J. exp. Biol. ***195****, 227–258 (1994)*
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INTEGRATED RESPONSES TO EXHAUSTIVE EXERCISE AND RECOVERY IN RAINBOW TROUT WHITE MUSCLE: ACID–BASE, PHOSPHOGEN, CARBOHYDRATE, LIPID, AMMONIA, FLUID VOLUME AND ELECTROLYTE METABOLISM

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Accepted 28 June 1994

Summary

White muscle and arterial blood plasma were sampled at rest and during 4 h of 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 levels, large elevations in haemoglobin concentration and haematocrit, red cell swelling, increases in the levels of most plasma electrolytes, but no shift of fluid out of the extracellular fluid (ECF) into the intracellular fluid (ICF) of white muscle. The decrease in white muscle pHi was comparable to that in pHe; both recovered by 4 h. Creatine phosphate and ATP levels 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 concentration mirrored those in creatine phosphate, whereas changes in IMP and ammonia concentration mirrored those in ATP. White muscle glycogen concentration was reduced 90 % primarily by conversion to lactate; recovery was slow, to only 40 % of resting glycogen levels by 4 h. 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, estimated from ICF lactate and pyruvate levels and pHi, remained unchanged from resting levels, challenging the traditional view of the 'anaerobic' production of lactate. Furthermore, the membrane potential, estimated from levels of ICF and ECF electrolytes using the Goldman equation, remained unchanged throughout, challenging the view that white muscle becomes depolarized after exhaustive exercise. Indeed, ICF K^+ concentration was elevated. Lactate was distributed well out of electrochemical equilibrium with either the membrane potential (E_m) or the pHe–pHi difference, supporting the view that lactate is actively retained in white muscle. In contrast, H^+ was actively extruded. Ammonia was distributed passively according to E_m rather than pHe–pHi throughout recovery, providing a mechanism for retaining high ICF ammonia levels for adenylate resynthesis *in situ*. Although lipid is not traditionally considered to be a fuel for burst exercise,

Key words: rainbow trout, glycogen, lactate, ammonia, fatty acids, carnitine, exercise, white muscle.

substantial decreases in free carnitine and elevations in acyl-carnitines and acetyl-CoA concentrations 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 mass in white muscle (Johnston, 1980; Stevens, 1968), the rainbow trout provides an ideal model in which 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 (e.g. 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*a*,*b*; 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 has 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 (i.e. creatine phosphate and ATP) are the fuel supply for strenuous exercise, while carbohydrates (i.e. glycogen and glucose) provide fuel reserves available upon the depletion of high-energy phosphogens (Milligan and Wood, 1986*b*; Dobson and Hochachka, 1987; Parkhouse *et al.* 1988; Pearson *et al.* 1990; Scarabello *et al.* 1991*a*; Schulte *et al.* 1992). Little is known about the effects of burst exercise on lipid metabolism in fish. Free fatty acids (FFAs), 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 the 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 phosphogen and

glycogen levels 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 (CoA-SH) and acetyl-CoA levels provide far more information than do measurements of just the available FFA pool and the absolute triglyceride (TG) levels in muscle. Changes in the levels of acyl-carnitines, in particular, reflect the true level of FFA metabolism in white muscle.

A third goal was specifically to 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 electrolyte levels 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 electrolyte levels in white muscle after exhaustive exercise. The distribution of electrolytes between intra- and extracellular 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 (e.g. 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 that 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* (Walbaum), 150–350 g] were purchased from a local trout hatchery (Rainbow Springs Trout Farm, Ontario) and held in a 800 l tank supplied with a continuous flow of aerated, dechlorinated Hamilton tapwater (composition as in Milligan and Wood, 1986*a*) for at least 2 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 (DA) catheters 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 water at 15 °C. DA catheters were flushed with heparinized Cortland saline (50 i.u. 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]-labelled PEG-4000 (polyethylene glycol; New England Nuclear, NET-405, M_r 4000, 2–8 mCi (=74–296 mBq) $mmol^{-1}$). 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 mmol l^{-1} NaCl and infused into the arterial bloodstream *via* the DA catheter (28 μCi (=1036 kBq)$^{-1}$ ml^{-1} kg^{-1} body mass) 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 our comparative study of different methods (Wang *et al.* 1994). The fish was killed 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. It was then immediately removed from the water; within 15 s, 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 (2–3 g) was taken from the same site to estimate white muscle ECFV, ICFV and water and ion contents.

Analytical protocols

Arterial blood pH (pHa) was determined with a Radiometer microelectrode (E5021) and PHM72 acid–base analyzer. Arterial blood $P_{a_{O_2}}$ was measured with a Radiometer P_{O_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 5000 ***g*** for 5 min in a sealed haematocrit capillary tube (Radiometer type D). True plasma was obtained by breaking the tube and was analyzed for total CO_2 on a Cameron Instruments Capni-Con total CO_2 analyzer (model II). Arterial plasma Pa_{CO_2} and $[HCO_3^-]$ were calculated by manipulation of the Henderson–Hasselbalch equation using appropriate constants (α_{CO_2} 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 (P_i) was measured by the method of Fiske and Subba Row (1925).

For measurement of ECFV, 100 μl of plasma was added to 10 ml of scintillation fluid (ACS; Amersham), and 50–100 mg of fresh muscle was digested in 2 ml of 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 neutralized digests. The samples were counted on an 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 (2–3 g) to a constant mass 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 pHi by the homogenization technique described by Pörtner *et al.* (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 of powder to 1 ml of 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 mol l^{-1} 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 were measured 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 CoA-SH 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, because of 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:

$$Carn_a + \text{CoA-SH} \rightleftharpoons \text{carnitine} + \text{acetyl-CoA},$$

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 – i.e. within the variability of step 1. Muscle total carnitine ($Carn_t$) and free carnitine ($Carn_f$) measurements were achieved by using thiol reagent (*N*-ethylmaleimide, NEM) to remove CoA-SH from the system to force the readily reversible reaction:

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

to proceed to completion in the right-to-left direction (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 carnitine), 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 (1 mol l^{-1}) at 40–50 °C for 48 h and the supernatant was then analyzed. Both plasma and muscle supernatant K^+, Na^+, Ca^{2+} and Mg^{2+} were measured with flame atomic absorption spectrometry (Varian AA-1275), while plasma and muscle Cl^- were determined on a chloride titrator (Radiometer CMT10).

Calculations and statistical analysis

The levels of metabolites and electrolytes in ECF were expressed per litre of plasma water (C_W). The detailed calculations of white muscle ICFV and ECFV have been

described by Munger *et al.* (1991). Owing to the processing methods of this study, ion and metabolite concentrations of muscle were initially expressed per unit dry mass. However, the final concentrations in muscle are presented per litre of ICFV, incorporating appropriate correction for the amount in trapped ECFV in the muscle. The conversions were performed as follows:

$$\text{Concentration (mmol kg}^{-1}\text{ wet mass)} = \text{Concentration (mmol kg}^{-1}\text{ dry mass)} \times [1 - \text{tissue } H_2O\text{ (l kg}^{-1}\text{ wet mass)}]\,. \quad (1)$$

For those metabolites or electrolytes that were measured in both plasma and muscle:

$$\text{Concentration (mmol l}^{-1}\text{ ICF)} = \frac{\text{Concentration (mmol kg}^{-1}\text{ wet mass)} - \text{Concentration (mmol l}^{-1}\text{ ECF)} \times \text{ECFV (l kg}^{-1}\text{ wet mass)}}{\text{ICFV (l kg}^{-1}\text{ wet mass)}}\,. \quad (2)$$

For those metabolites that were not measured in plasma (and most likely to be negligible in ECF – i.e. Gly, PCr, Cr, ATP and IMP):

$$\text{Concentration (mmol l}^{-1}\text{ ICF)} = \frac{\text{Concentration (mmol kg}^{-1}\text{ wet mass)}}{\text{ICFV (l kg}^{-1}\text{ wet mass)}}\,. \quad (3)$$

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 (1986*a*):

$$\Delta H^+_m = [HCO_3^-]_1 - [HCO_3^-]_2 - \beta(pH_1 - pH_2)\,, \quad (4)$$

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

$$\beta = -1.271 \times [\text{Haemoglobin}] - 2.31\,. \quad (5)$$

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

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

$$E_m = \frac{\boldsymbol{R}T}{F} \ln \frac{PK^+[K^+]_{ECF} + PNa^+[Na^+]_{ECF} + PCl^-[Cl^-]_{ICF}}{PK^+[K^+]_{ICF} + PNa^+[Na^+]_{ICF} + PCl^-[Cl^-]_{ECF}}\,, \quad (6)$$

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 (i.e. a simple Nernstian distribution). For example, for Amm (the sum of NH_4^+ and NH_3):

$$E_m = -\frac{\boldsymbol{R}T}{zF}\ln\frac{[NH_4^+]_{ICF}}{[NH_4^+]_{ECF}}$$

$$= -\frac{\boldsymbol{R}T}{zF}\ln\frac{[Amm]_{ICF} - [NH_3]_{ICF}}{[Amm]_{ECF} - [NH_3]_{ECF}}, \quad (7)$$

where $\boldsymbol{R}$, T, z and F have their usual meaning. For Amm and Lac, it was also possible to use measured pHi and pHe to predict the distribution ratio making an alternative assumption that the distribution was governed solely by the difference in pH between intra- and extracellular compartments (i.e. a simple distribution according to the laws of weak acids/bases; Jacobs and Stewart, 1936). For example, for Amm:

$$\frac{[Amm]_{ICF}}{[Amm]_{ECF}} = \frac{1 + 10^{(pK-pHi)}}{1 + 10^{(pK-pHe)}}. \quad (8)$$

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 (i.e. $[NAD^+]/[NADH]$ ratio) was estimated from the apparent equilibrium of the lactate dehydrogenase reaction using measurements of intracellular Pyr, Lac and pHi (for $[H^+]$):

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

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 analysis of variance (ANOVA) followed by *post hoc* comparison by means of Duncan's multiple range and critical range test ($P \leqslant 0.05$) (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 s 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 variables were typical of exhaustive exercise. $P_{a_{O_2}}$ exhibited a significant 40 % decrease immediately after exercise but returned to resting

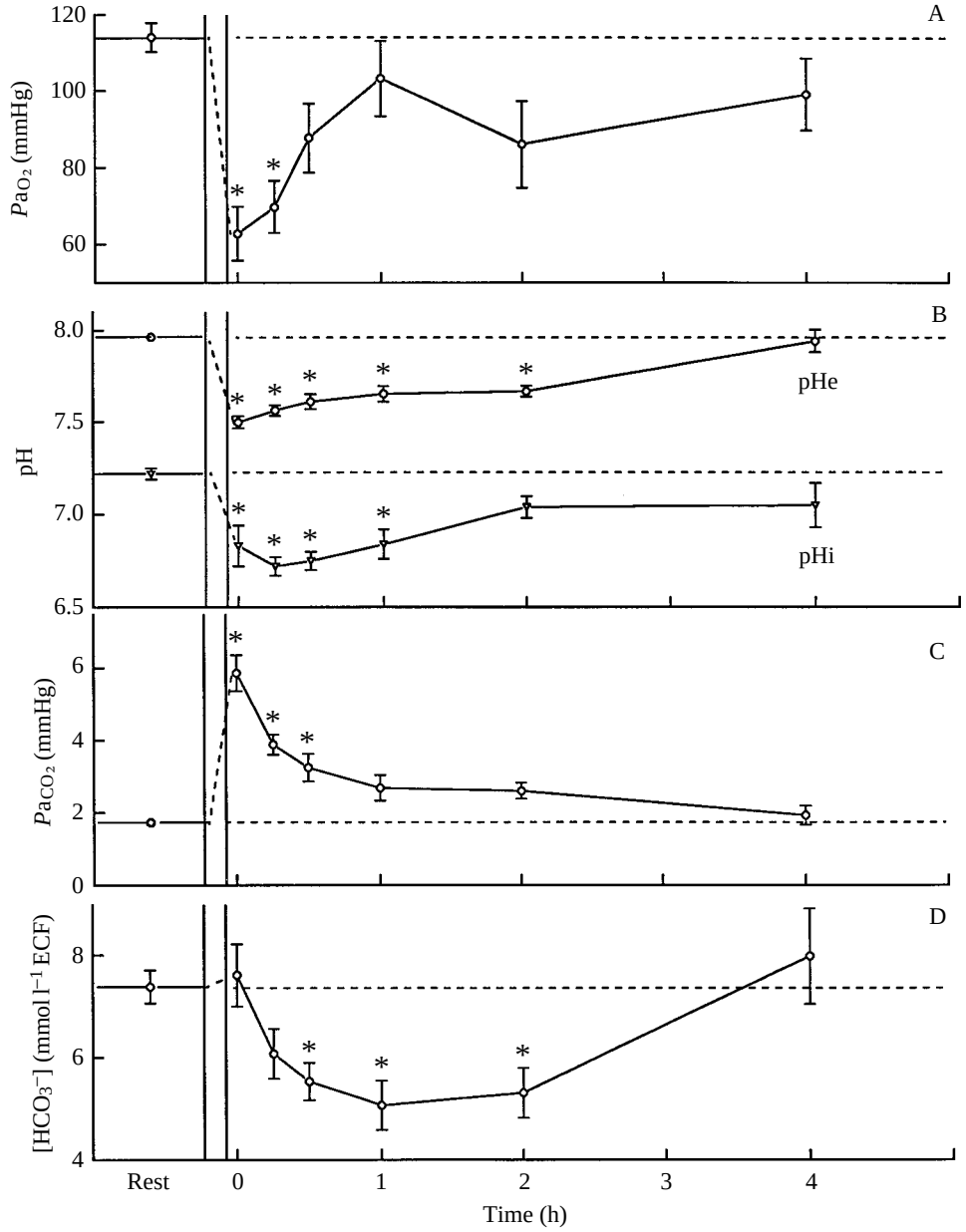

Fig. 1. Changes in (A) arterial P_{aO_2}, (B) arterial plasma pHe and white muscle intracellular pHi, (C) P_{aCO_2} and (D) $[HCO_3^-]$ prior to and after exhaustive exercise in the rainbow trout. Bar indicates 6 min of exercise, 0 immediately after exercise. Dashed line represents the mean resting value. 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. An asterisk indicates a significant (P<0.05) difference from the corresponding resting value. Values are means ± S.E.M.

levels within 1 h (Fig. 1A). There were significant decreases in both arterial plasma (i.e. extracellular) pHe and white muscle intracellular pHi (approximately 0.5 unit) after exercise and the correction of both acidoses was completed within 4 h (Fig. 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 threefold increase in arterial blood $P_{a_{CO_2}}$ (1.73 to 5.85 mmHg) immediately after exercise, contributing a large respiratory component to the extracellular acidosis (Fig. 1C). Although pHe was not completely corrected until 4 h, $P_{a_{CO_2}}$ returned to resting level within 1 h. In contrast to the rapid changes in $P_{a_{CO_2}}$, 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 % of resting level) at 1 h and returned to the resting value by 4 h (Fig. 1D).

Associated with this post-exercise acidosis was an almost 50 % increase in haematocrit (Hct, Fig. 2A), a 30 % increase in [haemoglobin] (Fig. 2A) and a 17 % decrease in mean cell haemoglobin concentration (MCHC, Fig. 2B), the latter indicative of red blood cell swelling. By 4 h, MCHC had returned to resting levels, while Hct and Hb both remained 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. 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. 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. 3B). Taking into account the relative sizes of the white muscle ICFV and the whole-animal ECFV (Milligan and Wood, 1986*a*,*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 (range 0.1–1.5 mmol l^{-1}; Fig. 3C). Both extracellular and intracellular Pyr continued to increase for some time after exercise; from 1 to 4 h, the extracellular level stabilized, whereas the intracellular level declined at this time. In consequence, the ECF/ICF gradient for Pyr was reversed at 2 h and 4 h. Glu exhibited yet another pattern, with extracellular levels (4–6 mmol l^{-1}) consistently greater than intracellular levels (1–2 mmol l^{-1}; Fig. 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 4 h; 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 to 2 h of recovery (Fig. 4A). The lower initial ΔH_m^+ (0 h), compared with ΔLac, was in concert with PCr breakdown 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

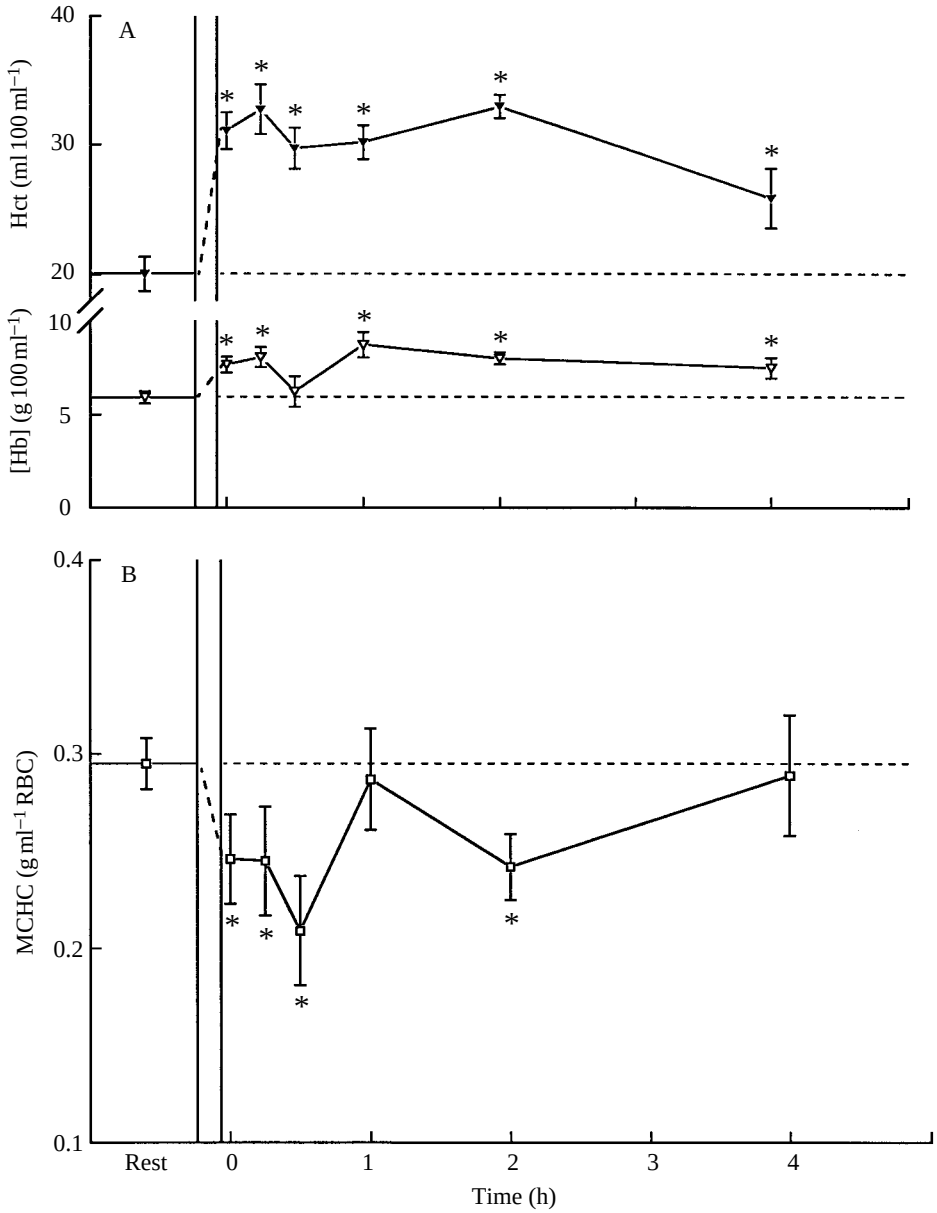

Fig. 2. Changes in (A) arterial blood haematocrit (Hct) and haemoglobin (Hb) and (B) mean cell haemoglobin concentration (MCHC) prior to and following exhaustive exercise. Other details as in the legend to Fig. 1.

patterns (Fig. 4B). ECF ΔLac always exceeded ΔH^+_m by at least 50 %, except during the 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.

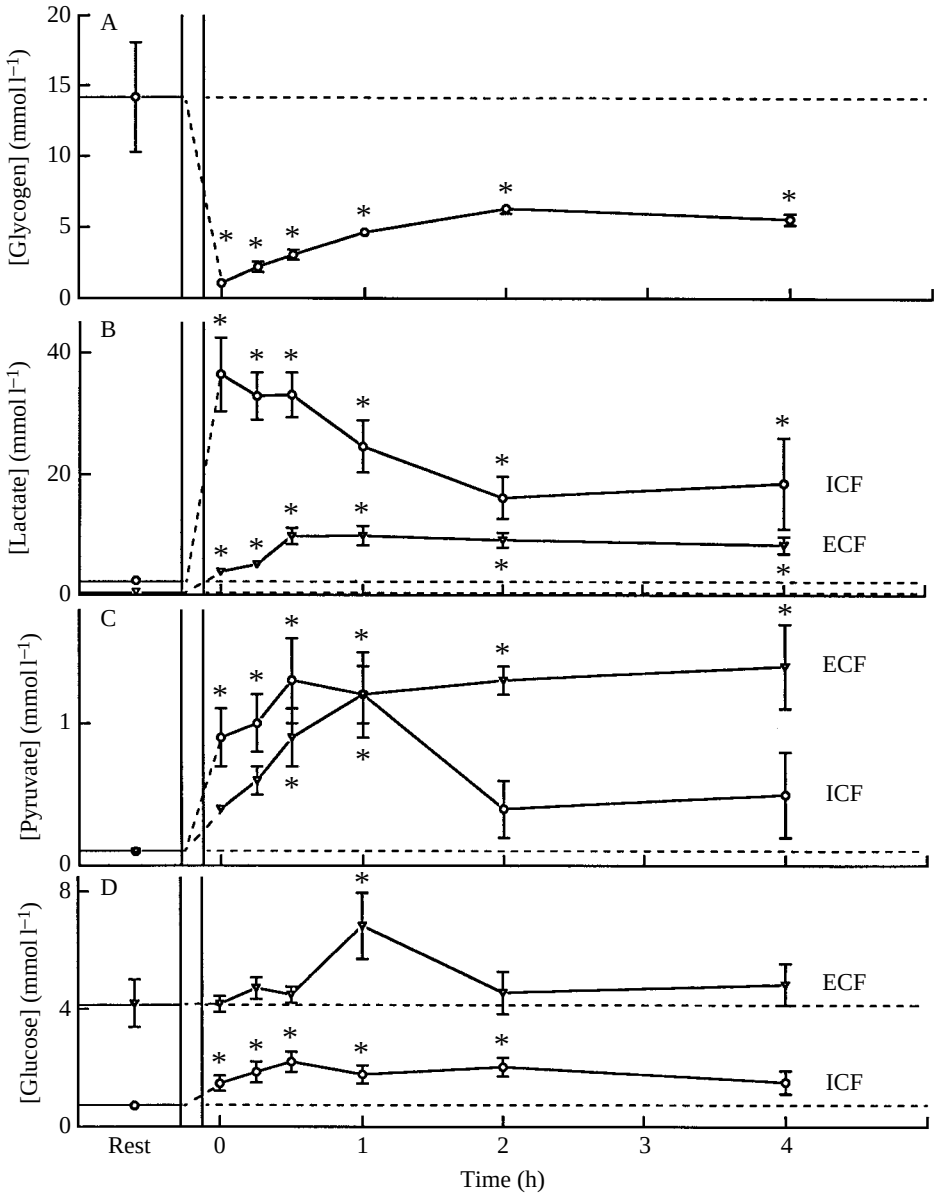

Fig. 3. Changes in concentration of (A) white muscle intracellular glycogen (Gly), (B) intra- and extracellular lactate (Lac), (C) pyruvate (Pyr) and (D) glucose (Glu) concentrations prior to and following exhaustive exercise. Values are means ± S.E.M. in mmol l^{-1} ICF or mmol l^{-1} ECF. Other details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

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. 5). 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

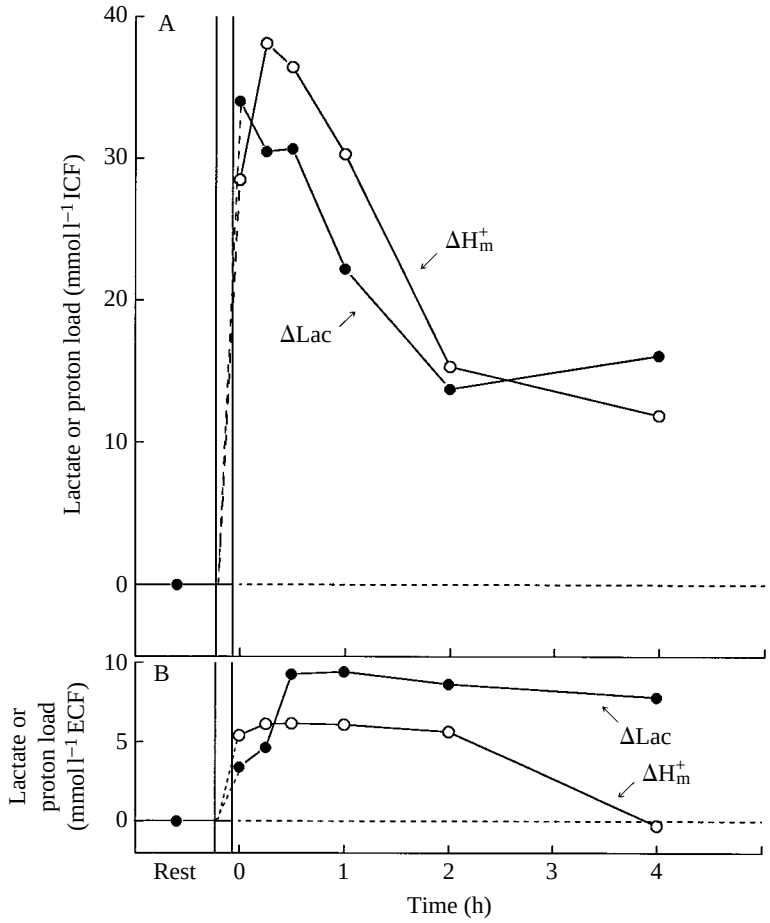

Fig. 4. (A) White muscle ICF and (B) ECF lactate (ΔLac) and metabolic acid (ΔH^+_m) loads after exercise. ΔLac and ΔH^+_m are zero, by definition, at rest. See text for detailed calculation. Values are calculated from the mean value at each time interval. Bar indicates exercise period.

course (Fig. 5). The increase in Cr matched the decrease in PCr stoichiometrically. Tissue total phosphate levels did not change significantly 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. 6A). Post-exercise reductions in ATP were mirrored by increases in intracellular IMP (Fig. 6A) and Amm (Fig. 6B). These two products of adenylate deamination demonstrated an almost identical pattern of changes, which was a 5–6 mmol l^{-1} 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 fivefold increase in ECF Amm immediately after exercise, followed by a slow recovery (Fig. 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}).

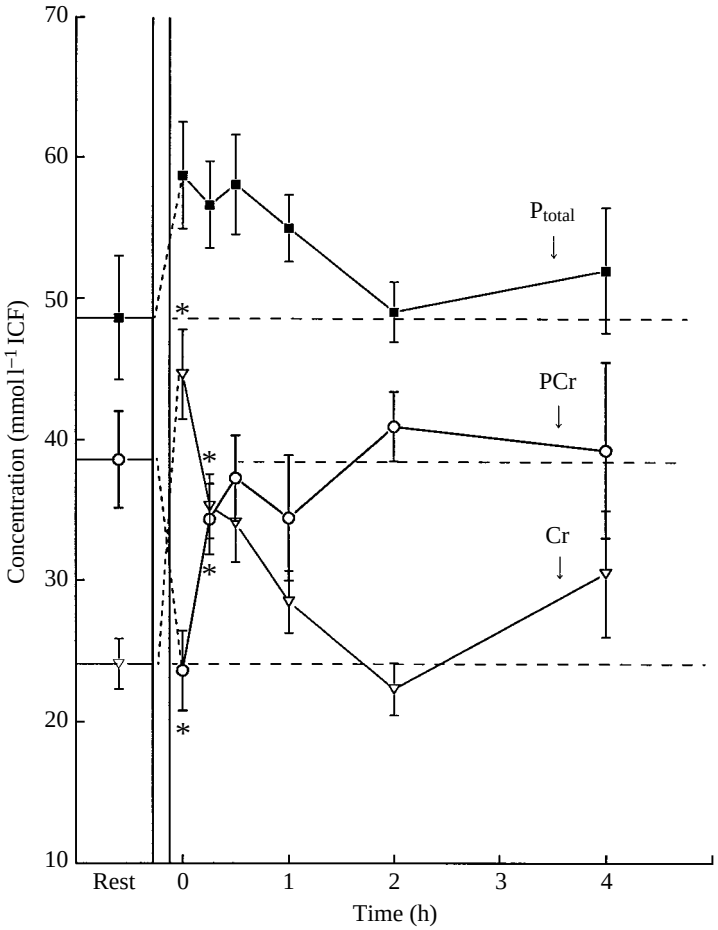

Fig. 5. Changes in white muscle intracellular total phosphate (P_{total}), creatine phosphate (PCr) and creatine (Cr) prior to and following exhaustive exercise. Other details as in the legend to Fig. 1.

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. 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. 7A). Acetyl-carnitine levels ($Carn_a$) were 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 $Carn_a$ (fourfold and sixfold, respectively). Both $Carn_s$ 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

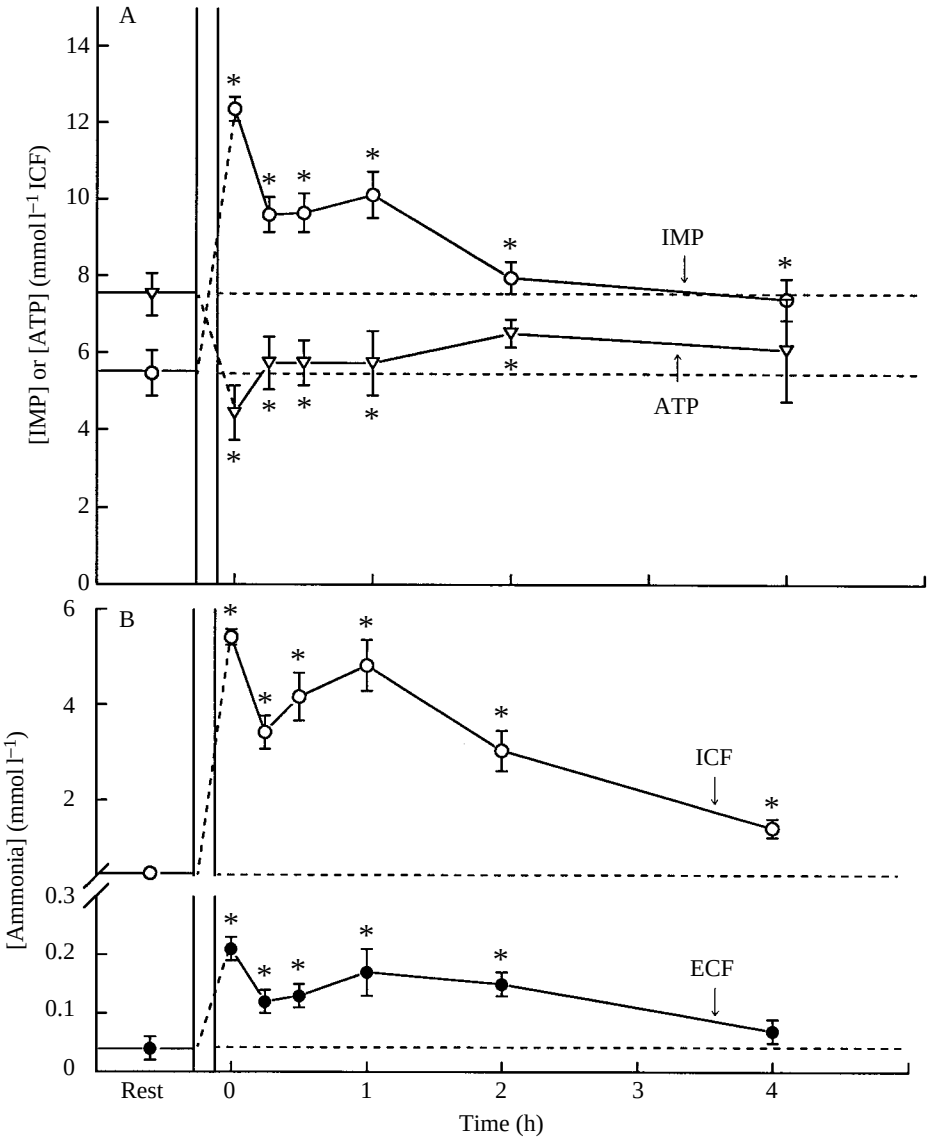

Fig. 6. Changes in (A) white muscle intracellular inosine monophosphate (IMP) and adenosine triphosphate (ATP) and (B) intra- and extracellular ammonia (Amm) concentrations. Other details as in the legend to Fig. 1.

total CoA pool of about 8–10 μmol l^{-1} (not shown). In this pool, about 90 % was coenzyme A (CoA-SH) and 10 % was acetyl-CoA at rest (Fig. 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 CoA-SH were observed.

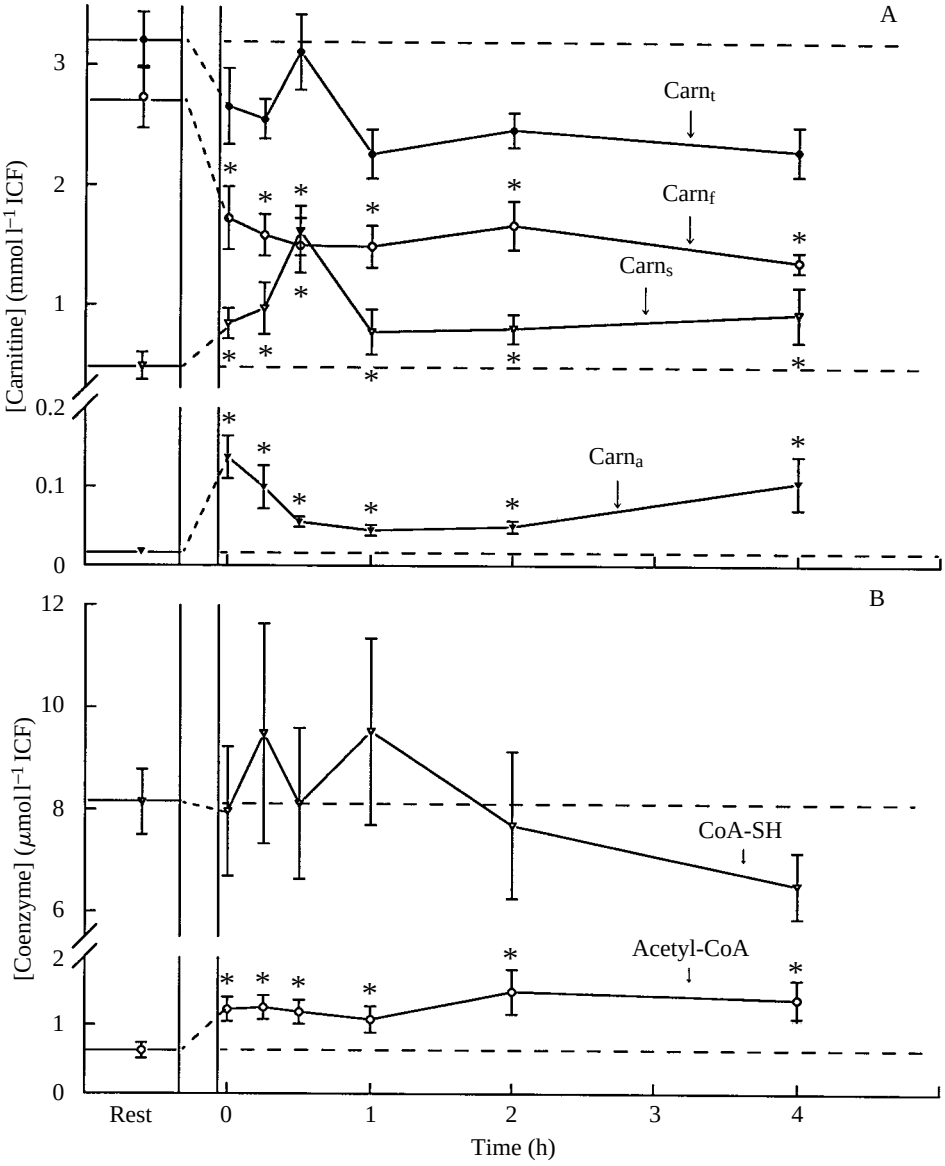

Fig. 7. (A) Changes in white muscle intracellular total carnitine ($Carn_t$), free carnitine ($Carn_f$), short-chain acyl-carnitine ($Carn_s$) and acetyl-carnitine ($Carn_a$) concentrations. (B) Changes in white muscle intracellular coenzyme A (CoA-SH) and acetyl coenzyme A (Acetyl-CoA) concentrations. Other details as in the legend to Fig. 1.

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 1). White muscle ECFV and ICFV remained at about 70 and 740 ml kg^{-1} wet mass, respectively. Muscle water content fluctuated

Table 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 (ml kg^{-1} wet mass)	ICFV (ml kg^{-1} wet mass)	ECFV (ml kg^{-1} wet mass)	C_{PP} (g l^{-1} plasma)
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 ± S.E.M. (N=8–13).
There are no significant differences between resting and post-exercise fish in all four categories.

slightly around 800 ml kg^{-1} wet mass. 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 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 until 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 2). In contrast to the cations, $[Cl^-]_{ICF}$ was not elevated until 2 h into the recovery period, when it increased by about 4 mmol l^{-1} and had recovered to its resting level at 4 h (Table 2). Lac is included as an anion in Table 2 because, with a 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 intracellular Ca^{2+} probably exists in the bound form, while the ratio of free Mg^{2+} to total Mg^{2+} is dependent on ATP levels. Nevertheless, it is evident from Table 2 that the

Table 2. *Electrolyte concentrations in intracellular fluid of rainbow trout white muscle at rest and during 4 h recovery from exhaustive exercise*

Treatment	$[K^+]_{ICF}$	$[Na^+]_{ICF}$	$[Ca^{2+}]_{ICF}$	$[Mg^{2+}]_{ICF}$	$[Cl^-]_{ICF}$	[Lactate]$_{ICF}$
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
0 h	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 ± S.E.M. (N=8–13) in mmol l^{-1} ICFV.
* indicates a value significantly different (P<0.05) from the corresponding resting value.

Table 3. *Electrolyte concentrations in extracellular fluid of rainbow trout at rest and during 4 h recovery from exhaustive exercise*

Treatment	$[K^+]_{ECF}$	$[Na^+]_{ECF}$	$[Ca^{2+}]_{ECF}$	$[Mg^{2+}]_{ECF}$	$[Cl^-]_{ECF}$	$[Lactate]_{ECF}$	$[P_i]_{ECF}$
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 ± S.E.M. (N=8–13) in mmol l^{-1} ECFV.
* indicates a value significantly different (P<0.05) from the corresponding resting value.
P_i, inorganic phosphate.

increases in Lac^- and Cl^- 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. 4A).

Extracellular electrolyte levels also varied significantly during recovery (Table 3). Levels of 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^+ increased by about 11 mmol l^{-1} within 15 min and stayed elevated through 2 h into recovery. The major extracellular anion Cl^- 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 ($[P_i]_{ECF}$; 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 $[Lac^-]_{ECF}$ 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. 4B).

Discussion

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

The pattern of blood gas and extracellular acid–base changes (Fig. 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, 1986*a*,*b*, 1987; Nikinmaa and Jensen, 1986; Tang and Boutilier, 1988*a*,*b*; McDonald *et al.* 1989; Wood *et al.* 1990); similar explanations probably apply. The cause of the $P_{a_{CO_2}}$ elevation, which contributes to the short-lived respiratory component of blood acidosis, remains

controversial (e.g. 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 Lac production and ATP hydrolysis in white muscle (Hochachka and Mommsen, 1983; Wood and Perry, 1985; Milligan and Wood, 1986*b*). The contribution of protons released from red blood cells may also be significant because of catecholamine-induced stimulation of Na^+/H^+ exchange on the red blood cell (RBC) membrane (e.g. Nikinmaa and Jensen, 1986; Wood *et al.* 1990). The significant decrease in MCHC (Fig. 2B) indicates RBC swelling as the result of a large net entry of Na^+ and Cl^- followed by water into RBC triggered by P_{CO_2} and catecholamine elevation (Ferguson and Boutilier, 1989). This clearly contributed to the large increase in Hct (Fig. 2A). However, the observation 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 1), the increase in the number of RBCs was probably 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 (1986*b*) and Parkhouse *et al.* (1987), there was no change in white muscle water content after exhaustive exercise (Table 1). However, in direct contrast to the results of Milligan and Wood (1986*b*), there was also no change in fluid volume distribution within the white muscle. Milligan and Wood (1986*a*,*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, i.e. 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 (1986*a*,*b*) actually did. In support of this view, the present fish exhibited no increase in C_{pp} (Table 1), while those of Milligan and Wood (1986*b*) 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 lactate concentrations after exercise in the trout of Milligan and Wood (1986*b*) and Wang *et al.* (1994): 44–48 mmol l^{-1} *versus* 35 mmol l^{-1} in the present study (Fig. 3B). Higher intracellular lactate concentrations in the white muscle would provide a greater osmotic gradient for fluid shift.

Changes in extracellular electrolyte levels after exhaustive exercise (Table 3) were qualitatively similar, but quantitatively smaller, than 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. 2B) and perhaps tissues other than white muscle, as well as ionic movements at the gills (e.g. 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 probably due to Cl^- losses across the gills (Holeton *et al.* 1983; Wood, 1988) following 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 intracellular K^+ predicted by Wood (1991), there was a significant increase (Table 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 2). The rise in white muscle $[K^+]_{ICF}$ (Table 2) at a time when $[K^+]_{ECF}$ was elevated (Table 3) and branchial and renal K^+ losses were all also probably 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 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 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 was statistically significant (Table 4).

'Metabolic acid' and lactate dynamics

Observed changes in pHi and pHe after exhaustive exercise (Fig. 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. 4; Turner *et al.* 1983; Milligan and Wood, 1986*b*; 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

Table 4. *Measured and estimated lactate, ammonia and H^+ distribution ratios across white muscle cell membrane according to transmembrane pH and voltage gradients*

	Estimated E_m (mV)	$[Lac]_{ICF}/[Lac]_{ECF}$			$[Amm]_{ICF}/[Amm]_{ECF}$			Measured $[H^+]_{ICF}/[H^+]_{ECF}$
		Measured	pH-estimated	E_m-estimated	Measured	pH-estimated	E_m-estimated‡	
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†
0.25 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†
0.5 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 (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).

‡Also applies to $[H^+]_{ICF}/[H^+]_{ECF}$.

* indicates a significant (P<0.05) difference from corresponding resting values; ‡ indicates a significant (P<0.05) difference from corresponding measured ratios; † indicates a significant (P<0.05) difference from corresponding E_m-estimated values.

remain, principal amongst which are the following. (i) What are the mechanism(s) of retention and why do the small amounts of H^+_m and Lac released apparently differ; i.e. different patterns of ΔH^+_m and ΔLac in the ECF (e.g. Fig. 4)? (ii) What are the relative fates of the metabolized 'lactic acid'; i.e. oxidation or glycogen resynthesis? (iii) In the latter case, what are the enzymatic pathways by which this could occur? This last question awaits resolution of 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 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 (i.e. pHi 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 distribute passively according to the pHi–pHe gradient rather than E_m (i.e. as a 'weak acid'; Jacobs and Stewart, 1936), Table 4 illustrates that this is not the case. The measured Lac distribution ratio at rest was again very different from that predicted by the measured pHi–pHe gradient.

After exercise, the measured H^+ distribution ratio did not change substantially and remained far lower than predicted by the E_m (Table 4). There is therefore no need to postulate a retention mechanism for H^+; on the contrary, H^+ must be actively extruded from white muscle, just as at rest, though the turnover rates are probably 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 pHe. The observed stabilization of ΔH^+_m at a lower value than ΔLac in the extracellular compartment (Fig. 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, 1986*a*; 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 4). The conclusion is that Lac must be actively retained and that 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 ninefold at this time (Milligan and McDonald, 1988). Maintenance of high $[Lac]_{ICF}$ may be particularly important in driving the process of glycogen resynthesis (see below). As in 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. 3B) may be contrasted with the low levels of $[Glu]_{ICF}$, which remained far lower than even $[Glu]_{ECF}$ after exercise (Fig. 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 would presumably 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). 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, 1986*b*; 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.* 1991*a*,*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.* 1991*a*), 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 pHi–pHe gradient; i.e. as a typical 'weak base', a pattern seen in many higher vertebrates. Table 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. 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 because of 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. 6). $[Amm]_{ICF}$ 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 4).

These results lead to several conclusions. First, 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 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

limits can occur passively, simply depending on the relative permeabilities of NH_3 (favours a pH-dictated ratio) and NH_4^+ (favours an E_m-dictated ratio). Second, 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 (e.g. 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 as a result of the very low plasma Amm concentrations. Third, it is apparent that an E_m-dictated distribution ratio will ensure a more efficient passive retention of intracellular Amm for greater intracellular buffering (Dobson and Hochachka, 1987; Mommsen and Hochachka, 1988) and for stoichiometric balance with IMP for ATP resynthesis (Fig. 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 approximately 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 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 (e.g. 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 $[H^+]$ (i.e. decline in pHi) was additionally factored into the

Table 5. *Measured intracellular [pyruvate]/[lactate] ratio and estimated redox state ($[NAD^+]/[NADH]$) in trout white muscle*

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

Redox values were estimated according to equation 9, using $K=2.106\times10^{-12}$ mol l^{-1}, based on correction of the value reported by Williamson *et al.* (1967) to 15 °C according to the van't Hoff equation (Williams and Williams, 1973) with a standard enthalpy $\Delta H°=52.5$ kJ mol^{-1} (=12550 cal mol^{-1}) from Somero and Siebenaller (1979).

Values are means ± S.E.M. (N=8–13).

* indicates a significant ($P<0.05$) difference from corresponding resting values.

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 (e.g. 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 levels 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, P_i, ADP and AMP, should activate glycolysis (i.e. 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 one, 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 (Figs 5, 6A), yet Gly reserves were more than 90 % reduced by equivalent conversion to Lac (Fig. 3A,B), so glycolysis was fully activated.

The depressed PCr recovered rather quickly (within 15 min) after exercise (Fig. 5), whereas ATP remained depressed for at least 2 h (Fig. 6A), and the slowly recovering Gly was still depressed by 60 % at 4 h (Fig. 3A). This differential time course agrees with most, but not all, previous studies (reviewed by Scarabello *et al.* 1991*a*). 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). The total creatine pool (about 70 mmol l^{-1} ICF) was comparable to those 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 was depleted to the extents reported in many previous studies (Milligan and Wood, 1986*b*; Dobson *et al.* 1987; Dobson and Hochachka, 1987; Pearson *et al.* 1990; Schulte *et al.* 1992). In addition to improved phosphogen 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 5). Nevertheless, activation of glycolysis clearly occurred, and stoichiometric increases in Amm and IMP accompanied the significant depletion of ATP (Fig. 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 to be a fuel for long-term sustainable 'aerobic' activity (see Introduction). However the results of Fig. 7 together with the 'aerobic' redox state of the tissue (Table 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 a 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 that persisted through 6 h 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 FFAs, especially the long-chain forms (C_{10}–C_{18}), are not very permeable through the inner mitochondrial membranes. In general, FFAs 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-CoAs 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, we elected to measure indirect indices of FFA metabolism rather than changes in absolute TG levels in muscle for several reasons. First, Milligan and Girard (1993) were unsuccessful in demonstrating significant changes in absolute levels because of great variability. Second, 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. 7A) was expected since carnitine serves only as a vehicle to shift FFAs 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. 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 0.5 h), 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-carnitine ($Carn_s$) levels were elevated (Fig. 7A). These increases in acyl-carnitine levels point to the utilization of FFAs 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. 7B) reinforce this conclusion since the β-oxidation spiral will generate acetyl-CoA. Acetyl-carnitine ($Carn_a$) levels, the shortest of the short-chain acyl-carnitines, increased dramatically after exercise (Fig. 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 pyruvate dehydrogenase in fish white muscle probably resulted in a low flux from Pyr to acetyl-CoA. It has also been reported in mammals that an increase in the availability of FFAs 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 that of CoA-SH in white muscle, carnitine acts as a receptor for acetyl groups to form acetyl-carnitine and to release CoA-SH (see Materials and methods). The advantages of carnitine acting as an acetyl unit buffer are twofold: (i) maintaining the available CoA-SH pool at certain level which is essential for FFAs to form acyl-carnitine, then being transported across the mitochondrial membrane; (ii) keeping available CoA-SH 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 CoA-SH pool throughout the experimental period 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 very quickly and they can then 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 $Carn_a$ made up less than 10 % of the carnitine pool.

The twice-daily injection of heparin may well have elevated the basal level of available FFAs in plasma. Lipoprotein lipase (LPL) attached to 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. 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 cyclic AMP 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 has been documented in humans (Essen, 1978; McCartney *et al.* 1986; Bangsbo *et al.* 1991), and the same now appears to be 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 probably helps power the contracting muscle, while during recovery, the ATP should be directed towards restorative processes. In view 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.* 1991*a*). In this regard, it is noteworthy that Moyes *et al.* (1992) reported that physiological levels of FFAs 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 FFAs are the major substrate oxidized during recovery from high-intensity exercise, despite the availability of high levels of Lac. However, at this stage, it is difficult to partition the FFA pool utilized for muscle activity and Gly replenishment in trout. There is a clear need for further studies on lipid utilization in fish in relation to exhaustive exercise.

We thank Russ Ellis and Tina Lukezic for their excellent assistance with various techniques in this study. This work was supported by NSERC operating grants to C.M.W. and MRC grants to G.J.F.H. G.J.F.H. is a Career Investigator for the Heart and Stroke Foundation of Ontario.

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