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

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We have investigated the effects of different sampling and processing methods on metabolite concentrations [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, killing 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) *v.* 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 killed 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.

Key words: biopsy; freeze-clamping; freeze-drying; carbohydrates; phosphagen; ammonia.

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 (e.g. Black *et al.*, 1962; see Wood, 1991, for 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

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without freezing (e.g. 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 unanaesthetized fish (e.g. Turner *et al.*, 1983; Milligan & Wood, 1986), while in others the fish were first killed by a cephalic blow (e.g. Dobson & Hochachka, 1987; Girard & Milligan, 1993) or decapitation (e.g. 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 (e.g. Pearson *et al.*, 1990; Scarabello *et al.*, 1991). Various types of anaesthetics (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 (e.g. 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 (e.g. 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 assess systematically the effects of various common sampling and processing techniques on adenylate and carbohydrate metabolites in white muscle of rainbow trout, *Oncorhynchus mykiss* Walbaum. For comparative purposes, measurements of these same parameters in trout muscle were tabulated from other recent studies. Our measurements focused 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 weighing 150–350 g, were obtained from a local hatchery (*Rainbow Springs Trout Farm, Ontario, Canada*) and held in a 800 l tank for at least 2 weeks prior to the experiments. The fish were acclimated to $15 \pm 1^{\circ}$ 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 implanted surgically 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 150 l cylindrical tank at this time, then chased manually 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). Our 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 drained rapidly by 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 capacity to take approximately 100 mg samples, though not all punctures were successful. Immediately after the sampling (about 5 s), 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 aluminium 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, six 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 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 aluminium 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 s. The glass homogenization method (see series III 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. 7 ml) containing 1·2 ml of 8% $HClO_4$ (PCA) was placed in ice water. Approximately 150 mg tissue was weighed in a 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 ground manually 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 1·5 ml 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 150 mg of frozen tissue powder was then transferred to a pre-weighed 1·5 ml centrifuge tube containing 1·2 ml ice-cold 8% PCA and the final weight was then determined. The tube was vortexed for 10 s 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 50 mg of dry powder was weighed into a bullet tube with 1 ml 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 $10\times$ dilution), and ground manually for 4 min as in the standard GH method. In the MDG treatment, approximately 0·5 g of frozen tissue was weighed (under liquid N_2) and then transferred to about $10\times$ volume of 8% PCA in a 17×100 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 analysed fluorometrically (Fluoro-microphotometer, American Instrument Co., Maryland, U.S.A.) for ATP and PCr (cf. Bergmeyer, 1983). Cr, Glu, Gly, Lac, and Pyr were assayed enzymatically (LKB UltralspecPlus 4053, LKB Ltd, Cambridge, U.K.) 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_{O_2} 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 analyser. True plasma total CO_2 was measured on a Cameron Instruments Capni-Con Total CO_2 analyser (Model II). Pa_{CO_2} and plasma HCO_3 were calculated via manipulation of the Henderson–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 haematocrit was measured by centrifuging 80 µl of blood in a sealed capillary tube (Radiometer type D) at 5000 *g* for 5 min. Haemoglobin 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 analysed enzymatically for Lac, Pyr, Glu, and Amm (Bergmeyer, 1983). Plasma inorganic phosphorus was measured by the method of Fiske & SubbaRow (1925).

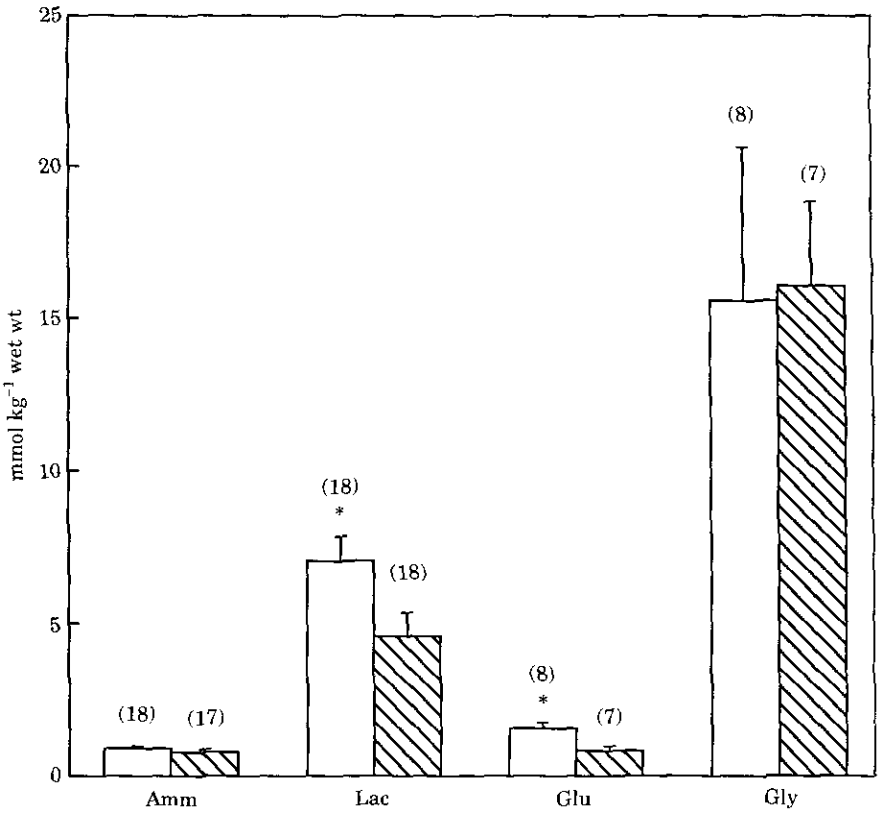

FIG. 1. Comparison of concentrations of ammonia (Amm), lactate (Lac), glucose (Glu), and glycogen (Gly) in resting rainbow trout white muscle sampled by needle biopsy and frozen in liquid N_2 by either the blow-out (open bars) or direct-freezing (hatched bars) methods. *Indicates significant difference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

STATISTICAL ANALYSIS

Data are reported as means ± 1 S.E. (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

The blow-out (BO) technique resulted in significantly greater Lac (53·6%) and Glu (88·7%) compared with the direct-freezing (DF) technique in white muscle samples of trout taken by needle biopsy (Fig. 1). 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 (Parkhouse *et al.*, 1987; Tang & Boutilier,

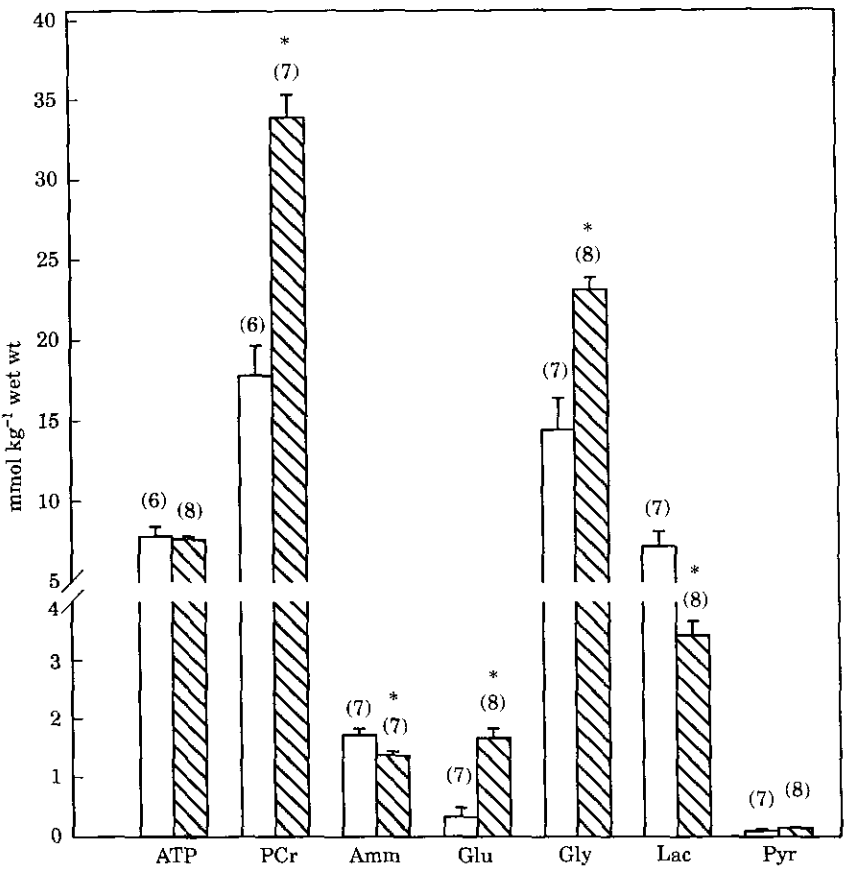

FIG. 2. Comparison of concentrations of ATP, creatine phosphate (PCr), ammonia (Amm), glucose (Glu), glycogen (Gly), lactate (Lac) and pyruvate (Pyr) in resting rainbow trout white muscle sampled by needle biopsy (with direct-freezing; open bars) or the MS-222 (with freeze-clamping; hatched bars) methods. *Indicates significant differences ($P<0{\cdot}05$) between the two techniques. Values are means ± 1 S.E. (number of fish) in mmol kg^{-1} wet wt.

1991), 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). 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).

TABLE 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.

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 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 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 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 were 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, corresponded with significant increases in both Lac and P_{CO_2} of arterial blood in the exercised fish (Table II). In parallel, exercised fish exhibited significantly decreased arterial P_{O_2}, elevated Hct, plasma protein, Pyr, Amm, and inorganic phosphorus, while HCO_3, Hb, and Glu did not change significantly (Table 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. 3(a)]. 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. 3(a)].

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. 3(a, b)]. Within the exercise treatment group, ATP, Gly, and

TABLE 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)*
P_{O_2} (Torr)	117·8 ± 3·3 (18)	102·4 ± 4·9 (7)*
P_{CO_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 100 ml^{-1})	6·56 ± 0·43 (13)	6·01 ± 0·59 (8)
Protein (g 100 ml^{-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).

Lac remained constant amongst the three processing methods [Fig. 3(b)]. 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. 3(b)]. 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 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. 4). Samples that were ground into fine powder under liquid N_2 and then immediately extracted in PCA (i.e. MP&GH) had Amm concentrations of approximately 0·7 mmol kg^{-1} wet weight, similar to the values in Table III. When these samples were allowed to sit at room temperature for only 30 s, Amm concentrations increased three-fold. Amm concentrations increased progressively for the first 10 min, stabilizing thereafter at approximately 6–8 mmol kg^{-1} wet weight (Fig. 4). These were comparable to levels measured in exhaustively exercised fish by GH or MP [Fig. 3(b)].

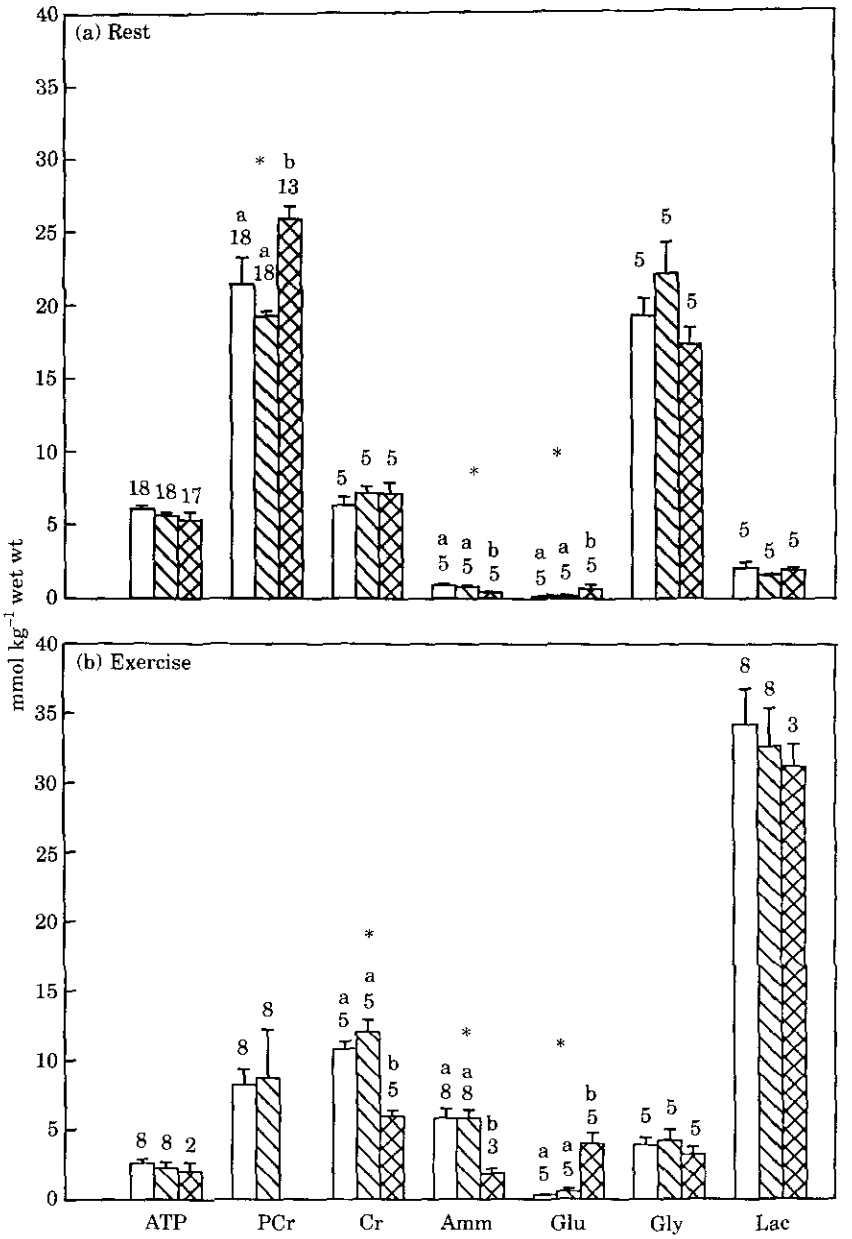

FIG. 3. Comparison of concentrations of ATP, creatine phosphate (PCr), creatine (Cr), ammonia (Amm), glucose (Glu), glycogen (Gly), and lactate (Lac) in rainbow trout white muscle processed by glass homogenization (open bars), mortar and pestle (hatched bars), and freeze-drying (crossed bars) methods. (A) Resting fish, (B) exhaustively exercised fish. *Indicates significant differences ($P<0{\cdot}05$) amongst the three methods (ANOVA). (a) Indicates no significant difference ($P>0{\cdot}05$) between the two groups, and (b) indicates significant differences ($P<0{\cdot}05$) from (a) (post-hoc comparison by Duncan's test). Values are means ± 1 S.E. (number of fish) in mmol kg^{-1} wet wt.

TABLE 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	0·89 ± 0·10 (5)
MP&GH	0·63 ± 0·09 (5)
MDG	0·53 ± 0·12 (5)

Values are means ± S.E.M. (number of fish) in mmol kg^{-1} wet weight. ANOVA indicated no significant differences ($P>0·05$).

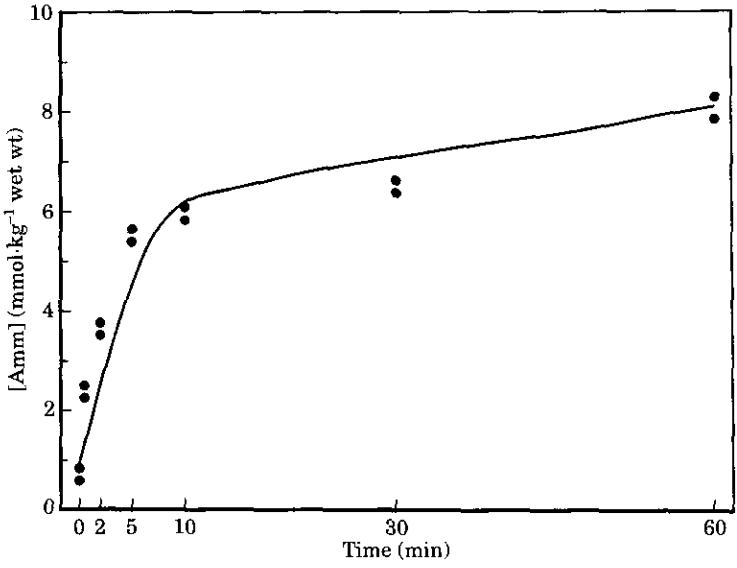

FIG. 4. White muscle ammonia (Amm) concentrations after thawing at room temperature (20° C) for different time periods (0–60 min). Values are duplicate measurements on tissue from a single resting fish, expressed in mmol kg^{-1} wet wt.

DISCUSSION

To place the present data in context, Table IV surveys other recent measurements of the same metabolites in the white muscle of rainbow trout at rest obtained by a variety of sampling, processing, and analytical methods. 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

TABLE 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
	31·50 ± 0·87	Decap	MP&GH	HPLC	2
	24·6 ± 1·0	Decap	MP&GH	HPLC	3
	19·36 ± 2·30	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
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
Gly	22·4 ± 1·68	Decap	MP&GH	Enzy	2
	23·3 ± 1·0	Decap	MP&GH	Enzy	3
	8·40 ± 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 ± 0·20	CB	MP&GH	Enzy	1
	1·02 ± 0·14	Decap	MP&GH	Enzy	10

TABLE IV. *Continued*

	Concentration (mmol kg^{-1} wet weight)	Sampling methods	Processing methods	Analytical methods	Source
Glu	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
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
	0·60 ± 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

Values are 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 and C. M. Wood (unpublished).

CB: Cephalic blow; Decap: decapitated; Somnotol Inj.: injection of somnotol; 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.* (1988*a*); 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.* (1988*b*); 11. Milligan & Girard (1993); 12. Turner *et al.* (1983); 13. Tang & Boutilier (1991); 14. Wright & Wood (1988).

the two freezing processes suggests that there was no elevation of adenylate deamination (Fig. 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 vapour 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 probably 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 IV), Pyr levels measured in the former study were far greater.

THE BIOPSY *V.* 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), support this conclusion. Indeed the present Gly levels are amongst the highest ever recorded in this type of study (Table IV). However, we were unable to obtain muscle Lac concentrations as low (Table 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). 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 and 3). ATP levels reported by various methods in the literature were also fairly uniform (Table IV). Indeed, a ^{31}P-NMR study of white muscle in goldfish *Carassius auratus* L. has shown that the initial levels of ATP are maintained for 1 h 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) was comparable to values reported in studies in which fish were killed by disturbing methods such as cephalic blow or decapitation (Table IV). Dobson & Hochachka (1987) have shown that one to four 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) 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 goldfish (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 s 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, *v.* biopsy (Fig. 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.*, 1988*a*).

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 I). If we assume, based on the results of series II (Fig. 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. 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. 3(a)]. 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 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. 3(a)] and exercised fish [Fig. 3(b)]. As we 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, our 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 at -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 we believed that the much lower Amm concentrations with FD than with GH or MP (Fig. 3) were real, reflecting better preservation of true *in vivo* levels. However, we 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 (e.g. 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 us 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 analysed for Amm. We 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. We 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. 4) 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 $-0{\cdot}8$ 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 probably 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 IV). In the present study, however, there was no evidence that MDG caused any elevation in Amm

(Table III). It therefore seems unlikely that artefacts 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 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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