl^--K^+ space" equation of Conway (see 7,56 for details), based on tissue C_w, muscle $[K^+]$ and muscle $[Cl^-]$, while the intracellular space (ICFV, ml g^{-1}) was given by the difference between C_w and ECFV. All concentrations of substances in the muscle tissue were corrected for the level existing in trapped ECF, and expressed per liter of ICFV (*i.e.* mmol l^{-1} ICFV).

The arteriovenous difference in "metabolic acid" (ΔH_m^+) was calculated according to the following equation (39):

$$\Delta H_m^+ = [HCO_3^-]_a - [HCO_3^-]_v - \beta \times (pH_a - pH_v) \tag{1}$$

where subscripts *a* and *v* denote measured values in arterial and venous saline, respectively.

The total lactate (Lac) is the sum of the ionic (Lac^-) and non-ionic (HLac) forms, and with a pK' ≈ 3.75, exists largely as Lac^- under physiological pH (6.0-8.0) according to the following equation:

$$pH = pK' + \log\frac{[Lac^-]}{[HLac]} \tag{2}$$

Based on the above relationship, [HLac] can be estimated as:

$$[HLac] = \frac{[Lac^-]}{1 + 10^{(pH - pK)}} \tag{3}$$

Therefore, at constant total Lac concentration, a decrease in pH results in a higher [HLac]. Under 'normal' resting and post-exercise conditions, there will always be an outwardly directed HLac gradient across the muscle cell membrane, owing to the ~0.6 unit lower pH_i compared to pH_e and a higher intracellular total [Lac].

If the muscle cell membranes are freely diffusible only to Lac^-, then the diffusive Lac-flux should be a linear function of the net driving force (NDF) on Lac^-:

$$NDF = E_m + \frac{RT}{ZF}\ln\frac{[Lac^-]_e}{[Lac^-]_i} \tag{4}$$

where R, Z, and F have their usual values, and T is the absolute temperature. As in our previous studies (56,57), the transmembrane potential (E_m) of the white muscle was estimated from measured intra- and extracellular [K^+], [Na^+], and [Cl^-] according to the Goldman-Hodgkin-Katz equation (21).

Statistics.

All values are presented as means ± S.E.M. (N) and significance was taken at $P \leq 0.05$ in all tests. Within each treatment group, Student's paired t-test was used to examine the differences between 30 and 60 min values. One-way ANOVA was used to test for significant differences among the means in the various treatments in the exercised and resting experiments, then *post hoc* comparison by means of Duncan's critical and multiple range test was performed between the control and each treatment at common times. The two-tailed Student's t-test for non-paired data was also used to test corresponding points in the L- and D-Lac series (Statistica, Statsoft Inc.). Linear regression was carried out on the data of the D-Lac experiment using the least-squares regression method. Curve fits to other data sets were accomplished using non-linear least-squares regression methods (SigmaPlot, Jandel Scientific).

RESULTS

Perfusion pressures (10-16 cm H_2O), and therefore vascular resistances, at constant flow = 2 ml 100 g^{-1} min^{-1}, were similar in resting and post-exercise series, and remained generally stable throughout the 60 min of perfusion. The rate of O_2 uptake of the preparation (0.8 to 1.0 mmol kg^{-1} h^{-1}) did not vary significantly over the course of the experiment, and there was no significant difference between the resting and exercised series (results not shown). Measured non-bicarbonate buffer capacities (β) of the perfusate used in different treatments are summarized in Table 6-1.

(1) Post-Exercise Series

The exhaustive exercise protocol used in this study introduced a pronounced intracellular acidosis, intracellular Lac accumulation, and partial depolarization in white muscle at 0 min post-exercise in all treatment groups (Table 6-2). Over the 60 min of perfusion, there was no correction of pH_i or reduction of $[Lac]_i$, but E_m repolarized slightly in all treatment groups (Table 6-2).

In control experiments, the presence or absence of 0.5% DMSO had no significant effect on post-exercise responses. However, control experiments for the two different batches of trout ("control-1" accompanied the CIN and SITS series; "control-2" accompanied the amiloride series) yielded quantitatively different results, and therefore have been presented separately. The trout of the second batch did not appear to exercise as intensively as the first batch, as evidenced by lower $[Lac]_i$ and higher pH_i in the muscle samples obtained both before and after perfusion (Table 6-2).

Lactate Efflux

Post-exercise intracellular muscle $[Lac]_i$ was about 75 mmol l^{-1} ICF in the control-1, CIN, and SITS treatments (Table 6-2). However, in the control-2 and amiloride treatments, post-exercise $[Lac]_i$ was only about 70% of this level (Table 6-2). In all five treatments, Lac efflux rates were similar (approximately 2 mmol kg^{-1} h^{-1}) after 30 min of perfusion (Fig. 6-2). After another 30 min of perfusion, Lac flux rate did not change in either of the control groups or the SITS group, while the CIN treatment resulted in a significant 40% decrease relative to its respective control group (control-1; Fig. 6-2). Although net Lac efflux tended to fall in the amiloride group in the second 30 min (Fig. 6-2), the difference was not statistically significant with respect to the relevant control group (control-2).

Metabolic Acid Flux

As in our previous study (57), the uncoupling of ΔH_m^+ and Lac movements was again very clearly demonstrated, because Lac *efflux* was always accompanied by ΔH_m^+ *influx* (Fig. 6-2, 3A). Post-exercise ΔH_m^+ flux was inwardly directed (net uptake into muscle) after both 30 and 60 min of perfusion in all treatments (Fig. 6-3A). However, the ΔH_m^+ influx was reduced by 50% after 60 min of perfusion in the control-1, control-2, and the CIN groups, whereas there was no such decrease after SITS treatment. In effect, ΔH_m^+ influx was significantly elevated by SITS relative to control-1 at 60 min. The amiloride treatment depressed ΔH_m^+ influx to a level not significantly different from zero or control-2 following 60 min of perfusion (Fig. 6-3A). Despite the constancy of O_2 uptake, Tco_2 efflux rate dropped over the post-exercise perfusion period in all treatments, but the values did not vary significantly among these treatments at 30 or 60 min (Fig. 6-3B). At 60 min, the acid-base status of the venous effluent perfusate and arteriovenous differences in Tco_2, Pco_2 and $[HCO_3^-]$ were very similar in the two control and all three experimental groups (Table 6-2).

(2) Resting Series

In resting preparations at 60 min, the white muscle pH_i (approximately 7.25) and $[Lac]_i$ (approximately 3 mmol l^{-1}) were generally similar throughout the various Lac concentrations in both the L-Lac (without inhibitors), and D-Lac series (Table 6-4). These values were not significantly different from their corresponding pre-perfusion levels at 0 min (data not shown) or from the *in vivo* resting values (55). In comparison with the 16 mM L-Lac group, $[Lac]_i$ increased about 75% and 80%, respectively, in the CIN and SITS treated groups (Table 6-4) despite the fact that their pre-perfusion $[Lac]_i$ were not different from the typical resting values reported above. In contrast, at 16 mM $[L\text{-}Lac]_a$, lower pH_a ("acid + 16") did not result in significant changes in $[Lac]_i$ (Table 6-4). Notably, these treatments which raised $[Lac]_i$ did not lower pH_i, and in the case of lower pH_a, actually caused a slight rise in pH_i.

Lactate Influx

As shown in Figure 4A, net L-Lac influx into resting white muscle was actually slightly negative (*ie.* efflux) at low extracellular concentrations of $[L\text{-}Lac]_a$ (0, 1, and 2 mM), in accord with the outwardly directed concentration gradients under these conditions (*cf.* Table 6-4). Net L-Lac influx became positive at higher $[L\text{-}Lac]_a$ levels. Influx started to level off at a rate of about 5 mmol kg^{-1} h^{-1} between the 16 mM and 32 mM $[Lac]_a$ points, suggesting saturation kinetics. Notably, this absolute net flux rate in the inward direction was more than twice that (about 2 mmol kg^{-1} h^{-1}; Fig. 6-2) seen in the outward direction in the post-exercise series. This difference occurred despite the fact that the inwardly directed [L-Lac] concentration and electrochemical gradients in these resting preparations were far lower (or even negative) relative to the large outwardly directed gradients in the post-exercise series.

For example, the simple chemical concentration gradient for [L-Lac] was approximately 50-70 mmol l^{-1} outward in the post-exercise series (Tables 2, 3), a difference which would be further increased by the highly negative value of E_m. These observations suggest that L-Lac transport is preferentially rectified in the inward direction in trout white muscle.

D-Lac net influx exhibited a somewhat different pattern from L-Lac influx. D-Lac influx was never negative, and increased in a simple linear fashion with extracellular concentration (Fig. 6-4A). Unlike L-Lac, D-Lac is not naturally present in animal tissues. Thus, theoretically $[\text{D-Lac}]_i$ was zero. Even after 30 min of D-Lac perfusion at various $[\text{D-Lac}]_a$ levels, muscle intracellular [D-Lac] was not detectable with the enzymatic assay employed in this study. However, $[\text{L-Lac}]_i$ values of the D-Lac series have been presented in Table 6-4 to illustrate the stable metabolic state of the preparation. The different levels of D-Lac in the extracellular fluid did not affect the negative net L-Lac "influx" which remained stable at -0.24 ± 0.05 mmol kg^{-1} h^{-1}. Thus, there must normally be a small lactate efflux occurring concurrently during lactate uptake.

When compared at a common extracellular concentration of 16 mM, D-Lac influx was 64% of net L-Lac influx, a significant difference (Fig. 6-4A). Inasmuch as the Lac^-/H^+ symport is considered to be stereo-specific for the L-Lac isomer, while other mechanisms are not (44), a simple interpretation is that 36% of L-Lac influx occurs by the symport, and 64% by anionic exchange (*i.e.* Lac^- *vs.* Cl^- or HCO_3^-) and/or diffusion. CIN treatment, reported to block both the symport and anionic exchange, resulted in a 75% inhibition of net L-Lac influx, suggesting a 25% contribution from diffusion (Fig. 6-4B). In accord with this breakdown, SITS treatment, reported to block only anionic exchange, reduced net L-Lac influx by 45% (Fig. 6-4B). Overall, these experiments suggests that the symport contributes 30-36%, the anion exchanger 39-45%, and diffusion 19-25%.

E_m did not vary significantly among any of the resting groups, and ranged from -80 to -91mV. In these experiments, net L-Lac influx was always directed against its electrochemical gradient (NDF, Eq. 4) and the influx increased exponentially after NDF became less negative than -80mV (Fig. 6-5A). A third order linear regression (hypobolic) provided a good fit through the mean values. The measured net Lac flux rate of the "pH=7.68+16mM" fitted well on the regression line (Fig. 6-5A).

Net L-Lac influx occurred at extracellular $[\text{L-Lac}]_a$ concentrations of 4 and 8 mM (Fig. 6-4A), even though the HLac gradient (Fig. 6-5B), as well as the NDF on L-Lac^- (Fig. 6-4B), were both outwardly directed. Not until $[\text{Lac}]_a$ reached 32 mM, did the L-HLac gradient become inwardly directed, while the D-HLac gradient was always inwardly directed owing to the lack of intracellular D-Lac (Fig. 6-5B). For both L-Lac and D-Lac fluxes, there were approximately linear relationships between net flux rate and the respective L-HLac and D-Hlac gradients, with the former displaced laterally to the left (Fig. 6-5B). The "pH= 7.68+16 mM" treatment, designed to make the L-HLac diffusion gradient more positive, did not result in an increase in net L-lac influx, but rather a significant decrease, and therefore plotted well off the regression line (Fig. 6-5B).

Metabolic Acid Flux

In both L- and D-Lac groups, pH_a was maintained between 7.9-8.0 in each treatment (with a grand mean of 7.920 ± 0.012, N = 83, data not shown), whereas pH_v averaged about 7.8 (with a grand mean of 7.806 ± 0.012, N = 83, data not shown).

ΔH_m^+ flux rates remained near zero in the L-Lac series as [L-Lac]$_a$ was raised from 0 to 8 mM, whereas significant influx occurred in the 16 and 32 mM [L-Lac]$_a$ treatment groups (Fig. 6-6).

At 16mM [L-Lac]$_a$, CIN completely blocked ΔH_m^+ influx, while SITS only partially blocked the influx (Fig. 6-6A). The ΔH_m^+ influx rate in the 16 mM+Acid group was comparable to that at 16 mM at normal pH. Unlike their L-Lac counterparts, in the D-Lac experiments, the high [Lac]$_a$-induced ΔH_m^+ influx did not occur. In fact, there was very little, if any, net ΔH_m^+ flux (Fig. 6-6A).

Tco_2 efflux rates in the resting groups with low or 0 [L-Lac]$_a$ (Fig. 6-6B) were about 50% of the rates measured in post-exercise preparations (Fig. 6-3B), despite the fact that O_2 consumption rates (data not shown) were the same as in post-exercise preparations. Unlike the post-exercise preparations, Tco_2 efflux rates remained unchanged over the 60 min of the perfusion period. The Tco_2 efflux rate was not affected by elevated [L-Lac]$_a$ up to 8 mM, but increased significantly in the 16 and 32 mM groups (Fig. 6-6B). However, at 16 mM [L-Lac]$_a$ with CIN or SITS, the Tco_2 efflux rates did not increase (Fig. 6-6B). Meanwhile, in the D-Lac series, the Tco_2 influx rate remained constant (Fig. 6-6B).

DISCUSSION

The Isolated-Perfused Tail-Trunk Preparation

In previous studies (56,57), I have assessed the physiological condition of the isolated-perfused tail-trunk preparation and its utility for the type of experiments performed here. I have concluded that the preparation is stable with respect to acid-base status (stable pH_i), metabolic status (stable O_2 consumption rate and intracellular lactate levels) and ionic status (stable intracellular electrolytes and fluid volumes) over the 1 h perfusion, and mimics *in vivo* values extremely well for these parameters in both resting and post-exercise situations. Of particular importance for the present experiments is the conclusion that post-exercise Lac efflux rates from the perfused preparation (58) are broadly similar to those measured *in vivo* by ^{14}C-lactate turnover (36).

However, quantitative differences were seen between the two batches of trout tested in the present study. The fish used in the control-2 and amiloride series displayed responses very similar to those of our previous studies (56,57). However, the trout used in the control-1, CIN, and SITS tests exhibited higher levels of intracellular [Lac] (~ 75 *versus* ~ 50 mmol l^{-1}), lower levels of pH_i (~ 6.45 *vs.* ~ 6.50), and partial depolarization of E_m, the latter associated with a reduction of intracellular [K^+] in the present preparations. As all these differences were present at time 0 min, *before* the start of perfusion but *after* the end of exercise, I attribute them to pre-existing differences. Presumably the first batch of fish had greater anaerobic capacity for lactic acid production, probably because of greater glycogen stores (10).

An isolated-perfusion preparation such as the present offers both advantages and limitations. It differs from vesicles, dispersed muscle cells, and isolated cells in better duplicating the true *in vivo* situation where perfusion and diffusion limitations may be important in determining Lac and H^+_m transfers (19,20). Thus, Lac and acid-base equivalents moving between muscle and blood/perfusate must transit both sarcolemmal and endothelial cell membranes as well as the interstitial fluid space. The [Lac] and acid-base composition of the blood/perfusate changes as it flows through the preparation. If movements of any of these substances are rapid relative to the flow rate, "equilibrium limitation" may occur, especially at the level of the venous capillary (19,20,44). Inasmuch as the perfusion rate chosen was comparable or higher than *in vivo* blood flow estimates in intact post-exercise trout (discussed in 56), vascular resistance was lower than *in vivo* (62), and the inflowing perfusate was lactate-free, perfusion limitation for Lac was likely less than *in vivo*. However, this may not be the case for H^+_m, because the perfusate lacked carbonic anhydrase activity and provided a non-HCO_3^- capacity (β) only about 40% of that of trout blood (38). Therefore, this study was not designed to define the *in vivo* rates of various Lac and H^+_m transport mechanisms, but rather to verify their presence or absence and relative importance.

The present study showed that O_2 uptake during post-exercise recovery does not significantly differ from the resting values despite an increase in CO_2 efflux (Fig. 6-3B, 6B, (57). The absence of a post-exercise increase in O_2 uptake together with unchanged $[Lac]_i$ over time (Table 6-3) suggests that Lac oxidation was negligible during this first hour of post-exercise recovery. The increase in Tco_2 efflux without the corresponding changes in O_2 uptake seen in the present study could result from an O_2 independent shunt to form acetyl-CoA from the exercise elevated acetyl-carnitine pool, as seen *in vivo* after exercise (55). Acetyl-CoA could then be metabolized in the Krebs cycle to produce CO_2 (18).

The Release of Lactate from Post-Exercise Muscle

The most important finding of the post-exercise study is that carrier-mediated transport and passive diffusion are both involved in Lac release from exercised trout white muscle. The significant 40% depression in Lac efflux caused by CIN, in combination with the insignificant effect of SITS treatment, clearly suggests the involvement of a Lac^-/H^+ co-transporter in post-exercise Lac release from fish white muscle (Fig. 6-2), whereas the role of Lac efflux through the Lac^- *vs.* Cl^- or HCO_3^- antiporter, if any, is minimal (Fig. 6-1A). Assuming that the CIN concentration was high enough to block all of the transporters on the cell membrane (61), the remaining part of Lac release (approximately 60%) occurs through free diffusion of either HLac or Lac^-. In the case of passive diffusion, Lac should move according to either the NDF for Lac^- (heavily influenced by E_m, Eq. 4), or the HLac concentration gradient (heavily influenced by the transmembrane pH gradient, Eq. 3). My previous study on Lac efflux from post-exercise trout muscle indicated the importance of electroneutral mechanisms, such as Lac^-/H^+ co-transport and/or HLac diffusion, and strongly discounted the importance of the free diffusion of Lac^- according to NDF (57). In particular, partial depolarization of membrane E_m by experimental elevation of $[K^+]_e$ did not result in the expected decrease in Lac efflux (58). Similar observations have been made on rat muscle sarcolemmal vesicles (49). This leaves the passive movement of HLac as the only diffusive mechanism, and this conclusion has been supported by many studies in higher vertebrates where changes in transmembrane pH gradient did impose a marked influence on lactate efflux (25,28,31,60). In my earlier study on post-exercise trout muscle (58), I found that net Lac efflux responded to the HLac gradient, but not in a proportionate manner. This finding is now understandable in view of my current finding that a carrier-mediated Lac^-/H^+ symport is also involved, and that the relatively small net Lac efflux is occurring against a background of simultaneous Lac uptake activity (see below).

Although, in the past, the passive diffusion of HLac or Lac^- was regarded as the predominant route for lactate efflux from muscle ICF to ECF (19,29), my results along with other studies on mammals and amphibians, indicate that a considerable portion of the Lac release from exercised muscle occurs through the saturable Lac^-/H^+ co-transporter, but not the Lac^-/anion exchanger (11,25,28,30,31,47). In an early study on the perfused trout-trunk, Turner and Wood (54) observed that SITS actually increased post-exercise Lac efflux, suggesting that Lac^-/Cl^--HCO_3^- antiporters may play a more important role in concurrent active Lac retention, rather than in the release process during post-exercise recovery. At first glance, this seems inconsistent with the lack of observed effect of SITS on net Lac efflux, in the present study. However, the lower perfusion rate (less than 40% of the rate used in the present study), and the larger portion of the trunk used by Turner and Wood (54), resulted in much higher Lac levels in the venous effluent. This higher extracellular $[Lac^-]$ may have facilitated Lac^- *uptake via* the band 3-mediated anion exchange. As a result, SITS blockade under these circumstances would increase net Lac efflux.

The SITS-triggered enhancement of ΔH_m^+ influx (equivalent to a depression of HCO_3^- efflux) observed in the present study (Fig. 6-3A) provides circumstantial evidence to support the idea that Lac uptake mediated by the Lac^-/HCO_3^- exchanger is occurring in the post-exercise preparation. If I assume the above scenario to be true, with CIN treatment completely eliminating Lac efflux *via* the symport (Fig. 6-1A, type A), and Lac uptake *via* the anion exchanger (Fig. 6-1A, type C, noting the exchange direction should be reversed), then the reduced Lac efflux which persists after CIN treatment (Fig. 6-2) should represent the "free diffusion portion" of Lac efflux. Under all other circumstances, simultaneous Lac uptake will usually result in an underestimated efflux rate.

Lactate transport, either by free diffusion of HLac or by carrier-facilitated mechanisms, is often pH gradient sensitive (25,28,31,48). Therefore, the Na^+/H^+ exchanger could serve as an important co-regulator for muscle pH_i and indirectly alter the apparent stoichiometry of Lac^-/H^+ transport. However, in the present preparation, the decreased Lac efflux in the amiloride group was not significant relative to the corresponding control-2 value at 60 min (Fig. 6-2). More importantly, however, amiloride did not result in a surge in Lac efflux as shown elsewhere (48). Although other studies have shown that local pH near the membrane could be disturbed (25,31,48,60), the amiloride-induced change in ΔH_m^+ flux (Fig. 6-3) was not enough to affect muscle pH_i (Table 6-2), probably because of the high muscle ICF buffer capacity (38). The lack of hormonal support in the present preparation may also have reduced the activity of the Na^+/H^+ exchanger (23).

Metabolic Acid Flux in Post-Exercise Muscle

The net ΔH_m^+ influx and its uncoupling from Lac efflux shown in Figure 3A were consistent with my earlier findings (57). However, the observation of net ΔH_m^+ *influx* was somewhat puzzling, as a release of metabolic protons is normally expected from post-exercise muscle. *In vivo*, this ΔH_m^+ efflux normally exceeds Lac efflux (37,38,61). It is important, however, to appreciate that ΔH_m^+ is a compound measurement, with an influx indicating a H^+ influx and/or HCO_3^- efflux. As pointed out in earlier studies, ΔH_m^+ flux is influenced by many factors, including the transmembrane pH gradient, the E_m (through its effect on NDF for HCO_3^- and H^+), the activity of symports and antiports for H^+ and HCO_3^-, and ECF buffer concentration (20,27,28,31,57). The latter may have had an important influence in the present study. As discussed earlier, the low value of β in the perfusate relative to whole trout blood, together with the absence of carbonic anhydrase, meant that the effective extracellular buffer concentration in my preparation was much lower than *in vivo*. Nevertheless, because my major interest was Lac release, I elected to duplicate normal *in vivo* post-exercise pH_a at the arterial inflow. As a result, non-equilibrium pH values in the venous capillary may have been exceedingly low, thereby resulting in "equilibrium limitation" and the reversal of electrochemical gradients for ΔH_m^+ movement. In support of this interpretation is my earlier finding that ΔH_m^+ became positive (efflux), and similar to Lac efflux, when an inflowing perfusate of abnormally high pH_a was used (57).

Lactate Uptake in the Resting Muscle

In the resting series, pH_i and $[L\text{-}Lac]_i$ in both pre-, and post-perfusion muscle samples (Table 6-4) were similar to *in vivo* resting muscle (26,55). My approach of elevating perfusate $[Lac]_e$ while maintaining normal pH_a under these resting conditions is an experimental strategy to study the potential Lac uptake capability of white muscle membranes. The situation is somewhat unnatural because the mammalian scenario does not apply in fish. As discussed earlier, all white muscle is activated simultaneously in fish, so inactive white muscle does not normally have the opportunity to act as a "lactate sink" for another set of white muscle which is active. Possibly however, the situation could occur in the later stages of post-exercise recovery (35).

In some ways, the most important finding is that Lac flux appears to be preferentially rectified in the *uptake* direction into white muscle. When $[L\text{-}Lac]_e$ was experimentally elevated to the blood levels (*eg.* 16 mM) normally seen after strenuous exercise *in vivo* (26,34-39,51, 55, 61), net L-Lac influx (*e.g.* Fig. 6-4) greatly exceeded net efflux measured in the post-exercise perfusion (*e.g*. Fig. 6-2). This occurred despite the fact that the passive gradients (NDF on Lac$^-$, HLac concentration gradients) strongly favor Lac efflux in the post-exercise situation (see 58 for detailed calculation), whereas the passive gradients for Lac uptake in resting preparations are either strongly opposed (highly negative NDF on Lac$^-$, Fig. 6-5A) or negligible (HLac concentration gradient, Fig 5B). The system appears to be designed to preferentially uptake and retain Lac in white muscle, and SITS-sensitive anion exchange appears to play a role in this process, as originally suggested by Turner and Wood (54).

The hyperbolic form of the L-lac uptake curve (Fig. 6-4A) suggests the involvement of a saturable or partially saturable component in lactate uptake. However, traditional methods (Lineweaver-Burk, Eadie-Hofstee plots) could not be applied to the L-Lac influx data to characterize the kinetics properties because of the relatively large linear component involved. Nonetheless, there appeared to be basic agreement with most previous studies on L-Lac transport kinetics in other systems, where K_m's ranging from 4 to 40 mM have been reported (22,31,32,47,48,60). My L-Lac uptake curve therefore seems to plateau within the reasonable range (approximately 30 mM). It is worth noting that L-Lac transport kinetics are reported to vary according to many factors. K_m decreased by 60% (from 10 to 4 mM) when pH was lowered from 7.35 to 6.8 (31), while V_{max} increased 1.5 times when temperature was raised from 25 to 37°C (49). Endurance and intensive training resulted in an increase in V_{max} and a decrease in K_m (33,42), and enhanced lactate removal from blood (9). In contrast, the linear D-Lac uptake curve did not display any indication of saturation kinetics. Since D-Lac does not move across cell membranes through the stereo-specific Lac$^-$/H$^+$ co-transporter (8), its flux likely represents movement through the anion exchanger and/or by free diffusion. In higher vertebrate systems, the Lac$^-$/HCO$_3^-$-Cl$^-$ exchanger is reported to be saturable only at extremely high concentration; it has very high capacity and low affinity ($K_m \approx$ 300 mM) (44,47-49). Thus, lactate uptake through this pathway should not show saturation kinetics at the [Lac] concentrations (both D- and L-form) used in the present study (4[illegible]). The linear relation of D-Lac influx against concentration is therefore the expected relationship (Fig. 6-5B). The difference in the lactate net flux between the two isomers may represent the portion transported by the symport. At 16 mM $[Lac]_e$, this accounted for about 36% of the observed uptake rate. CIN treatment, which should block both symport and the anion exchanger caused a 75% inhibition, whereas SITS treatment, which should block only the anion exchanger caused a 45% inhibition. These experiments are internally consistent, indicating that the symport contributes 30-36%, the anion exchanger 39-45%, and diffusion 19-25%. It is likely, however, that the diffusion component has been overestimated and the symport component underestimated for the following reasons. At an [L-Lac] of 16 mM, the NDF on L-Lac was strongly outward (Fig. 6-5A), whereas, the inward HLac gradient was negligible due to the presence of appreciable intracellular [L-Lac]. However, such an inward HLac concentration gradient did exist at [D-Lac] of 16 mM, so the difference in the net influx rates between the L- and D-Lac experiments would underestimate the symport contribution.

The rationale for decreasing pH_e in the 16mM+acid group was to examine whether improving the HLac for D-Lac gradient from essentially zero to the inward direction would lead to an increase in lactate influx. Moreover, lower pH_e can sometimes facilitate lactate uptake *via* Lac^-/H^+ cotransport according to other studies (25,31,44). As shown in Figure 5B, lower pH_e (pH_a=7.68 +16 mM), shifted the gradient to the inward direction only slightly because the actual $[Lac]_a$ achieved was only 80% of the nominal 16 mM (*cf.* Table 6-4). However, the important point is that relative to the regression relationship established by the other L-Lac data at higher pH_e ($pH_a = 7.92$), there was no increase in Lac influx, and indeed a decrease occurred. The reason for this is unknown, but it reinforces the conclusion that the contribution of HLac diffusion to influx is small or non-existent.

The significant increases in ΔH_m^+ influx in the 16 and 32 mM $[L\text{-}Lac]_a$ groups, although still substantially lower than L-lac influx, were in accord with the lactate flux data (Fig. 6-4A, 6A). Together with the CIN- and SITS-induced decrease in ΔH_m^+ influx, these results support the proposed lactate uptake mechanisms (Lac^-/H^+ co-transport, Lac^-/HCO_3^- exchange), and perhaps a very small contribution by the free diffusion of HLac. In the case of 16 mM $[Lac]_a$, the possibility of HLac diffusion was minimized as mentioned previously. Furthermore, the significant increases in Tco_2 efflux in the 16 and 32 mM $[L\text{-}Lac]_a$ groups, indicated that either lactate oxidation was accelerated due to greater lactate influx, which caused higher CO_2 excretion, or else greater HCO_3^- efflux occurred as the result of increased Lac^-/HCO_3^- exchange (Fig. 6-6B). The CIN- and SITS-triggered decreases in Tco_2 efflux support either of these possibilities (Fig. 6-6B), but the latter is favored by the constancy of O_2 uptake in the various treatments, as well as by the acid-base status of the venous effluent perfusate. Venous pH_v's were significantly higher in the 16 and 32 mM L-Lac treatments than at lower concentrations (data not shown), implying a decrease in lactate and CO_2 efflux, and/or an increase in HCO_3^- efflux. The inhibitory effects of CIN and SITS on Tco_2 efflux could also be due to inhibition of mitochondrial pyruvate transport; lactate oxidation through the Krebs cycle would be impeded, which would explain the observed accumulation of $[Lac]_i$ in muscle (Table 6-4).

In the D-Lac series, Tco_2 and ΔH_m^+ efflux rates remained constant, despite the rising D-Lac influx (Fig. 6-4A, 6A, B). Venous pH also remained unchanged (data not shown).

Since D-Lac is not metabolized in muscle, the enhanced D-Lac uptake would not provide more fuel for lactate oxidation to elevate CO_2 production. As the result of rising D-Lac influx and unchanged HCO_3^- efflux, Lac uptake mediated by band 3 may be limited when there is an inwardly directed HLac gradient. In mammalian muscle, it has been suggested that lactate uptake *via* passive diffusion increases with [Lac] or lactate gradient, and it eventually exceeds transport by the carrier system at high concentration (30 mM, 14,15).

At low or zero $[\text{L-Lac}]_e$, as in the D-lac experiments, the simultaneous L-Lac efflux (*e.g.* Fig. 6-4A) was likely due to diffusion along the outward HLac gradient. Normally, this would lead to an underestimate of the true lactate influx rate effected by the carrier-mediated transporters (Fig. 6-4A).

Transmembrane lactate transport in fish white muscle operates at a very low level in comparison with its higher vertebrate counterparts. The lactate flux rate in trout muscle were only about 1/10 and 1/5 of those observed in rats and frogs, respectively (28,60). This study clearly demonstrates the existence of lactate uptake in resting fish muscle and provides evidence to support the idea that fish white muscle has the potential to actively retain lactate *via* the anionic exchanger during post-exercise recovery (54). Unlike the situation in mammals and amphibians, which exhibit either minor or no effects of SITS on lactate uptake (14,16,31,32,60), the involvement of the Lac^- *vs.* HCO_3^- or Cl^- exchanger (by no means the only carrier-mediated transporter) was clearly demonstrated. In conjunction with specific blockers, further studies with elevated and isotopically labeled lactate in the extracellular space would provide direct evidence for the simultaneous lactate uptake in exercised fish muscle. Nonetheless, the present study suggested that fish muscle is capable of regulating lactate and H^+ flux in the resting and even post-exercise state, and the "equilibrium limitation" may not necessarily dictate the situation when lactate and H^+ are both distributed out of equilibrium and the flux rates are very low.

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Figure 6-1.

Schematic models of lactate and H^+ transport and distribution across white muscle cell membranes. Type A is the Lac^-/H^+ co-transporter; Type B is the free diffusion of non-dissociated HLac and/or dissociated Lac^-; Type C is the Lac^-/Cl^--HCO_3^- anion exchanger; and Type D is the Na^+/H^+ exchanger. Model A shows the post-exercise scenario when the intracellular space is loaded with lactate and net lactate efflux occurs. Model B shows the resting scenario when extracellular perfusate is experimentally loaded with various concentrations of lactate.

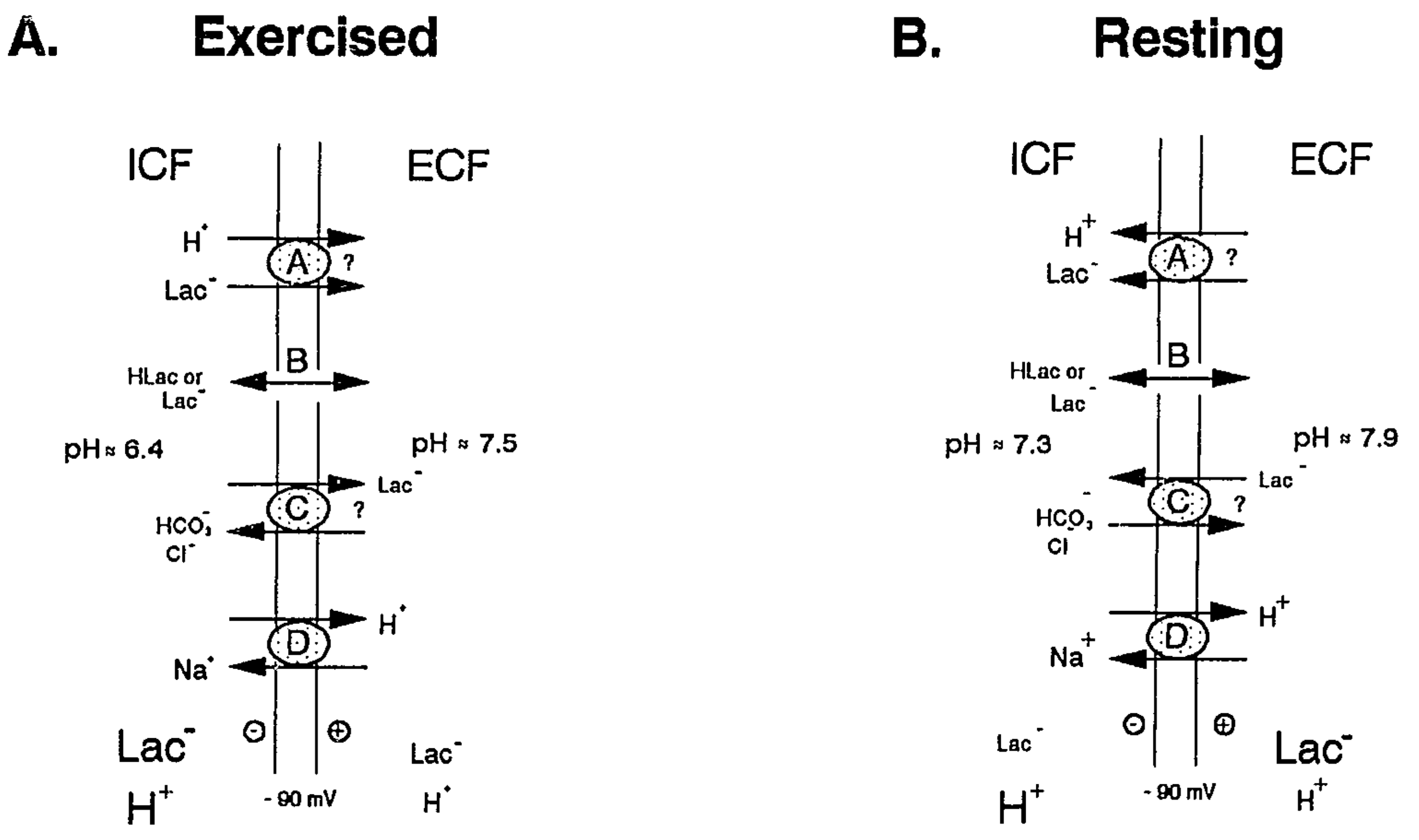

A.
Exercised
ICF
ECF
H⁺
A
?
Lac⁻
B
HLac or Lac⁻
pH ≈ 6.4
pH ≈ 7.5
Lac⁻
C
?
HCO₃⁻, Cl⁻
H⁺
D
Na⁺
Lac⁻
H⁺
⊖
⊕
-90 mV
Lac⁻
H⁺
B.
Resting
ICF
ECF
H⁺
A
?
Lac⁻
B
HLac or Lac⁻
pH ≈ 7.3
pH ≈ 7.9
Lac⁻
C
?
HCO₃⁻, Cl
H⁺
D
Na⁺
Lac⁻
H⁺
⊖
⊕
-90 mV
Lac⁻
H⁺

Figure 6-2.

Net efflux rates of Lac from the post-exercise perfused tail-trunk preparation in the various treatment groups after 30 and 60 min of perfusion. Control-1 accompanied the CIN and SITS series; control-2 accompanied the amiloride series. Efflux rates were expressed as mmol kg^{-1} wet weight of the tail-trunk. The tail-trunks were taken from exhaustively exercised trout. Values are means ± S.E.M., N = 8, 9, 10, 9, 11 for the control-1, CIN, SITS, control-2, and amiloride groups, respectively. * indicates significant difference ($P \leq 0.05$) from the corresponding control value.

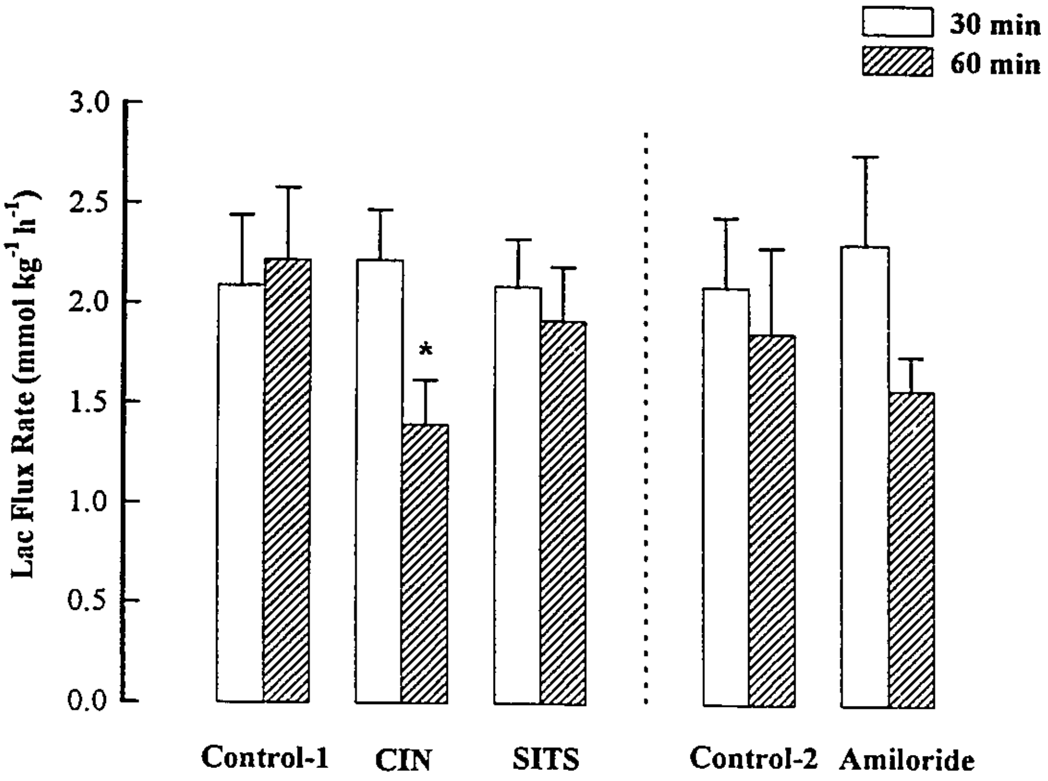

30 min
60 min
Lac Flux Rate (mmol kg^{-1} h^{-1})
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Control-1
CIN
SITS
Control-2
Amiloride

Figure 6-3.

A. ΔH_m^+ flux rates (Eq. 1) across the post-exercise perfused tail-trunk preparation after 30 min (open bars) and 60 min (striped bars) of perfusion in the control-1, CIN, SITS, control-2, and amiloride groups.

B. Tco_2 efflux rates across the preparation after 30 min (open bars) and 60 min (striped bars) of perfusion in the various post-exercise treatment groups. Other details are as in the legend of Figure 2. * indicates significant difference ($P \leq 0.05$) from the corresponding control values. ‡ indicates significant difference ($P \leq 0.05$) from the values at 30 min.

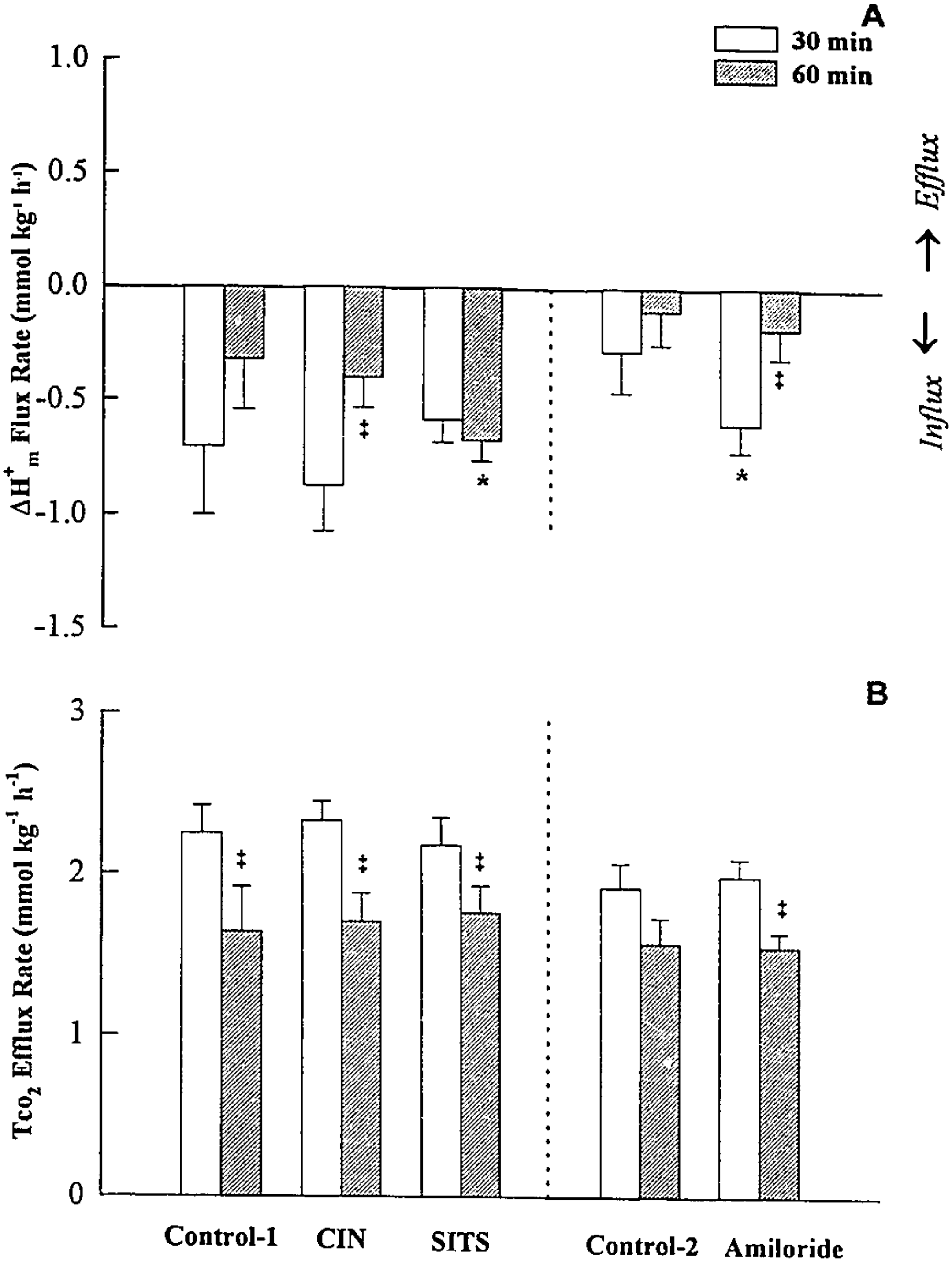

A
30 min
60 min
1.0
0.5
0.0
-0.5
-1.0
-1.5
ΔH^+_m Flux Rate (mmol kg^{-1} h^{-1})
Efflux
Influx
‡
*
‡
*
B
3
2
1
0
Tco_2 Efflux Rate (mmol kg^{-1} h^{-1})
‡
‡
‡
‡
Control-1
CIN
SITS
Control-2
Amiloride

Figure 6-4.

A. Net uptake rates of L-Lac (open circles) and D-Lac (filled triangles) after 60 min of perfusion, as a function of measured inflowing arterial L- or D-Lac concentrations ($[\text{Lac}]_a$). * indicates significant difference between the corresponding L- and D-Lac flux rates. Numbers of trunk preparation in each treatment are as in Table 6-4.

L-Lac flux presented non-linear kinetics:

L-Lac Flux = $-5.04e^{-3} \times [\text{L-Lac}]_a^2 + 0.35[\times \text{L-Lac}]_a - 0.61$, $r^2=0.99$.

D-Lac flux presented linear kinetics:

D-Lac Flux = $0.144 \times [\text{D-Lac}]_a + 0.179$, $r^2= 0.99$.

B. A comparison of the net L-Lac (open bars) and D-Lac (stippled bars) influx rates with 16 mM Lac (L- or D-) in the inflowing arterial perfusate. The effects of CIN and of SITS with 16 mM [L-Lac] in the perfusate are also illustrated, together with a tentative breakdown of the relative contributions of the transport mechanisms involved. * indicates a significant difference ($P \leq 0.05$) from the L-Lac flux rate at 16 mM $[\text{Lac}]_a$ without inhibitor treatment. Numbers of trunk preparation in each treatment are as in Table 6-4.

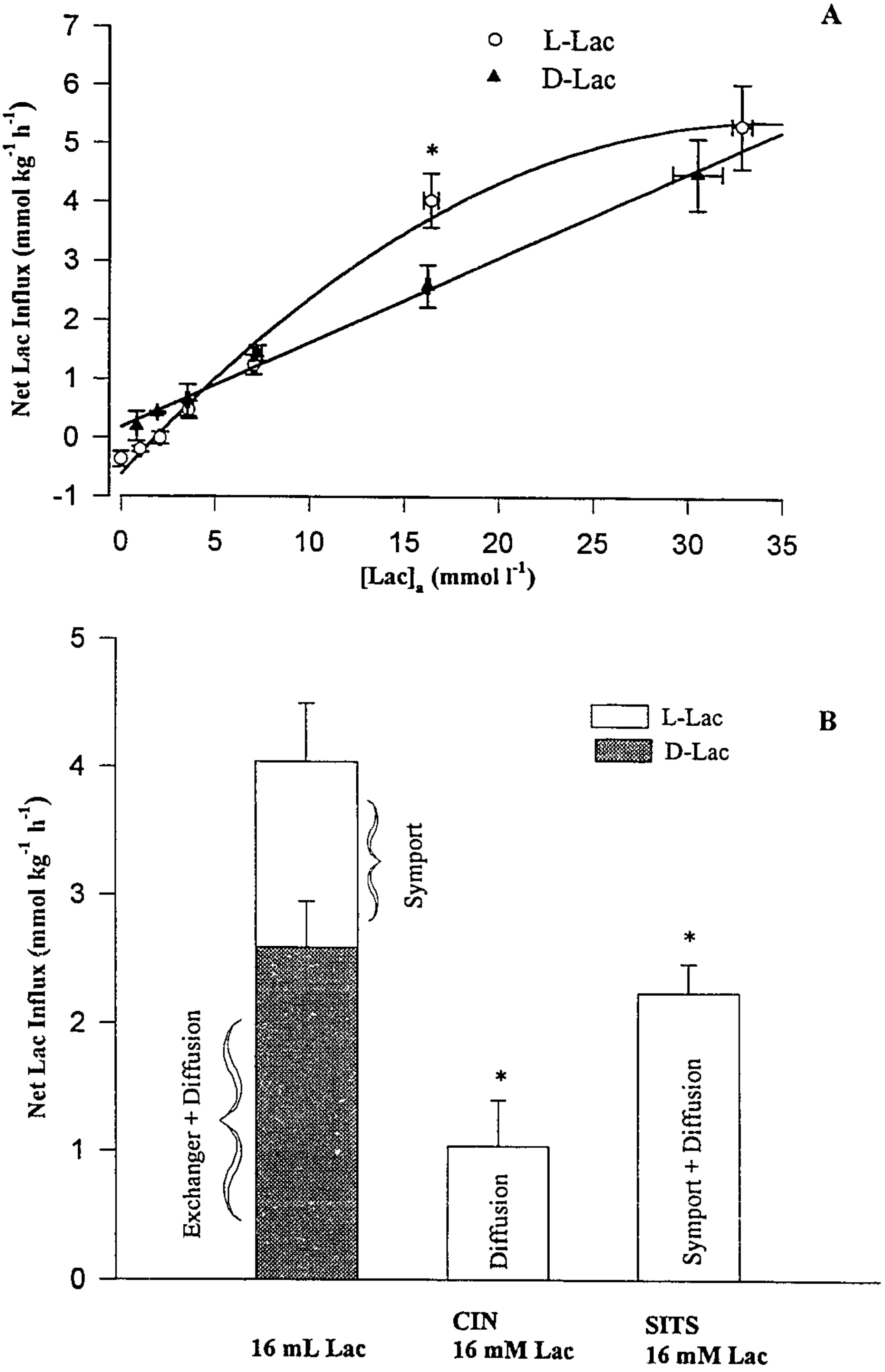

A
L-Lac
D-Lac
Net Lac Influx (mmol kg^{-1} h^{-1})
7
6
5
4
3
2
1
0
-1
*
0
5
10
15
20
25
30
35
[Lac]$_a$ (mmol l^{-1})
B
L-Lac
D-Lac
Net Lac Influx (mmol kg^{-1} h^{-1})
5
4
3
2
1
0
Symport
Exchanger + Diffusion
Diffusion
Symport + Diffusion
*
*
16 mL Lac
CIN
16 mM Lac
SITS
16 mM Lac

Figure 6-5.

A. Net L-Lac influx rates as a function of the calculated net driving force (NDF) on Lac$^-$ (Eq. 4). The NDF on Lac$^-$ was based on the measured transmembrane Lac$^-$ gradient between arterial inflow perfusate and muscle intracellular fluid after 60 min of perfusion. The curve is a third order polynomial regression line:

Lac Flux = $3.98e^{-6} \times NDF^3 + 1.80e^{-3} \times NDF^2 + 0.27 \times NDF + 13.42$, $r^2 = 0.97$.

Note: negative NDF on Lac$^-$ opposes Lac$^-$ moving into ICF *via* free diffusion.

B. Net L-Lac (open circle) or D-Lac (filled triangle) flux rates as functions of the corresponding L-HLac or D-HLac concentration gradient between arterial perfusate and muscle intracellular fluid after 60 min of perfusion. Linear regression lines have been fitted:

L-Lac Flux = $3.22 \times HLac + 3.09$, $r^2 = 0.92$

D-Lac Flux = $2.8 \times HLac - 0.13$, $r^2 = 0.99$.

The negative values indicate an outwardly directed HLac gradient, while positive values suggest an inwardly directed gradient. Note that net L-Lac uptake can occur even in the presence of an outwardly directed gradient.

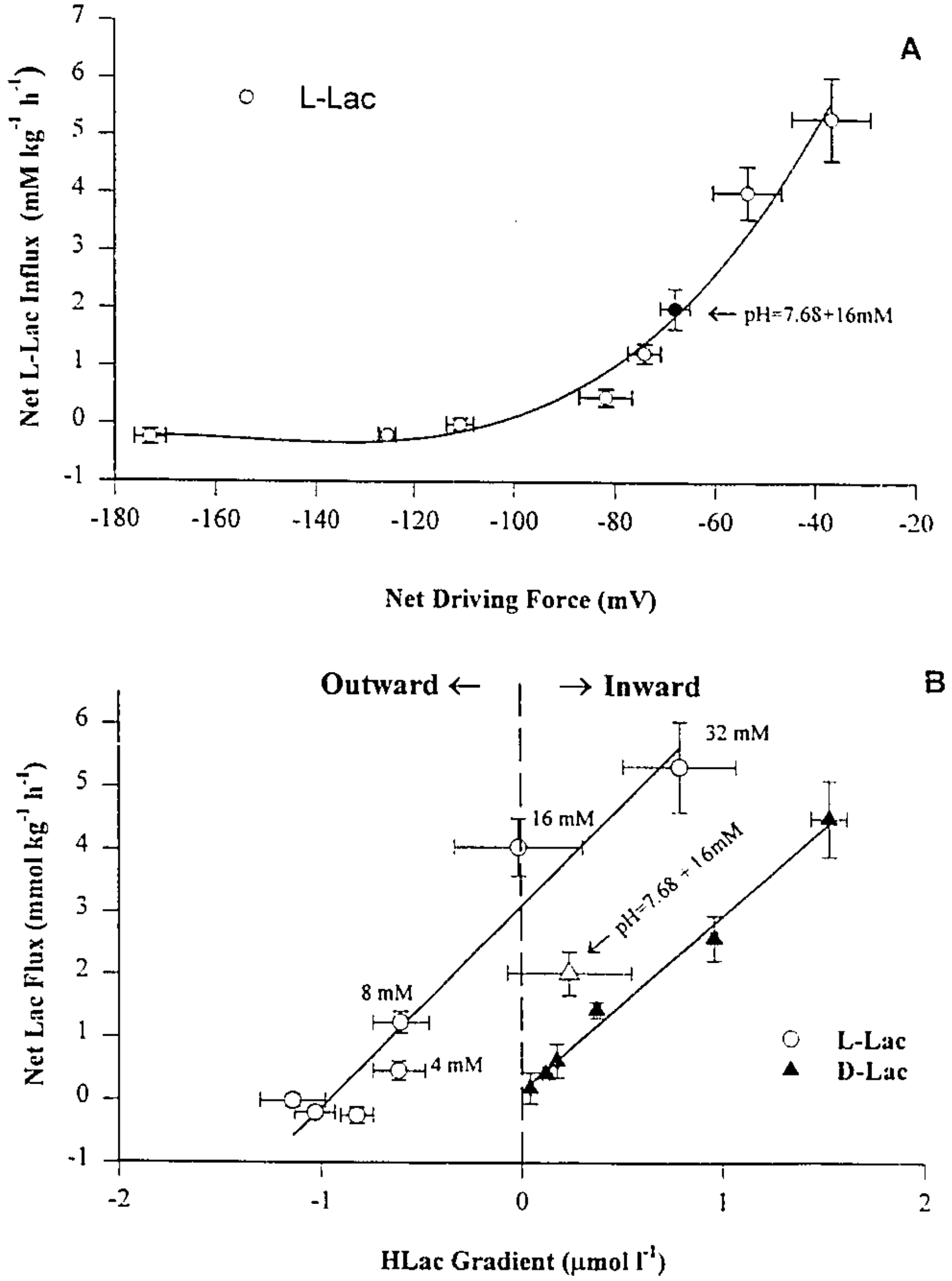
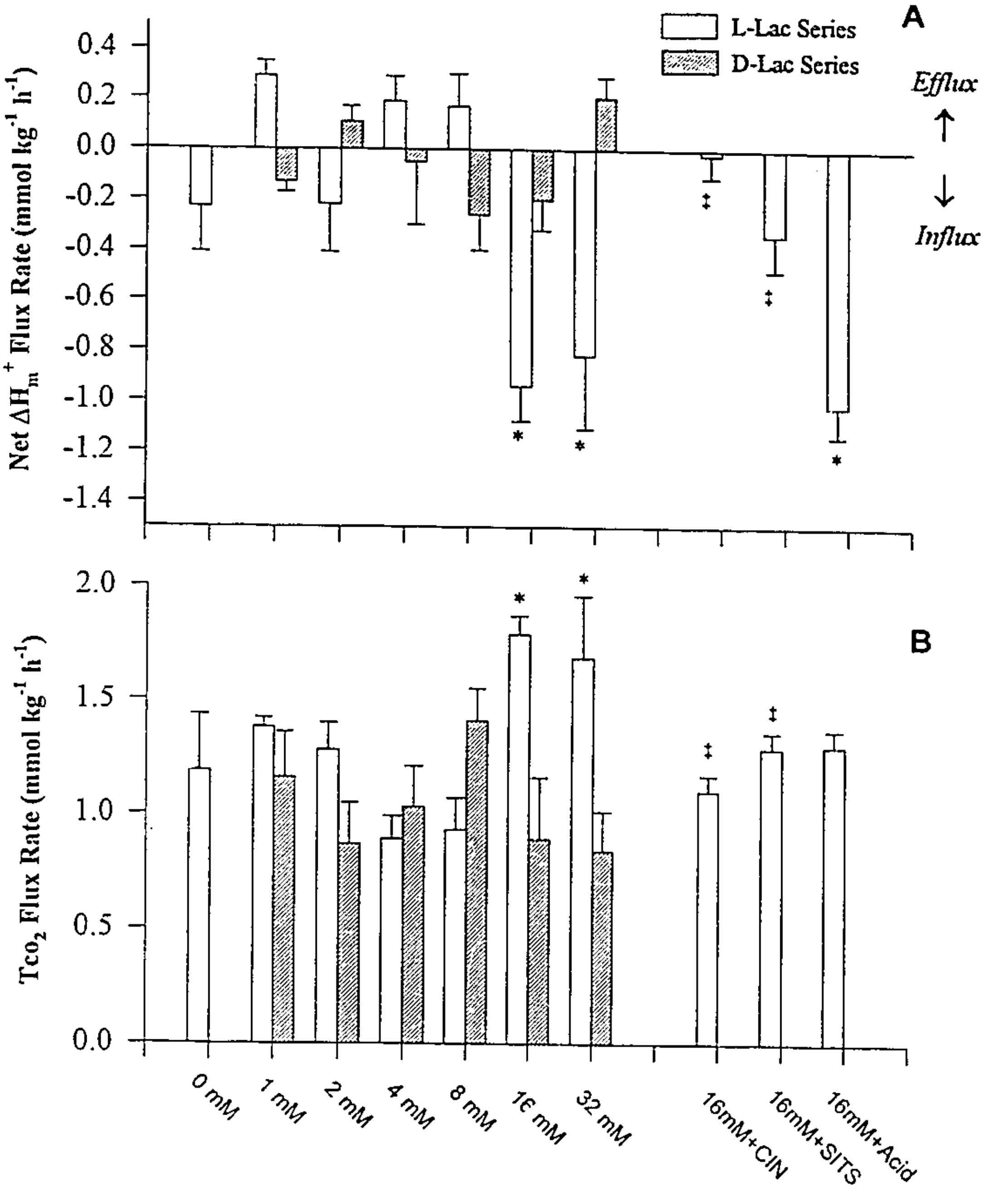

A
L-Lac
Net L-Lac Influx (mM kg⁻¹ h⁻¹)
7
6
5
4
3
2
1
0
-1
← pH=7.68+16mM
-180
-160
-140
-120
-100
-80
-60
-40
-20
Net Driving Force (mV)
B
Outward ←
→ Inward
Net Lac Flux (mmol kg⁻¹ h⁻¹)
6
5
4
3
2
1
0
-1
32 mM
16 mM
8 mM
4 mM
pH=7.68 + 16mM
L-Lac
D-Lac
-2
-1
0
1
2
HLac Gradient (μmol l⁻¹)

Figure 6-6.

A. Net ΔH_m^+ flux rates across white muscle cell membranes in the L-Lac (open bars) and D-Lac (striped bars) series of the resting perfused tail-trunk preparation after 60 min of perfusion.

B. Tco_2 efflux rates across white muscle cell membranes in the L-Lac (open bars) and D-Lac (striped bars) series of the resting perfused tail-trunk preparation after 60 min of perfusion. Positive values indicate efflux and negative values indicate influx. The flux rates are expressed as mmol kg^{-1} wet tail weight h^{-1}. The L-Lac series (open bars) consists of treatments with different [L-Lac] in the inflowing perfusate: 0, 1, 2, 4, 8, 16, and 32 mM, 16 mM + CIN, 16mM + SITS, and 16 mM + acid ($pH_a = 7.68$). The D-Lac series (striped bars) consists only of treatments with different [D-Lac] in arterial inflowing saline: 1, 2, 4, 8, 16, and 32 mM. * indicates significant difference ($P \leq 0.05$) from the corresponding value in the 0 mM treatment. ‡ indicates significant difference from the corresponding value in the 16 mM treatment. Numbers of trunk preparation in each treatment are as in Table 6-4.



A
L-Lac Series
D-Lac Series
Efflux
Influx
Net ΔH_m^+ Flux Rate (mmol kg^{-1} h^{-1})
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
-1.4
*
*
*
‡
‡
B
Tco$_2$ Flux Rate (mmol kg^{-1} h^{-1})
2.0
1.5
1.0
0.5
0.0
*
*
‡
‡
0 mM
1 mM
2 mM
4 mM
8 mM
16 mM
32 mM
16mM+CIN
16mM+SITS
16mM+Acid

Table 6-1. Summary of the various treatments and perfusate non-bicarbonate buffer capacities (β) in the exercised and resting tail-trunk perfusion studies.

Treatment		Nominal $[\mathrm{Lac}]_e$ (mmol l^{-1})	β ($[\mathrm{HCO_3^-}]$ mM · pH^{-1})
Exercised	Control with DMSO	0	-3.99
	Control without DMSO	0	-5.40
	CIN (5 mM)	0	-5.98
	SITS (0.5 mM)	0	-4.01
	Amiloride (0.1 mM)	0	-4.40
Resting	L(+)-Lac	0, 1, 2, 4, 8, 16, 32	-6.61
	D(-)-Lac	1, 2, 4, 8, 16, 32	-6.61
	CIN (5 mM)	16 L-Lac	-5.98
	SITS (0.5 mM)	16 L-Lac	-4.01
	Low pH_e ($\approx$7.68)	16 L-Lac	-6.61

Table 6-2. The white muscle intracellular pH (pH_i), transmembrane potential (E_m), and lactate concentration ($[Lac]_i$) prior to, and after, 60 min of perfusion in the various post-exercise treatments.

	pH_i	E_m	$[Lac]_i$
Control-1 (N=8)			
0 min	6.453±0.057	-69.9±1.3	74.05±4.31
60 min	6.444±0.077	-74.6±1.2*	74.05±5.66
CIN (N=9)			
0 min	6.413±0.029	-62.5±3.8	71.08±7.08
60 min	6.415±0.045	-75.6±1.8*	74.45±8.62
SITS (N=10)			
0 min	6.450±0.028	-71.4±1.2	75.41±5.28
60 min	6.431±0.031	-76.4±2.5*	78.89±6.36
Control-2 (N=9)			
0 min	6.488±0.057	-68.8±1.80	46.04±4.61
60 min	6.452±0.061	78.4±1.84*	50.76±5.88
Amiloride (N=11)			
0 min	6.530±0.036	-68.3±0.9	51.52±7.71
60 min	6.508±0.039	-80.0±1.3*	52.44±3.22

Note: Values are means ± S.E.M. (N). Control-1 accompanied the CIN and SITS series, control-2 accompanied the amiloride series. $[Lac]_i$ values are expressed in mmol l^{-1} ICF water. The 0 min values were corrected for lactate concentrations in the ECF and ECFV obtained from a previous *in vivo* study (55). E_m values were calculated according to the Goldman-Hodgkin-Katz equation (see 21,56 for equations) and expressed as mV. * indicates significant difference ($P \leq 0.05$) from the corresponding pre-perfusion value.

Table 6-3. Levels of pH, total CO_2 (Tco_2), partial pressure of CO_2 (Pco_2), bicarbonate (HCO_3^-), and lactate ([Lac]) in the arterial and venous perfusate after 60 min of perfusion in the five treatments post-exercise series.

	pH	Tco_2	Pco_2	$[HCO_3^-]$	[Lac]
Control-1					
Arterial	7.543 ± 0.007	2.83 ± 0.17	2.26 ± 0.16	2.71 ± 0.17	0
Venous	7.317 ± 0.039	4.20 ± 0.30	5.87 ± 0.75	3.88 ± 0.27	1.85 ± 0.30
CIN					
Arterial	7.495 ± 0.019	2.73 ± 0.11	2.43 ± 0.12	2.38 ± 0.21	0
Venous	7.341 ± 0.020	4.15 ± 0.16	5.33 ± 0.35	3.07 ± 0.17	1.17 ± 0.18
SITS					
Arterial	7.513 ± 0.032	2.49 ± 0.21	2.09 ± 0.09	2.38 ± 0.21	0
Venous	7.331 ± 0.040	3.96 ± 0.17	5.26 ± 0.44	3.67 ± 0.17	1.60 ± 0.23
Control-2					
Arterial	7.472 ± 0.025	3.10 ± 0.17	2.99 ± 0.25	2.97 ± 0.15	0
Venous	7.264 ± 0.039	4.35 ± 0.24	7.05 ± 0.91	3.78 ± 0.08	1.55 ± 0.36
Amiloride					
Arterial	7.447 ± 0.027	2.87 ± 0.11	2.97 ± 0.32	2.71 ± 0.10	0
Venous	7.236 ± 0.026	4.16 ± 0.09	6.88 ± 0.49	3.78 ± 0.08	1.32 ± 0.14

Note: Values are mean ± S.E.M. (See table 2 for N). Control-1 accompanied the CIN and SITS series, control-2 accompanied the amiloride series. Tco_2, $[HCO_3^-]$, and [Lac] values are expressed in mmol l^{-1} ECF water, Pco_2 values are in torr.

Table 6-4. The muscle intracellular pH (pH_i), intra- ($[Lac]_i$) and extracellular L-lactate concentration ($[Lac]_e$) after 60 min of perfusion in the various resting treatments.

Treatment		pH_i	$[L\text{-}Lac]_i$	$[Lac]_e$
L-Lac				
0	(N=5)	7.291±0.025	3.04±0.59	0
1	(N=8)	7.231±0.021	3.25±0.20	1.02±0.04
2	(N=8)	7.207±0.014	3.73±0.37	2.07±0.05
4	(N=8)	7.259±0.015	2.67±0.38	3.58±0.07
8	(N=7)	7.226±0.011	3.19±0.36	7.08±0.12
16	(N=7)	7.209±0.020	3.74±0.73	16.42±0.41
32	(N=5)	7.301±0.014	3.06±0.94	32.90±0.50
CIN+16	(N=6)	7.235±0.016	6.50±0.95*	16.12±0.49
SITS+16	(N=7)	7.237±0.031	6.78±0.45*	14.80±0.44*
Acid+16	(N=7)	7.333±0.032*	4.90±0.65	12.80±0.38*
D-Lac				
1	(N=6)	7.306±0.011	2.61±0.46	0.83±0.03
2	(N=4)	7.325±0.025	2.52±0.62	1.93±0.03
4	(N=5)	7.388±0.020	2.89±0.50	3.53±0.08
8	(N=10)	7.318±0.012	4.01±0.50	7.27±0.22
16	(N=5)	7.330±0.012	3.43±0.62	16.25±0.18
32	(N=5)	7.246±0.025	3.06±0.96	30.56±1.29

Note: Values are mean ± S.E.M. (N). Intracellular [Lac] values are expressed in mmol l^{-1} ICF water. Extracellular [Lac] values are expressed in mmol l^{-1} ECF water. * indicates significant difference from the corresponding values in the 16 mM $[Lac]_e$ treatment.