ENERGY METABOLISM IN CARP WHITE MUSCLE

by

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ABSTRACT

The myotomal muscle of fish is largely composed of two tissue types, usually referred to as the red and white fibers. On the basis of histochemical and biochemical properties it is generally accepted that white muscle has a metabolism which is predominantly anaerobically based, utilizing glycogen as its fuel source and that red muscle functions largely aerobically burning fats and/or carbohydrates. In carp (Cyprinus carpio L.) the red fibers are found as a thin superficial layer below the skin and the white fibers make up the mass of the underlying myotome. Thus, in this species it is possible to rapidly obtain an homogenous sample of white muscle allowing the analysis of labile metabolites. This study investigates the control of glycolysis in white muscle and the source and function of anaerobic NH_4^+ production by white muscle.

The concentrations of key metabolites were determined in muscle before exercise and after maximal activity. During exercise there was an increase in levels of glucose-6-phosphate, fructose-6-phosphate and fructose-1-6-diphosphate which, along with a decrease in ATP levels, could account for the increase in glucolytic flux by activation of phosphofructokinase and pyruvate kinase. It was found that after maximal activity the concentration of ATP decreased by about 65%, ADP decreased slightly, while AMP remained low and unchanged. Consequently the level of the free adenylate pool decreased. Simultaneously there was an increase in the concentration of IMP (inosine monophosphate) and NH_4^+. The increase in IMP level and the decrease in adenylate pool were essentially in 1:1 stoichiometry, a result showing that the adenylate pool was decreased by the reaction catalyzed by 5' AMP deaminase. However, the increase in free NH_4^+ was less than the decrease in the adenylate

pool. The concentration of free amino acids was also determined in white muscle, before and after severe environmental hypoxia. During the hypoxic period the total amount of nitrogen in the amino acid pool increased and there was a tendency for an increase in the total number of free amino acids.

On the basis of this study it is possible to construct a fairly comprehensive metabolic scheme for nitrogen metabolism in carp white muscle during anaerobic work. The energy required for work is ultimately derived from the hydrolysis of ATP. When ATP levels cannot be maintained the content of ADP increases and as this occurs the level of AMP also increases due to the equilibrium relationship of the adenylates. As the work load on the tissue exceeds its aerobic capabilities GTP (guanosine triphosphate) levels drop, 5' AMP deaminase is activated and the adenylate pool is decreased. NH_4^+ released from AMP is subsequently incorporated into the free amino acid pool.

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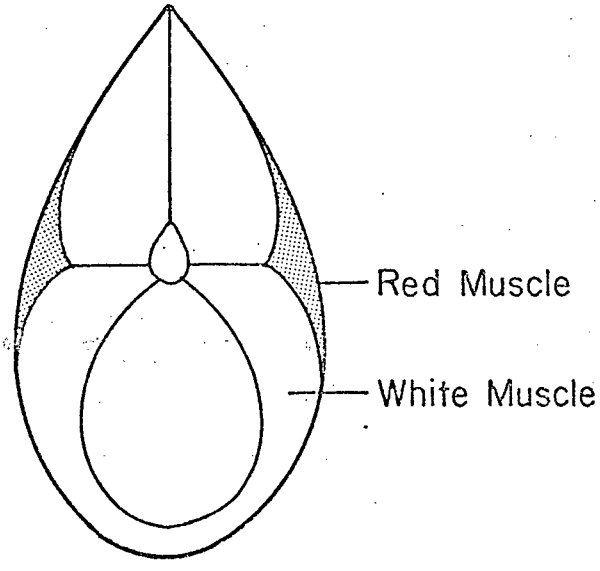
CHAPTER I.

INTRODUCTION

The skeletal muscle of vertebrates is composed of a number of characteristically different fiber types. At the extreme ends of the spectrum of fiber types are those which make up the red and white muscles. In mammals, red, white, and intermediate type fibers usually occur in complex mixed muscle masses. In some instances, such as the cat soleus, a relatively pure red muscle may be obtained, but in mammalian systems, white muscle *per se*, does not exist as a discrete tissue. However, it is possible to study white muscle by utilizing lower vertebrates. In many fish species the white fibers, which constitute 80-95% of the swimming musculature, exist as a discrete, easily separable tissue mass. Where this occurs, the red fibers are found as a thin superficial layer below the skin forming a thicker triangle of muscle at the level of the lateral line, with the white fibers making up the mass of the underlying myotome. The myotomal musculature of carp (*Cyprinus carpio* L.) is structured in this manner (Figure 1). Although there may be gradations in the size of the fibers (Boddeke *et al*, 1962), all of the available histochemical evidence indicates that white muscle of carp is an homogenous tissue in terms of energy generating properties (Ogata and Mori, 1964; Brotchi, 1968). In this species a small number of intermediate type fibers are found, but these occur between the red and white muscle masses. Thus it is possible with very little difficulty to obtain a relatively pure preparation of white muscle from carp.

It is now well established that the red and white muscle of fish have markedly different metabolic capabilities. On the basis of biochemical and histological properties it is generally accepted that white muscle has a metabolism which is predominantly anaerobic, whereas the red muscle functions largely aerobically. Thus, red muscle is characterized by a higher content of mitochondria (Buttkus, 1963), myoglobin (Hamoir *et al*, 1972), haemoglobin

Figure 1. Schematic representation of carp (Cyprinus carpio) in cross-section at the level of the posterior margin of the dorsal fin.


Red Muscle
White Muscle

(Hamoir et al, 1972), lipid (Bone, 1966; Lin et al, 1974), lipolytic enzymes (George, 1962), Krebs cycle enzymes (Bostrom and Johansson, 1972), and cytochrome oxidase (Bostrom and Johansson, 1972). These properties are reflected by a higher vascular supply to the tissue (Stevens, 1968) and a greater capacity to consume oxygen (Lin et al, 1974; Wittenberger and Diaciuc, 1965). White muscle, on the other hand, is poorly vascularized (Stevens, 1968), shows a low oxygen consumption rate (Lin et al, 1974; Wittenberger and Diaciuc, 1965), and is biochemically geared for anaerobic metabolism. Consequently, this tissue has a high content of glycolytic enzymes (Hamoir et al, 1972) and an extremely active lactate dehydrogenase designed to channel pyruvate into lactate (Bostrom and Johansson, 1972). Studies on mammalian systems are in total agreement with the above metabolic findings and have been reviewed by Pette and Staudte (1973) and Keul et al (1972).

The control of blood flow to skeletal muscle in fish is still poorly understood. Satchell (1971) has reviewed this area of literature with respect to both exercise and hypoxia. Exercise evokes an overall reduction in peripheral resistance and an increased blood flow through the trunk region. Satchell argues that since the white muscle makes up such a large portion of the body musculature, blood flow to this tissue increases during activity. During hypoxia however, peripheral vasoconstriction occurs (Satchell, 1971). It is well established that during hypoxia blood flow to skeletal muscle in mammals in reduced (Irving, 1964); although not unequivocally established, it appears that the same mechanism functions in fish. Moreover, in light of the poor circulation of white muscle under aerobic conditions, it is probable that this tissue approximates a closed system during hypoxia.

The metabolic characteristics of red and white muscle are reflected by

a functional difference between these two tissues. Electrophysiological studies show that during slow swimming the propulsive force is derived entirely from the red musculature. At the highest swimming velocities the white muscle becomes maximally active and together with the red muscle provides the power for locomotion (Bone, 1966; Hudson, 1973). White muscle was previously thought to be utilized only during periods of burst activity (Bone, 1966); however, it is now generally accepted, that at least in the teleost, this tissue plays a role over a much wider range of swimming speeds. All of the current data indicate that at some level in the transition from low to high swimming speed there is increasing recruitment of the white fibers. This is based on: (a) the accumulation of lactate in the white muscle of trout (Black et al, 1962), carp (Johnston and Goldspink, 1973a), coalfish (Johnston and Goldspink, 1973b), and mackerel (Pritchard et al, 1971) worked at intermediate velocities; (b) hypertrophy of white muscle fibers in coalfish forced to swim at moderate speeds for extended periods of time (Walker and Pull, 1973); (c) the repayment of an oxygen debt by salmon during recovery from swimming at all elevated speeds (Brett, 1964) and (d) the observation that in goldfish the mass of the red muscle fibers alone is not great enough to meet the overall power output of the fish (Smit et al, 1971).

During exercise carp white muscle generates a large portion of its energy by anaerobic glycolysis (Wittenberger and Diaciuc, 1965); thus, during work there is a decrease in glycogen with a concomitant increase in lactate. The quantitative aspects of this phenomenon, in numerous species, have recently been reviewed by Bilinski (1974) and need not be repeated here. All of the available evidence indicates that glycogen is metabolized in fish by the classical Embden, Myerhof, Parnas pathway; for each mole of glycogen-derived

glucose mobilized, 2 moles of lactate are formed with a net gain of 3 moles of ATP. Again this area of literature has been reviewed in depth by Hochachka (1969) and Tarr (1972). In many respects the control of glycolysis in fish white muscle appears similar to other systems studied. Thus, glycogen mobilization is initiated by glycogen phosphorylase, a regulatory enzyme, which in most tissues in under a variety of controls including hormonal agents such as epinepherine and norepinepherine, the adenylates, and free Ca^{++} (Drummond, 1971). In fish white muscle, Ca^{++} appears to be the primary if not the sole regulator of glycogen phosphorylase since blood flow and hence hormonal signals are minimal (Pocinwong et al, 1974). The next site of control in the glycolytic pathway occurs at the reaction catalyzed by phosphofructokinase (PFK) which catalyzed the conversion of fructose-6-phosphate plus ATP to fructose-1,6-diphosphate plus ADP. The reaction catalyzed by PFK represents the first unique step in glycolysis; hence, it is not surprising that the enzyme is precisely regulated by various metabolites in a manner that controls the rate of glycolysis in accord with the cells' need for energy or glycolytic intermediates. In a variety of tissues, both substrates and products of the PFK reaction, as well as other factors, are allosteric modifiers of the enzyme. Thus, activators of PFK include AMP, fructose-6-phosphate, fructose-1,6-diphosphate, P_i, and NH_4^+; whereas ATP, citrate and creatine phosphate are inhibitors (Mansour, 1972). To complete the activation of muscle glycolysis, the activity of PFK is integrated with the next major control site in the pathway: pyruvate kinase (PyK). PyK catalyzes the conversion of phosphoenolpyruvate plus ADP to pyruvate plus ATP. An integration of the rate controlling enzymes is achieved in two ways. Firstly, ADP formed in the PFK reaction serves as a substrate for PyK, and secondly, at least in

the lower vertebrates, fructose-1,6-diphosphate functions as a feed forward activator of PyK (Hochachka and Somero, 1973). The kinetic properties of both PFK and PyK from fish muscle have been studied (Freed, 1971; Somero and Hochachka, 1968; Mustafa et al, 1971); however, the mechanism controlling glycolytic flux in vivo in this tissue had not been verified. Thus, it seemed worthwhile to determine the concentration of metabolites known to regulate this pathway under varying conditions of energy demand.

There is now a substantial amount of evidence which suggests that glycolysis is not the only anaerobic metabolic pathway in white muscle. At least three studies have shown the mobilization of nitrogenous compounds in fish white muscle during exercise. Thus, with exercise to fatigue, the white muscle content of NH_4^+ increases from about 4 to 7 μmoles/gm in cod (Fraser et al, 1966) and from about 2.5 to 7 μmoles/gm in Triachis scyllium (Suyama et al, 1960). Kutty (1972) has hypothesized, on the basis of oxygen consumption and NH_4^+ excretion by Tilapia mossambica, that at least a portion of the NH_4^+ produced in the muscle during activity is of anaerobic origin. Support for this contention arises from the observations that goldfish expire increased amounts of NH_4^+ during hypoxia (Dejours et al, 1968) and that the blood level of NH_4^+ in carp increases during hypoxia (Pequin and Serfaty, 1962). It would be of interest to ascertain if there are reactions in white muscle, functional under anaerobic conditions, which could provide a source of NH_4^+. There appear, on the basis of our knowledge of intermediary metabolism, to be only two likely origins of anaerobic NH_4^+: the free adenylate pool and the amino acid pool.

It is recognized that the enzyme 5' AMP deaminase is instrumental in the controlled release of NH_4^+ in skeletal muscle (Lowenstein, 1972). 5' AMP

deaminase catalyzes the degradation of AMP to IMP (inosine monophosphate) plus NH_4^+ and occurs in particularly high titres in skeletal muscle. Furthermore, the activity of this enzyme is at least 15 times higher in carp white muscle than in red muscle or heart (Fields, personal communication). This reaction was thus considered a likely candidate for the production of anaerobic NH_4^+. AMP, however, is in equilibrium with ATP and ADP by the adenylate kinase reaction:

$$2\ ADP \longrightarrow AMP + ATP$$

Therefore if 5' AMP deaminase is responsible for the production of anaerobic NH_4^+ this should be reflected by a decrease in the entire adenylate pool. In insect flight muscle, a tissue with no anaerobic capacity, the adenylate pool in fact remains constant during activity (Gerez and Kirsten, 1965) or imposed hypoxia (Ford and Candy, 1972). But in vertebrate muscle the situation is more complex probably due to the varying content of red and white fibers in different mammalian musculatures. In working conditions when oxygen is not limiting the adenylate pool does not decrease in either skeletal muscle (Edington et al, 1973) or heart (Shafer and Williamson, 1973), whereas after an hypoxic stress it is found to decrease with a concomitant increase in IMP plus its degradation products (Imai et al, 1964; Chaudry et al, 1974; Deuticke and Gerlach, 1966). In fish, Jones and Murray (1960) found a 1:1 stoichiometric relationship between depletion of adenine nucleotides and accumulation of IMP in cod muscle after the animal had been fatigued. In light of the apparent discrepancies in this area, a study of adenylate pool alterations in an organism, the carp, possessing a large homogenous white muscle mass was initiated.

In a study of anaerobic NH_4^+ production, the possibility of the fermenta-

tion of amino acids must also be considered. Many invertebrates which are facultative anaerobes mobilize both carbohydrates and amino acids anaerobically and this apparently provides them with additional means of generating ATP over and above classical glycolysis. Figure 2 is a simplified representation of what is thought to occur during periods of oxygen deprivation by invertebrate facultative anaerobes (Hochachka et al, 1973). The reaction scheme for carbohydrates is the same as that which occurs in vertebrate tissue as far as the level of phosphoenolpyruvate. However, instead of phosphoenolpyruvate being directly converted to pyruvate it is first carboxylated to form oxaloacetate which is quickly reduced to malate. Malate apparently has two metabolite fates. One route involving the reversal of Krebs cycle reactions leads to the formation of succinate and the concomitant production of ATP. Succinate may accumulate as an end product or be further catabolized to proprionate, a volatile acid, with the further production of ATP and CO_2. The other destination of malate is conversion to pyruvate followed by transamination with glutamate to form alanine. Invertebrates have particularly high levels of free amino acids and it is thought that these are initially transaminated with α-ketoglutarate to form their respective α-ketoacids. The α-ketoacids formed from amino acids such as leucine and valine are further catabolized to volatile acids again with the production of ATP and CO_2.

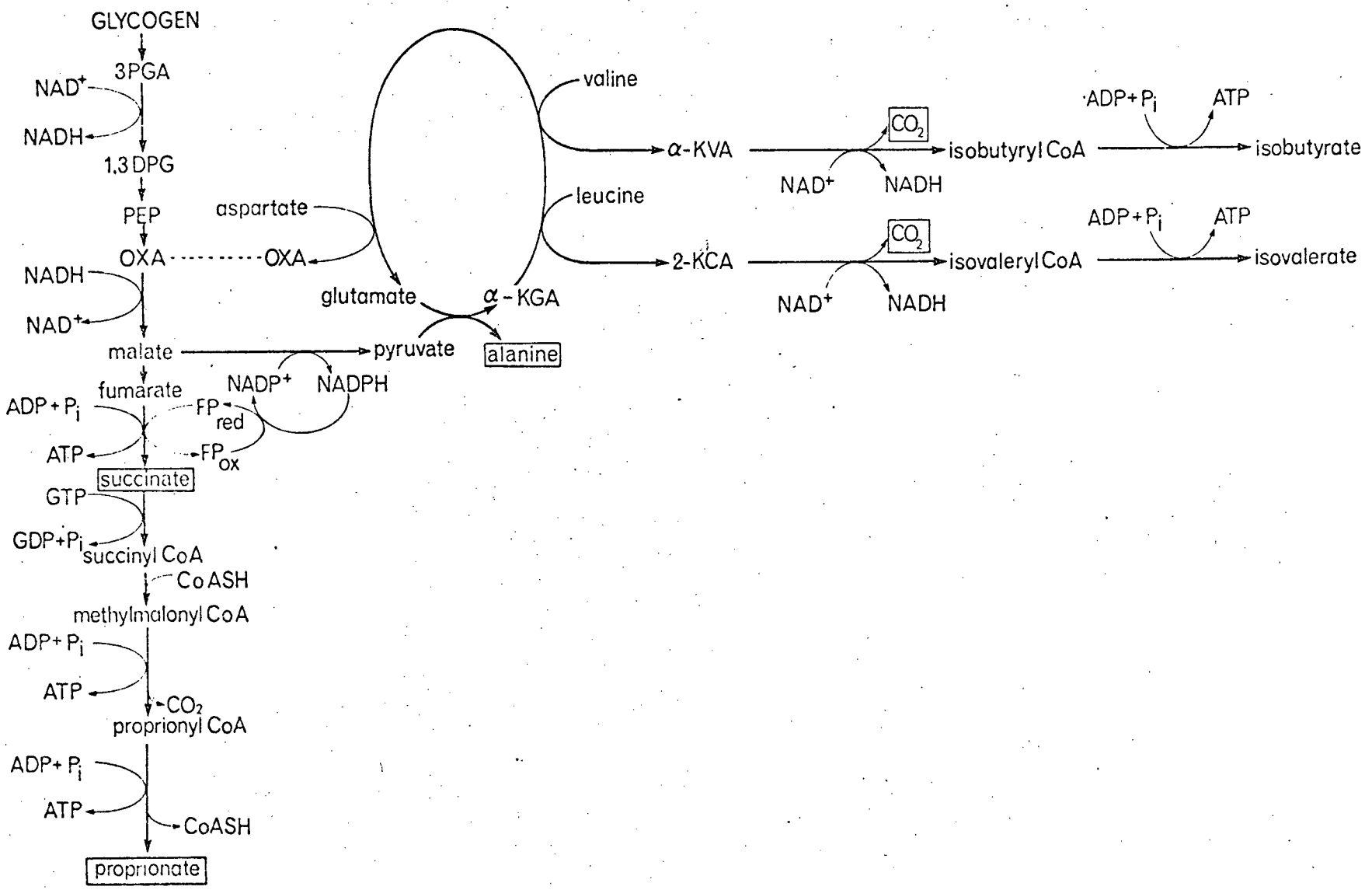
Interestingly enough, there are data which lend credulence to the hypothesis that such a reaction scheme may occur in fish tissues. The anaerobic production of CO_2 by fish has been reported several times. Thus, goldfish injected with labelled glucose produce labelled CO_2 under anoxic conditions (Hochachka, 1961) and gill tissue incubated under anaerobic conditions produces CO_2 of metabolic origin (Ekberg, 1962). This concept of anaerobic

CO_2 production is supported by numerous reports of respiratory quotients greater than 1 during both hypoxia and swimming (Kutty, 1968, 1972; Morris, 1967; Mathur, 1967). Furthermore, the anaerobic production of unidentified volatile acids has been noted on at least two occasions (Blazka, 1958; Blazka and Kopecky, 1961). Perhaps the observations of the anaerobic production of NH_4^+, CO_2 and volatile acids can be explained singularly, but when all of them are considered in toto they suggest that something is unaccounted for in our current theory of anaerobic metabolism in fish and that the problem should by no means be considered closed. In particular, the above unusual observations suggest (1) that lactate may not be the sole end product of anaerobic glycogenolysis, and/or (2) glycogen is not the only anaerobic energy source utilized.

Although white muscle generates much of its energy by anaerobic means, it also has an aerobic component to its metabolism. Moreover, during work this tissue has the capacity to increase its oxygen consumption to a small degree (Wittenberger and Diaciuc, 1965). In the mammalian heart and skeletal muscle the total amount of Krebs cycle intermediates increases as the work load increases (Shafer and Williamson, 1973; Edington et al, 1973); however, it had never been ascertained whether or not white muscle per se has the capacity to do so. Consequently, this work also investigates the ability of white muscle to augment the size of its Krebs cycle pool during increased energy demands.

In the present thesis, two types of experiments were carried out to examine energy metabolism in carp white muscle. In one set of experiments, animals were exercised in order to elucidate anaerobic control mechanisms and Krebs cycle alterations. In another study carp were subjected to hypoxic stress and changes in the free amino acid pool were examined.

Figure 2. Anaerobic metabolism in invertebrate facultative anaerobes. Abbreviations not indicated in text: 1,3 DPG, 1,3 diphosphoglycerate; 2-KCA, 2-ketoisocaproate; α-KGA, α-ketoglutarate; α-KVA, α-ketoisovalerate; OXA, oxaloacetate; PEP, phosphoenolpyruvate; 3 PGA, 3 phosphoglycerate. Modified from Hochachka *et al* (1973).


GLYCOGEN
3PGA
NAD+
NADH
1,3 DPG
PEP
aspartate
OXA
OXA
NADH
NAD+
malate
pyruvate
glutamate
α-KGA
alanine
NADP+
NADPH
fumarate
ADP+Pi
FP red
ATP
FP ox
succinate
GTP
GDP+Pi
succinyl CoA
CoASH
methylmalonyl CoA
ADP+Pi
ATP
CO2
proprionyl CoA
ADP+Pi
ATP
CoASH
proprionate
valine
α-KVA
CO2
NAD+
NADH
isobutyryl CoA
ADP+Pi
ATP
isobutyrate
leucine
2-KCA
CO2
NAD+
NADH
isovaleryl CoA
ADP+Pi
ATP
isovalerate

CHAPTER II

MATERIALS AND METHODS

Animals

Carp (Cyprinus carpio L.) were seined from local ponds in southern British Columbia. Fish employed in the swimming experiments were between 12 and 15 cm in length and weighed between 40 and 60 gm. These animals were maintained at 12 ±1°C in aerated running water, under a simulated natural photoperiod and fed corn ad lib. The carp utilized in the hypoxia experiment were about 30 cm in length and weighed about 1000 gm. These animals were held out of doors in running aerated water at 12 ±3°C and during the holding period of not longer than three weeks were not fed.

Exercise Experiments

Fish were exercised in a swim tunnel similar to that described by Brett (1964). Fish were introduced into the tunnel and forced to swim at 8.6 cm/sec for 1 hr. Following the introductory phase the fish were subjected to 10 min periods of swimming at fixed velocities after which the velocity was rapidly increased. The velocity increment was approximately 7 cm/sec. The experiment was terminated either after successful completion of the 2nd velocity level or when the fish was unable to remove itself from an electrified grid (15 v AC) at the downstream end of the tunnel. This later behaviour was defined as fatigue and usually occurred during the 4th speed increment. The water was maintained at 11°C and 100% air saturation. Critical velocity was calculated using the empirical formula of Brett (1964) such that the last velocity that the fish successfully maintained was added to the velocity at which the fish fatigued, multiplied by the proportion of the 10 minute period that it was able to sustain this final speed. The mean critical velocity for the animals of this experiment was 45 cm/sec.

Failure of a fish to meet the imposed velocity is due to the limitation of the oxygen delivery system and not exhaustion of the white muscle (Jones,

1971; Brett, 1964). After the experimental period an animal is still able to perform burst activity if forced to do so. Thus, in the present study there is no reason to believe that the white muscle was fatigued.

Hypoxia Experiments

The day before an experiment a fish was removed from the holding tank and placed in a sealed chamber which was just slightly larger than the animal. Aerated water, about 1 C^{o} higher than the holding temperature, flowed through the chamber at 180 ±10 ml/min. The test chamber was covered with black plastic in the evening and uncovered the following morning. The entire apparatus was immersed in a water bath which served to maintain the temperature. In the morning, on the day of an experiment, the O_2 content of the inflowing water was reduced by nitrogen gas to about 10% air saturation as measured by a micro Winkler technique (Kent and Hall, personal communication). Within 45 minutes the O_2 content in the chamber fell to this level and continued to drop for the duration of the experiment which was terminated after 4 hours. For control fish which were kept in the chamber for the same length of time as the experimentals the O_2 content never fell below 60% of the air saturated level.

In an experiment of this type it is difficult to quantitate the degree of hypoxic stress to which the animals were exposed. Observations of a qualitative nature, however, indicated beyond question that the experimental fish were indeed subjected to severe hypoxia. Thus, preliminary studies showed that there was mortality in animals subjected to experimental conditions for periods any longer than four hours; whereas, the control fish could survive for a second day and probably longer. Furthermore, animals subjected to the reduced oxygen level demonstrated an extreme hyperventilation and frequently lost equilibrium. Neither of these behaviour patterns was observed in the

control group. During the test period both the control and the experimental fish demonstrated very little activity.

Preparation of Tissue for Biochemical Analysis

In the exercise experiments, the fish were removed from the holding tank or the swim tunnel and immediately decapitated. In the hypoxia experiment, the animals were removed from the experimental chamber and stunned by a blow to the head. In both studies, a portion of white muscle weighing approximately 1 gm was dissected from immediately below the dorsal fin, starting at the posterior margin and going anteriorly. The tissue sample was then frozen in liquid nitrogen, within 20 sec after removal of the fish from the water. In the hypoxia experiment, a second sample of tissue, weighing about 30 gm, was also dissected out and frozen in liquid nitrogen. This tissue sample was used for volatile acid analysis.

Extraction of All Metabolites Except Volatile Acids

The frozen tissue was powdered with mortar and pestle which had been previously cooled and then the sample was placed in a 40 ml plastic centrifuge tube into which a Teflon pestle could fit snugly. The test tube contained an aliquot of cold $HClO_4$ (8% w/v) in 40% ethanol. The sample was mixed quickly with a glass rod and the amount of $HClO_4$ was taken up to 3.5 ml/gm tissue. The tissue was homogenized for 2 min at high speed with a Virtis (#23) mixer during which time the test tube was maintained in a dry-ice ethanol bath. The homogenate was spun at 25,000 g ($-4^{o}C$) for 10 min to precipitate protein. The supernatant solution was saved and the precipitate was resuspended in the same volume of $HClO_4$ as previously used. After a further centrifugation the supernatant solutions were combined and neutralized to pH 5.5-6.0 with 3 M K_2CO_3 containing 0.5 triethanolamine. The precipitated $KClO_4$ was removed by

centrifugation and the supernatant solution was stored at -20°C (Williamson and Corkey, 1969).

Extraction of Volatile Acids

Volatile acids were isolated by steam distillation (Baker, 1957). The larger piece of frozen tissue was broken into small pieces and placed in a 500 ml double neck distilling flask. Ten volumes of H_2O and 2 drops of Antifoam A concentrate (Sigma) (Ackman and Noble, 1973) were added and the pH was adjusted to 2.0-2.5 with 10 N H_2SO_4. Between 10 and 11 times the volume of fluid originally present in the distilling vessel was collected in 25 ml KOH pH=8. The pH of the distillate was maintained between 8.0-8.5 with 0.1 N KOH. The distillate was flash evaporated to dryness and taken up in 200 µl of acetate free formic acid.

Enzymatic Analysis of Metabolites

All of the metabolites, with the exception of the amino acids and the volatile acids, were measured enzymatically. Furthermore, each of the enzymatic analyses, except the procedures for formate and IMP, was based on the absorbance changes of the pyridine nucleotides at 340 mµ. The change in amount of the pyridine nucleotides in µmoles is calculated from the equation (Δ optical density$_{340}$)(volume of assay mixture)/Σ, where Σ = 6.22 cm^2/µmole, the extinction coefficient of the pyridine nucleotides at 340 mµ. This value represents the number of µmoles of metabolite present per the amount of protein free neutralized extract added, if in the analysis there is a 1:1 ratio between the content of measured metabolites and the oxidation or reduction of the pyridine nucleotides. Assays were carried out at 37°C on a Unicam SP 1800 dual beam spectophotometer connected to a strip chart recorder. All enzymes were purchased from Sigma, St. Louis, Mo.

Alanine assay:

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{lactate dehydrogenase}} \text{lactate} + \text{NAD}^+$$

$$\text{alanine} + \alpha\text{-ketoglutarate} \xrightarrow[\text{transaminase}]{\text{glutamate-pyruvate}} \text{pyruvate} + \text{glutamate}$$

Reagents

Buffer: 0.5 M tris pH 8.1

NADH: 8 mM in 1% $KHCO_3$ (w/v)

α-ketoglutarate: 0.1 M in 0.1 M tris pH 7.4

Lactate dehydrogenase: source - beef heart

Glutamate-pyruvate transaminase: source - pig heart

Procedure

LDH is added to a cuvette containing buffer, NADH (0.17 mM), and neutralized protein free extract to remove endogenous pyruvate. When the reaction is complete α-ketoglutarate (0.2 mM) is added. Finally glutamate-pyruvate transaminase is added and the decrease in OD_{340} is recorded. The reaction is extremely slow, thus it is preferable to plot an alanine standard curve and interpolate to ascertain the content of alanine in the extract. Reaction times - pyruvate, 1 min; alanine, 10 min (Lowry and Passonneau, 1972).

Aspartate assay:

$$\text{oxaloacetate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{malate dehydrogenase}} \text{malate} + \text{NAD}^+$$

$$\text{aspartate} + \alpha\text{-ketoglutarate} \xrightarrow[\text{transaminase}]{\text{glutamate-oxaloacetate}} \text{oxaloacetate} + \text{glutamate}$$

Reagents

Buffer: 50 mM imidazole pH 7

NADH: 8 mM in 1% $KHCO_3$ (w/v)

α-ketoglutarate: 0.1 M in 0.1 M tris pH 7.4

Malate dehydrogenase: source - pig heart

Glutamate-oxaloacetate transaminase: source - pig heart

Procedure

Malate dehydrogenase is added to a cuvette containing buffer, NADH (0.17 mM), α-ketoglutarate (0.2 mM), and neutralized protein free extract. When the reaction is complete glutamate-oxaloacetate is added and the decrease in OD_{340} is recorded. Reaction time - 10 min (Lowry and Passonneau, 1972).

Citrate assay:

$$\text{oxaloacetate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{malate dehydrogenase}} \text{malate} + \text{NAD}^+$$

$$\text{citrate} \xrightarrow{\text{citrate lyase}} \text{oxaloacetate} + \text{acetate}$$

Reagents

Buffer: 0.1 M tris pH 7.6

NADH: 8 mM in 1% $KHCO_3$ (w/v)

$ZnCl_2$: 1.2 mM in H_2O

Malate dehydrogenase: source - pig heart

Citrate lyase: source - Aerobacter aerogenes

Procedure

Malate dehydrogenase is added to a cuvette containing buffer, NADH (0.17 mM), $ZnCl_2$ (40 µM) and neutralized protein free extract. When the reaction is complete citrate lyase is added and the decrease in OD_{340} recorded to determine citrate content. Reaction time - 3 min (Lowry and Passonneau, 1972).

Formate assay:

$$\text{formate} + \text{ATP} + \text{tetrahydrofolic acid} \xrightarrow{\text{tetrahydrofolic acid formylase}} \text{N (10)-formyl-tetrahydrofolic acid} + \text{ADP} + P_i$$

$$\text{N (10)-formyl-tetrahydrofolic acid} \xrightarrow{H^+} \text{5,10-methenyl-tetrahydrofolic acid}$$

Reagents

Buffer: 1.0 M triethanolamine pH 8.0

Tetrahydrofolic acid: 0.01 M pH 7.0 in 1 M 2-mercaptoethanol

ATP: 0.05 M in 1.0 M triethanolamine

$MgCl_2$: 0.1 M in H_2O

Percholic acid: 2% (w/v) in H_2O

Tetrahydrofolic acid formylase: source - Clostridium cylindrosporum

Procedure

Tetrahydrofolic acid formylase is added to a centrifuge tube containing buffer, tetrahydrofolic acid (0.4 mM), ATP (1 mM), $MgCl_2$ (5 mM) and neutralized protein free extract. After 2 min at 37°C 1 volume of perchloric acid is added and the mixture centrifuged to remove protein. The difference in OD_{340} between a blank and a sample is determined. The extinction coefficient

of 5,10-methenyl-tetrahydrofolic acid at 350 mμ is 24.9 cm^2/ μmole (Rabinowitz and Pricer, 1965).

Fructose-6-phosphate and fructose-1,6-diphosphate assay:

$$\text{glucose-6-phosphate} + NADP^+ \xrightarrow[\text{dehydrogenase}]{\text{glucose-6-phosphate}} \text{6-phosphogluconate} + NADPH + H^+$$

$$\text{fructose-6-phosphate} \xrightarrow[\text{isomerase}]{\text{phosphoglucose}} \text{glucose-6-phosphate}$$

$$\text{fructose-1,6-diphosphate} \xrightarrow{\text{fructose-1,6-diphosphatase}} \text{fructose-6-phosphate} + P_i$$

Reagents

Buffer: 1.0 M tris pH 8.8

$NADP^+$: 10 mM in H_2O

$MgCl_2$: 0.1 M in H_2O

EDTA: 1.2% (w/v) in H_2O

Glucose-6-phosphate dehydrogenase: source - yeast

Phosphoglucose isomerase: source - yeast

Fructose-1,6-diphosphatase: source - rabbit liver

Procedure

Glucose-6-phosphate dehydrogenase is added to a cuvette containing buffer, $NADP^+$ (0.33 mM), $MgCl_2$ (7 mM), EDTA (10 mM), and neutralized protein free extract. Glucose-6-phosphate dehydrogenase is added to remove endogenous glucose-6-phosphate. When the reaction is complete phosphoglucose isomerase is added and the increase in OD_{340} is recorded to determine the content of fructose-6-phosphate. Finally fructose-1,6-diphosphatase is added to determine the

level of fructose-1,6-diphosphate. Reaction times - glucose-6-phosphate, 1 min; fructose-6-phosphate, 5 min; fructose-1,6-diphosphate, 30 min (Racker, 1965).

Glucose-6-phosphate and ATP assay:

$$\text{glucose-6-phosphate} + NADP^{+} \xrightarrow[\text{dehydrogenase}]{\text{glucose-6-phosphate}} \text{6-phosphogluconate} + NADPH + H^{+}$$

$$ATP + \text{glucose} \xrightarrow{\text{hexokinase}} ADP + \text{glucose-6-phosphate}$$

Reagents

Buffer: 0.05 M triethanolamine pH 7.5

$NADP^{+}$: 10 mM in H_2O

$MgCl_2$: 60 mM in H_2O

Glucose: 30 mM in H_2O

Glucose-6-phosphate dehydrogenase: source - yeast

Hexokinase: source - yeast

Procedure

Glucose-6-phosphate dehydrogenase is added to a cuvette containing buffer, $NADP^{+}$ (0.17 mM), $MgCl_2$ (1 mM), and neutralized protein free extract to determine the content of glucose-6-phosphate. When the reaction is complete glucose (1.0 mM) is added. Finally hexokinase is included and the increase in OD_{340} recorded to determine the level of ATP. Reaction times - glucose-6-phosphate, 1 min; ATP, 8 min (Lamprecht and Trautschold, 1965).

α-glycerophosphate assay:

$$\alpha\text{-glycerophosphate} + NAD^+ \xrightarrow[\text{dehydrogenase}]{\text{glycerophosphate}} \text{dihydroxyacetonephosphate} + NADH + H^+$$

Reagents

Buffer: glycine-hydrazine pH 9.2 (prepared by Sigma - stock #826-6)

NAD^+: 10 mM in H_2O

α-glycerophosphate dehydrogenase: source - rabbit muscle

Procedure

α-glycerophosphate dehydrogenase is added to a cuvette containing buffer, NAD^+ (0.17 mM), and neutralized protein free extract and the increase in OD_{340} is recorded. The reaction is extremely slow, thus it is preferable to plot a standard curve and interpolate to ascertain the content of α-glycerophosphate in the extract. Reaction time - 10 min (Lowry and Passonneau, 1972).

Inosine monophosphate assay:

$$\text{hypoxanthine} + 2O_2 + 2\ H_2O \xrightarrow{\text{xanthic oxidase}} \text{uric acid} + 2\ H_2O_2$$

$$\text{inosine} + P_i \xrightarrow{\text{nucleoside phosphorylase}} \text{hypoxanthine} + \text{ribose-5-phosphate}$$

$$IMP + H_2O \xrightarrow{5' \text{ nucleotidase}} \text{inosine} + P_i$$

Reagents

Buffer: 0.05 M KH_2PO_4 pH 7.4

EDTA: 0.1 M in H_2O

xanthic oxidase: source - buttermilk

nucleoside phosphorylase: source - calf spleen

5' nucleotidase: source - Crotalus adamanteus venom

Procedure

The course of the reaction is followed at 293 mμ. Xanthic oxidase is added to a cuvette containing buffer, EDTA (33 mM), and neutralized protein free extract. When the reaction is complete nucleoside phosphorylase is added to remove any endogenous inosine. Finally 5' nucleotidase is included to determine the content of IMP. The extinction coefficient for uric acid at 293 mμ is 12 $cm^2/\mu mole$. Reaction times - hypoxanthine, 1 min; inosine, 1 min; IMP, 20 min (adapted from Coddington, 1965).

α-ketoglutarate assay:

$$\alpha\text{-ketoglutarate} + NH_4^+ + NADH + H^+ \xrightarrow[\text{dehydrogenase}]{\text{glutamate}} \text{glutamate} \pm NAD^+$$

Reagents

Buffer: 0.5 M tris pH 8.0

NADH: 8 mM in 1% $KHCO_3$ (w/v)

$(NH_4^+)_2SO_4$: 35 mM in H_2O

Glutamate dehydrogenase: source - bovine liver

Procedure

GDH is added to a cuvette containing buffer, NADH (0.17 mM), $(NH_4^+)_2SO_4$ (5 mM), and neutralized protein free extract and the decrease in OD_{340} recorded. Reaction time - 8 min (Bergmeyer and Bernt, 1965).

Pyruvate, ADP and AMP assay:

$$\text{pyruvate} + \text{NADH} + \text{H}^{+} \xrightarrow{\text{lactate dehydrogenase}} \text{lactate} + \text{NAD}^{+}$$

$$\text{ADP} + \text{phosphoenolpyruvate} \xrightarrow{\text{pyruvate kinase}} \text{ATP} + \text{pyruvate}$$

$$\text{AMP} + \text{ATP} \xrightarrow{\text{adenylate kinase}} 2\ \text{ADP}$$

Reagents

Buffer: 0.05 M triethanolamine pH 7.5

NADH: 8 mM in 1% $KHCO_3$ (w/v)

$MgCl_2$: 120 mM in H_2O

KCl: 750 mM in H_2O

Phosphoenolpyruvate: 15 mM in 0.05 M triethanolamine pH 7.5

ATP: 6 mM in 0.05 triethanolamine pH 7.5

Lactate dehydrogenase: source - beef heart

Pyruvate kinase: source- rabbit muscle

Adenylate kinase: source - rabbit muscle

Procedure

LDH is added to a cuvette containing buffer, NADH (0.17 mM), $MgCl_2$ (2 mM), KCl (75 mM), and neutralized protein free extract to determine the content of pyruvate. When the reaction is complete phosphoenolpyruvate (0.25 mM) is added followed by pyruvate kinase to determine the content of ADP. When the second reaction is complete ATP (0.1 mM) is included and the decrease in OD_{340} recorded after the addition of adenylate kinase. For each mole of AMP two moles of NADH are oxidized. Reaction times - pyruvate, 1 min; ADP, 2-4 min; AMP, 6 min (Lowry and Passonneau, 1972).

Succinate assay:

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{lactate dehydrogenase}} \text{lactate} + \text{NAD}^+$$

$$\text{phosphoenolpyruvate} + \text{ADP} \xrightarrow{\text{pyruvate kinase}} \text{pyruvate} + \text{ADP}$$

$$\text{succinate} + \text{ATP} + \text{CoA} \xrightarrow{\text{succinate thiokinase}} \text{succinyl CoA} + \text{ADP} + P_i$$

Reagents

Buffer: 0.05 M triethanolamine, 10 mM $MgSO_4$, 5 mM EDTA pH 7.4

NADH: 5 mM in 0.1 M triethanolamine pH 8.2

Phosphoenolpyruvate: 0.1 M in 0.05 M triethanolamine pH 7.4

ATP: 10 mM in 0.05 M triethanolamine pH 7.4

CoA, lithium salt: 5 mM in H_2O

Lactate dehydrogenase: source - beef heart

Pyruvate kinase: source - rabbit muscle

Succinate thiokinase: source - E. coli

Procedure

LDH is added to a cuvette containing buffer, NADH (0.17 mM), and neutralized protein free extract to remove endogenous pyruvate. When the initial reaction is complete phosphoenolpyruvate (1.5 mM) and pyruvate kinase are added to the cuvette to remove endogenous ADP. When the second reaction is complete, lithium CoA (0.8 mM) and ATP (0.15 mM) are added. Finally succinate thiokinase is added and the decrease in OD_{340} is recorded. Reaction times - pyruvate, 1 min; ADP, 3 min; succinate, 30 min (Williamson and Corkey, 1969).

Lactate assay:

$$\text{lactate} + \text{NAD}^+ \xrightarrow{\text{lactate dehydrogenase}} \text{pyruvate} + \text{NADH} + \text{H}^+$$

Reagents

Buffer: glycine-hydrazine pH 9.2 (prepared by Sigma-stock #826-6)

NAD^+: 10 mM in H_2O

Lactate dehydrogenase: source - beef heart

Procedure

LDH is added to a cuvette containing buffer, NAD^+ (0.33 mM), and neutralized protein free extract and the increase in OD_{340} recorded. Reaction time - 45 min (Sigma bulletin #826).

Malate assay:

$$\text{oxaloacetate} + \text{glutamate} \xrightarrow[\text{transaminase}]{\text{glutamate-oxaloacetate}} \text{aspartate} + \alpha\text{-ketoglutarate}$$

$$\text{malate} + NAD^+ \xrightarrow{\text{malate dehydrogenase}} \text{oxaloacetate} + NADH + H^+$$

Reagents

Buffer: glycine-hydrazine pH 9.2 (prepared by Sigma-stock #826-6)

NAD^+: 10 mM in H_2O

Glutamate: 88 mM in 0.5 M tris

Glutamate-oxaloacetate transaminase: source - pig heart

Malate dehydrogenase: source - pig heart

Procedure

Glutamate-oxaloacetate transaminase is added to a cuvette containing buffer, NAD^+ (0.17 mM), glutamate (10 mM), and neutralized protein free extract. When the reaction is complete malate dehydrogenase is added and the increase in OD_{340} is recorded. Reaction time - 60 min (Lowry and Passonneau, 1972).

NH_4^+ assay:

$$NH_4^+ + \alpha\text{-ketoglutarate} + NADH + H^+ \xrightarrow{\text{glutamate dehydrogenase}} \text{glutamate} + NAD^+$$

Reagents

Buffer: 0.5 M tris pH 8.0

NADH: 8 mM in 1% $KHCO_3$ (w/v)

α-ketoglutarate: 0.1 M in 0.1 M tris pH 7.4

Glutamate dehydrogenase: NH_4^+ free, source - bovine liver

Procedure

GDH is added to a cuvette containing buffer, NADH (0.17 mM), α-ketoglutarate (10 mM), and neutralized protein free extract and the decrease in OD_{340} recorded. Reaction time - 10 min (Kun and Kearney, 1970).

Amino Acid Analysis

Prior to amino acid analysis the protein free extract was separated from interfering substances by absorption on a column (1 x 15 cm) of Amberlite IR-120 which had been previously washed with 5% HCl. 2.0 ml of protein free extract adjusted to pH 2-3 with 10 N H_2SO_4 were applied to the column. The column was washed with 40 ml of distilled water and the amino acids were eluted with 40 ml 2 N NH_4OH (Williamson et al, 1967). The eluant was evaporated to dryness in a 1 litre flask taken up in 5 ml H_2O and transferred to a 50 ml flask. The original 1 litre collecting flask was washed with 2 ml H_2O which was placed in the 50 ml flask. The amino acid extract was again evaporated to dryness and stored at -20°C. Immediately prior to analysis the sample was taken up in citrate buffer pH 2.2.

Amino acids in 200-500 µl aliquots were separated on a Beckman 120C amino acid analyzer. The ion exchange resin was a sulfonated polystyrene-divinyl benzene copolymer. The operating temperature was 55°C. Lysine, histidine and arginine were separated on a 16 cm column by elution with 0.35 N Na citrate pH 5.25. The acidic amino acids were separated on a 46 cm column by elution

with 0.2 N Na citrate pH 3.25. All amino acids were detected by ninhydrin reagent. Internal standards, α-amino-β-guanidinopropionic acid for the basics column and norleucine for the acidics column, were employed so that a correction could be made for the aging of the ninhydrin reagent. The number of moles of each amino acid residue applied was determined by comparison with a standard chromatogram.

Volatile Acid Analysis

Volatile acids were separated by gas liquid chromatography. A Varian Aereograph (#1700) equipped with a flame ionization detector was used. The columns were 6 ft by 1/8 in stainless steel packed with Chromsorb 101 80/100 mesh, and were maintained at 130°C. The carrier gas was N_2 with a flow rate of 56 ml/min. No attempt was made to accurately quantitate the content of volatile acids.

Calculations

Both the amino acid analysis and the enzymatic analysis provide data in μmoles of specified substance per aliquot of protein free neutralized extract. The aliquot of neutralized protein free extract is converted to the corresponding value in gm fresh tissue by multiplying by

$$\frac{(V_c)(W)}{(V_c + V_d)(V_a + V_b)}$$

where, V_a = total volume of $HClO_4$ added during the extraction

V_b = amount of water in the sample of tissue powder

V_c = volume of aliquot used for neutralization

V_d = volume of K_2CO_3 added to neutralize the above aliquot V_c

W = fresh weight of tissue sample in grams

The water content of carp white muscle was determined to be about 80%.

In the results, where appropriate, values are expressed as ± standard error of the mean. Results were analyzed statistically by a two sampled t-test for data collected in the swimming experiment and by the Mann-Whitney U test for data of the hypoxia experiment. In all cases a probability of less than 0.05 was considered to be significant.

CHAPTER III

RESULTS

Swimming Experiment Carried Out in Spring 1974

The concentrations of all of the glycolytic intermediates measured with the exception of pyruvate increased during activity (Table I). There was also a tendency for α-glycerophosphate, a metabolite associated with the glycolytic pathway, to increase. The greatest change occurred in lactate levels which increased by about 10 μmoles/gm. The mass action ratio of the phosphofructokinase reaction, that is (ADP)(fructose-1,6-diphosphate)/(ATP)(fructose-6-phosphate), was 1.82 in the rested fish and 2.78 in the exercised group. These values were displaced from the equilibrium constant of reaction by about two orders of magnitude (Mahler and Cordes, 1966), and support the concept of a regulatory role of phosphofructokinase in this tissue.

The results of this study with respect to the adenylate pool are summarized in Tables II and III. The content of ATP decreased with activity. When the animals were exercised, ATP concentrations were reduced by about 65%. Levels of ADP also decreased a small but significant amount; however, AMP concentrations remained low and unchanged. Thus in this complex way, the total free adenylate pool decreased during the exercise period. Concomitant with this decrease was an increase in IMP concentration. The increase in IMP level and the decrease in the adenylate pool were essentially in 1:1 stoichiometry, a result clearly showing that the adenylate pool was decreased by the conversion of AMP to IMP and NH_4^+. It is interesting to note that although NH_4^+ concentration increased in working white muscle, the change is not as large as for IMP. The energy charge, [ATP] + 0.5 [ADP]/[ATP] + [ADP] + [AMP], as defined by Atkinson (1968a), was high in both groups of animals, 0.89 in the rested fish, and 0.83 in the exercised group (Table III). The apparent equilibrium constant of the adenylate kinase reaction was 0.3 in both the rested and exercised animals.

Of the two amino acids measured, aspartate levels decreased by a small but significant amount while there was a tendency for an increase in the level of alanine (Table IV). Citrate, malate, α-ketoglutarate and oxaloacetate, compounds associated with the Krebs cycle, did not increase during activity, and in fact there was a tendency for citrate to decrease. It therefore appears that carp white muscle has a limited capacity to augment the size of its Krebs cycle pool.

Table I. Concentrations of glycolytic intermediates in white muscle of carp under two well defined conditions: resting and maximally active.

Metabolite	Resting	Maximally active
Glucose-6-phosphate	0.67 ± 0.05	1.33 ± 0.10*
Fructose-6-phosphate	0.11 ± 0.01	0.18 ± 0.03*
Fructose-1,6-diphosphate	0.85 ± 0.08	1.28 ± 0.10*
Pyruvate	0.11 ± 0.03	0.12 ± 0.03
Lactate	3.71 ± 0.17	12.58 ± 1.18*
α-glycerophosphate	2.52 ± 0.60	4.59 ± 1.30

All values are expressed in micromoles/gm of fresh tissue (±S.E.).

N = 7.

*Statistically significant difference between groups.

Table II. Concentrations of the adenylates and related metabolites in white muscle of carp under two well defined conditions: resting and maximally active.

Metabolite	Resting	Maximally active
ATP	4.12 ± 0.18	1.87 ± 0.08*
ADP	0.97 ± 0.05	0.73 ± 0.18*
AMP	0.07 ± 0.02	0.08 ± 0.02
IMP	1.38 ± 0.26	4.01 ± 0.16
NH_4^+	3.00 ± 0.30	4.10 ± 0.25*

All values are expressed in micromoles/gm of fresh tissue (±S.E.M).

N = 7.

*Statistically significant difference between groups.

Table III. Energy charge and changes in concentrations of the adenylate pool and related metabolites in white muscle of carp under two well defined conditions: resting and maximally active.

	Resting	Maximally active	Difference
Adenylate pool	5.1	2.68	-2.48
IMP	1.38	4.01	+2.63
NH_4^+	3.00	4.10	+1.10
Energy charge	0.89	0.83	-0.06

Adenylate pool, IMP and NH_4^+ are expressed in micromoles/gm of fresh tissue. N = 7.

Table IV. Concentrations of Krebs cycle intermediates and related metabolites in white muscle of carp under two well defined conditions: resting and maximally active.

Metabolite	Resting	Maximally active
Citrate	0.50 ± 0.06	0.34 ± 0.04
Malate	1.12 ± 0.28	1.16 ± 0.09
Oxaloacetate	Undetectable	Undetectable
α-ketoglutarate	<0.10	<0.10
Aspartate	0.24 ± 0.03	0.14 ± 0.03*
Alanine	2.62 ± 0.46	3.12 ± 0.47

All values are expressed in micromoles/gm of fresh tissue (±S.E.).

N = 7.

*Statistically significant difference between groups.

Swimming Experiment Carried Out in Summer 1974

Results of a second study in which fish were also exercised at an intermediate speed are shown in Table V. Although it is now well recognized that white muscle is active at intermediate speeds, these data, however, are difficult to interpret since the degree of activity of individual fibers is unknown. For this reason the experiment was terminated after only a few individuals were sampled. In light of the paucity of results all of the raw data are presented. Even though the data are limited they are interesting in a number of respects. The concentration of lactate in the control and maximally exercised groups was similar to that found in the spring study. Lactate reached a maximum of about 12 μmoles/gm in both swimming studies. Lactate concentration in white muscle sampled from fish worked at moderate speeds was in most cases intermediate in level relative to that found in resting and maximally active muscle. NH_4^+ concentration increased with activity and in one individual case reached 95 μmoles /gm. The level of NH_4^+ in the summer sampled fish was 5-6 times greater than that in the spring sampled animals. As expected both ATP and ADP generally decreased with activity, the lowest levels occurred in the maximally exercised group. There was a tendency for AMP to increase in the most strenuously worked muscle. It is interesting to note that the energy charge remained high and relatively constant at all three work loads. The level of IMP was highest in the maximally exercised group although there was much individual variability in this component. The high content of IMP in the one rested fish is not unexpected in light of the low level of ATP; however, the low level of IMP in two of the fish exercised at intermediate speeds remains an enigma. This finding may be due to experimental error since in all other cases the level of the adenylate pool and the concentration of IMP are inversely proportional.

Table V. Concentrations of metabolites and the energy charge value in white muscle of carp after various levels of activity.

Level of activity	Animal number	Metabolite						Energy charge
		Lactate	NH_4^+	ATP	ADP	AMP	IMP	
Rested	1	1.70*	16.96	5.17	1.01	0.02	0.78	0.92
	2	3.26	16.52	2.31	0.72	0.02	2.59	0.87
Intermediate	3	2.36	25.26	3.99	0.93	0.03	0.38	0.90
	4	4.54	23.13	1.98	0.49	0.02	0.52	0.89
	5	7.17	19.12	2.77	0.70	0.02	1.24	0.89
Maximally active	6	9.90	25.11	1.62	0.67	0.06	3.29	0.83
	7	12.66	95.10	1.07	0.64	0.14	4.06	0.75

*All values expressed in µmoles/gm fresh tissue.

Hypoxia Experiment

The concentration of lactate in white muscle of carp exposed to severe environmental hypoxia was about 12 μmoles/gm (Table VI). This value was similar to that found after maximal activity by white muscle. The content of lactate in the control fish of the hypoxia study was almost as high as that of the experimentals. In light of previous observations (Tables I and V) that the lactate level in white muscle of rested fish was about 3 μmoles/gm, it is probable that the experimental fish, in the present study, were subjected to some degree of anaerobic stress. Nevertheless, many positive conclusions may be made from this work.

It has been clearly shown that the volatile acids, acetate, proprionate, butyrate, or valerate (Table VI) were not produced as anaerobic end products in the white muscle of carp. Accumulation of formate would not be detected with the analytical techniques employed here; however, using an enzymatic assay it had been shown that this acid was not produced in white muscle of carp during strenuous exercise. Furthermore, the data show that succinate was not a quantitatively important anaerobic end product in carp white muscle.

The content of free amino acids may be found in Table VII. Since the control animals were, on the basis of lactate concentration, subjected to some degree of anaerobic stress, any alteration in the free amino acid pool will be minimal and consequently difficult to pick up. Regardless, it may be said with some confidence that no one amino acid was a quantitatively important anaerobic end product such as alanine is in invertebrate animals. No single amino acid was markedly altered by the hypoxic conditions; although, there was a tendency for an increase in the total free amino acid pool and all of the amino acids with the exception of glycine. The most significant finding in relation to amino acid metabolism was the amount of nitrogen that was

locked up in the free amino acid pool. This increased by about 6 μmoles/gm (Table VII) and was in agreement with the tendency for free NH_4^+ to decrease (Table VI) and the total free amino acid pool to increase. A lack of 1:1 stoichiometry between the increase in amino acids and the increase in nitrogen incorporated into the amino acid pool occurs since histidine, lysine, and arginine contain more than 1 nitrogen atom each. The amount of nitrogen incorporated into the free amino acid pool was far in excess of the decrease in NH_4^+. When these findings are considered along with the lack of production of volatile acids it may be safely concluded that there was not an active and general amino acid fermentation. Glycine may, however, be an exception to this general rule since it was the only amino acid to decrease during hypoxia.

Table VI. Metabolite concentrations in carp white muscle before and after hypoxic stress.

	Control		Hypoxic	
	Mean	Range	Mean	Range
Lactate	9.60*	6.23-13.50	12.02	6.90-15.12
NH_4^+	4.32	2.18-8.24	3.09	1.90-5.63
Succinate	<0.30		<0.30	
Acetate Proprionate Butyrate Valerate	$<0.2 \times 10^{-3}$		$<0.2 \times 10^{-3}$	

*All values are expressed in µmoles/gm of fresh tissue. N = 4.

Table VII. Free amino acid concentration in carp white muscle before and after hypoxic stress.

	Control		Hypoxic	
	Mean	Range	Mean	Range
Asp	0.15*	(0.08-0.21)	0.09	(0.07-0.11)
Thr	0.47	(0.21-0.70)	0.48	(0.28-0.63)
Ser-Gln	0.73	(0.46-1.09)	1.00	(0.76-1.42)
Glut	0.22	(0.11-0.31)	0.26	(0.19-0.32)
Prol	0.17	(0.08-0.23)	0.25	(0.25-0.37)
Gly	6.17	(3.69-9.82)	5.42	(4.31-6.94)
Ala	0.84	(0.42-1.08)	1.41	(0.80-1.99)
Val	0.29	(0.14-0.48)	0.44	(0.29-0.69)
Met	0.08	(0.05-0.12)	0.11	(0.09-0.16)
Isoleu	0.26	(0.12-0.43)	0.38	(0.21-0.38)
Lec	0.37	(0.23-0.57)	0.55	(0.34-0.83)
Tyr	0.06	(0.02-0.11)	0.09	(0.07-0.12)
Phe	0.08	(0.04-0.12)	0.33	(0.08-0.73)
Lys	1.22	(0.64-2.12)	1.97	(0.63-3.40)
Hist	3.71	(2.83-4.66)	4.74	(3.56-6.02)
Arg	0.10	(trace-0.16)	0.23	(0.13-0.32)
Total amino acids	14.93	(13.12-18.18)	17.75	(17.31-18.67)
Nitrogen in amino acid pool	23.74**	(21.16-26.24)	29.90**	(27.63-31.92)

*All values are expressed in μmoles/gm fresh tissue. N = 3.

**Statistically significant difference between means.

CHAPTER IV

DISCUSSION

Control of Glycolysis

The situation with respect to the control of glycolysis in white muscle seems relatively straight forward. Concentrations of glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-diphosphate and lactate rise during the exercise period indicating that, as expected, the glycolytic contribution to energy production is increased during high work rates. Activation of the two key regulatory enzymes of glycolysis, phosphofructokinase and pyruvate kinase, may be explained on the basis of the known kinetic properties of these enzymes. Thus substrate and product activation (Freed, 1971) of phosphofructokinase (by fructose-6-phosphate and fructose-1,6-diphosphate, respectively) with concomitant fructose-1,6-diphosphate feed forward activation of pyruvate kinase, commonly observed in fish muscle pyruvate kinases (Somero and Hochachka, 1968; Mustafa et al, 1971), could readily account for the observed increase in glycolytic rate. Moreover, deinhibition of these two enzymes would be expected as a consequence of falling levels of ATP (Freed, 1971; Mustafa et al, 1971), and of creatine phosphate (Storey and Hochachka, 1974), both processes being facilitated by fructose-1,6-diphosphate. In these control characteristics, carp white muscle glycolysis appears to be similar to other more commonly studied systems. Two inconsistencies with the literature, however, deserve mention. Firstly, it is evident from the data that the energy charge is essentially identical at both levels of muscle metabolism and muscle work. Although in vitro both phosphofructokinase (Shen et al, 1968) and pyruvate kinase (Purich and Fromm, 1973) are stimulated by a decrease in this parameter, in vivo it is clear that energy charge plays only a modest role in sustaining the high glycolytic rates that support extreme muscle work. Secondly, there is no evidence whatever that AMP constitutes an uniquely important metabolite

signal to glycolysis in white muscle, as suggested by Newsholme (1972) for heart, because its concentration is similar at the widely differing glycolytic rates. In contrast, if there is a single adenylate signal that is important to a sustained high level of glycolysis it presumably is ATP, since its overall concentration change is the greatest. However, as shall be argued later, in order to take advantage of this metabolic "signal" the organism must tolerate an overall reduction in the adenylate pool.

Lactate and Other Potential End Products

It is interesting to note that in the three individual experiments reported here the maximal level of white muscle lactate is consistently about 12 µmoles/gm. However, when carp white muscle was electrically stimulated until the muscle itself fatigued, lactate reached 33 µmoles/gm (Wittenberger and Diaciuc, 1965). The present data may indicate an upper limit that lactate approaches in vivo for this species. Data which support the concept of a limit to which lactate is allowed to normally accumulate have been obtained with rainbow trout. Thus, in trout strenuously exercised for 5 minutes muscle lactate increased from 3 to 47 µmoles/gm. Yet in animals sampled after 9 and 15 minutes of strenuous exercise, there was no further increase in muscle lactate above that found in the animals worked for 5 minutes (Black et al, 1962). Stevens and Black (1966) and Hammond and Hickman (1966) also obtained results of a similar nature with trout.

All of the available biochemical and histological evidence suggests that carp white muscle is particularly well designed for anaerobic metabolism. In fact, on the basis of hypoxia studies, one would normally consider carp to be a "good anaerobe". The extreme resistance of this animal to low O_2 environments was demonstrated by Mazeaud (1973) who induced anoxia in carp by expos-

ing them to air for periods up to 2 hours with only infrequent short returns to water. Even at temperatures as high as 10°C the lower lethal level of dissolved O_2 for carp was observed to be about 0.5 mg/l (≃5% air saturation) (Downing and Merkins, 1957). Species closely related to carp also show an inordinate tolerance to hypoxia. For instance, Basu (1949) found no deaths in goldfish held for 9 hours in water with an O_2 content of only 0.6 mg/l at 28°C. This particular species can in fact survive total interruption of oxidative phosphorylation by cyanide poisoning (Fry, personal communication). A further extreme case is demonstrated by Crucian carp which live in small ponds that become ice locked, gradually grow anoxic and remain O_2 free for up to 2 months (Blazka, 1958). These findings are very much different from those that have been obtained with many other fish species. For instance, the salmonids, which are the most actively studied, have been repeatedly shown to succumb between 1 and 2 mg/l dissolved O_2 (see Doudoroff and Shumway, 1970, for numerous references). Studies of this nature, of course, do not provide evidence of the anaerobic capacity of white muscle per se. However, it is possible to estimate the anaerobic capabilities of the swimming musculature with forced exercised experiments.

The aerobic efficiency of a working muscle, that is the energy converted to useful work/energy available from consumed oxygen, is considered to be about 20-30% (Hill, 1950); any values higher than this are thus indicative of anaerobic metabolism. The percentage aerobic working efficiency of swimming goldfish (Figure 3) has been determined by Smit et al (1971). At low swimming speeds the goldfish demonstrates a low aerobic efficiency, as the animal swims faster the percentage efficiency increases, and at velocities above 6 lengths/sec the working efficiency is greater than can be accounted for by aerobic means alone.

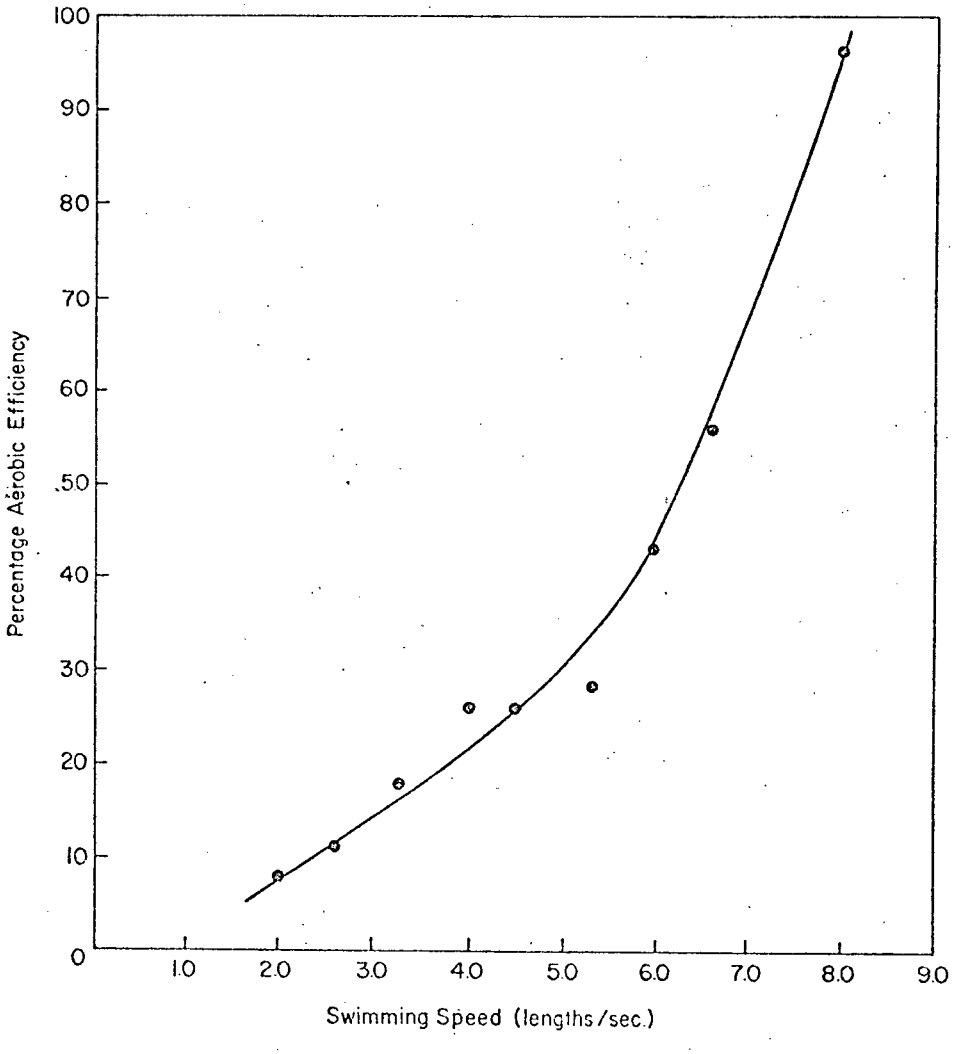
At the highest speeds the goldfish can attain the working efficiency reaches almost 100%. Thus, it may be said with some certitude that much of the energy required for intense swimming by the goldfish is generated by anaerobic means and in the extreme case approaches 80% of the energy output of the animal. In a comparable study employing rainbow trout, Webb (1971) has shown that the overall contribution of anaerobic metabolism to intense swimming is negligible. Yet in trout, exercised strenuously for only a few minutes, the concentration of muscle lactate increases to 40-50 μmoles/gm from a resting value of about 3 μmoles/gm (Black et al, 1962; Stevens and Black, 1966; Hammond and Hickman, 1966). Even in mammalian muscle, the content of lactate normally reaches 35 μmoles/gm during activity (Edington et al, 1972). Since carp white muscle is apparently capable of performing high levels of anaerobic work one would predict that it would also produce high amounts of lactate. Yet, despite the anaerobic capabilities of carp white muscle, lactate accumulation in this tissue is low by vertebrate standards.

The phenomenon of a lack of proportionality between the amount of energy which must be generated anaerobically and the accumulation of lactate has been observed for other species. For instance, Blazka and Kopecky (1961) claimed that after 4 hours of anoxia the Crucian carp accumulated only 0.5 μmoles of lactate/gm. During hypoxic excursions (ending in anoxia) bullheads accumulated only about 0.7 μmoles lactate/gm of muscle; by comparison lactate levels increased by about 15 and 30 μmoles/gm in rainbow and brown trout, respectively, although the hypoxic stress in the latter two cases was much less severe (Burton and Spehar, 1971). Clearly, white muscle of some species has a high anaerobic capability but it does not accumulate an extraordinary amount of lactate. It is known that lactate is not excreted during anaerobic work

(Prosser et al, 1957) and in light of peripheral vasoconstriction during hypoxia (Satchell, 1971) it is unlikely that there could be an effective deposition in other tissues such as the liver. These findings therefore suggest that something is yet unanswered about the manner in which carp white muscle deals with low oxygen availability.

The data of the present study show that carp white muscle is not the site of metabolic pathways which have evolved in facultative anaerobes. The production of volatile acids anaerobically by trout (Blazka, 1958) and Crucian carp (Blazka and Kopecky, 1961) had been suggested; however, this finding was refuted for muscle and liver by Burton and Spehar (1971) who considered Blazka's finding to be an artifact. This study confirms the work of Burton and Spehar (1971) at least for muscle; however at the present time the formation of these products in tissues other than muscle and liver cannot be ruled out. The failure to find volatile acids argues strongly against the possibility of an active amino acid fermentation, since during anaerobic work in invertebrates it is believed that volatile end products are derived from the catabolism of amino acids (Hochachka et al, 1973). The possibility of an active amino acid fermentation is further negated by the observation that during hypoxia there is a tendency for the free amino acid pool to increase not decrease. Furthermore, there is not a reorganization of the amino acid pool which could indicate a preferential utilization of some amino acids. Glycine may be an exception to this rule since there is a tendency for this amino acid to decrease during hypoxia. It is known though that fish have an active glycine anabolism under both aerobic and hypoxic conditions (Demael-Suard et al, 1974) and that under some circumstances the concentration of free glycine in carp white muscle may be as high as 30 μmoles/gm (Creach, 1966). Certainly the role of glycine

Figure 3. The percentage aerobic swimming efficiency versus swimming speed of goldfish. Aerobic efficiency is defined as energy required to develop power/energy available from consumed oxygen. The graph is plotted from the data of Smit *et al* (1971).


100
90
80
70
60
50
40
30
20
10
0
Percentage Aérobic Efficiency
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
Swimming Speed (lengths/sec.)

metabolism should be the subject of future work. This study also shows that succinate is not a significant anaerobic end product in fish white muscle whereas in the oyster heart even after a short anoxic period it reaches 5 μmoles /gm (Collicut, personal communication). In the invertebrates, succinate is formed anaerobically by a reversal of the Krebs cycle. One would expect this to occur only under conditions of complete anoxia; this was not the case in the present study, and it is doubtful whether this ever occurs as a normal course of events in the carp.

In conclusion, it would be of extreme interest to ascertain if under anaerobic conditions glycogen is quantitatively converted to lactate in carp white muscle. Stevens and Black (1966) have provided statistical evidence that lactate is the sole end product of anaerobic glycogen catabolism it trout; however, similar data do not exist for any other species.

$\underline{NH_4^+}$ Levels

The results of the swimming experiment carried out in spring 1974 show quite conclusively that the adenylate pool is a source of anaerobic NH_4^+ production in carp white muscle. In that particular experiment, the increase in NH_4^+ content in muscle during activity was less than the adenylate pool decrease or IMP increase. One explanation for the lack of 1:1 stoichiometry between IMP and NH_4^+ increase is that some NH_4^+ is being released into the blood. This would provide an explanation for the observed phenomenon of anaerobic NH_4^+ production by swimming fish (Kutty, 1972). However, on the basis of the hypoxia study, there appears to be another fate of NH_4^+ released from the free adenylate pool. This aspect shall be discussed under amino acid metabolism.

The NH_4^+ content of carp white muscle sampled in the exercise experiment

carried out in summer 1974 is most intriguing. The values are extremely high and in fact appear to be the highest ever reported in the literature for skeletal muscle. It is well known that carp can mobilize their muscle proteins to serve as an energy source (Creach and Serfaty, 1974) and this is probably what the animals in the present study are doing. This concept is supported by the observation that NH_4^+ content increases quite markedly during activity. The data further suggest that carp muscle has the capacity to totally utilize some amino acids directly as an energy source *in situ* and that prior conversion to carbohydrates in other tissues is not necessary. It is not clear why the animals were mobilizing their protein stores in the present study. One possibility is that even though the fish were fed on a daily basis, the supplied diet may have been inadequate. Otherwise these fish may undergo a seasonal switch in fuel source as is thought to occur in salmonids during smoltification (Saunders, personal communication). Clearly this problem warrants further consideration.

Adenylate Pool Size

The stoichiometric relationship between adenylate pool depletion and IMP accumulation clearly shows that the adenylate pool is reduced by the reaction catalyzed by 5' AMP deaminase. The regulatory nature of this enzyme from carp white muscle has been well characterized (Fields, personal communication; Purzycha-Preis and Zydowo, 1969). The enzyme is activated by ADP (K_a about 0.5 mM) and potently inhibited by GTP (guanosine triphosphate) (K_i about 50 μM). From the present study the enzyme appears to be controlled largely by the removal of GTP inhibition. GTP levels are initially low in fish muscle (Jones and Murray, 1960; Gras *et al*, 1967) and as demands for high energy phosphates increase during activity GTP levels must fall, for GTP is only formed in

essentially two ways, firstly by transphosphorylation with ATP and secondly by the Krebs cycle reaction catalyzed by succinate thiokinase. When ATP levels are reduced the rate of GTP production by the former reaction must also be reduced. Furthermore, as energy demands are placed on white muscle, glycolysis is activated far more than Krebs cycle activity (Wittenberger and Diaciuc, 1965), consequently the proportion of triphosphorylated nucleotides that GTP represents must decrease. In fact, Jones and Murray (1960) were unable to detect GTP in muscle of fatigued cod.

Be that as it may, the question still remains of the physiological significance of the reduced adenylate pool during high muscle work rates. One simple explanation may be that 5' AMP deaminase functions in concert with the adenylate kinase reaction to maximize ATP production by a mass action effect. However, there may be a more important thermodynamic explanation for the observation. Thus, as ATP levels drop during muscle work the ratio change of $[ADP][P_i]/[ATP]$ could drastically reduce the free energy of ATP hydrolysis according to the following relationship:

$$\Delta G = \Delta G^{o} + RT \ln \frac{[ADP][P_i]}{[ATP]}$$

During muscle work, control of this ratio may become increasingly difficult since not only is there a change in the ADP/ATP ratio, there also occurs an increase in P_i concentrations (Hammond and Hickman, 1966). These considerations emphasize that in the absence of external controlling mechanisms large drops in ATP concentrations could not be tolerated because they would occur concomitantly with increasing ADP levels of comparable magnitude, a situation that is clearly prevented. In this connection the regulation of energy charge may be less critical to glycolytic control than to the maintenance of a suit-

able relationship between ADP and ATP levels. That relationship could be adjusted by the concerted action of adenylate kinase and AMP deaminase, the AMP formed from the adenylate kinase reaction being removed by AMP deaminase in order to minimize ADP accumulation.

Amino Acid Metabolism

The results of the hypoxia study show that during anaerobic metabolism nitrogen is incorporated into the free amino acid pool. The source of this nitrogen is most likely free NH_4^+ liberated from the adenylate pool. The only known mechanisms for fixing free NH_4^+ in muscle are by the reactions catalyzed by glutamate dehydrogenase and glutamine synthetase. The latter enzyme catalyzes the formation of glutamine from glutamate and NH_4^+; however, since the concentration of glutamine is quite low in fish muscle this can be ruled out as a major nitrogenous sink. It thus seems likely, that glutamate dehydrogenase, which has been shown to occur in fish muscle (McBean et al, 1966), functions to fix free NH_4^+ into the amino acid pool by the following reaction:

$$NH_4^+ + \alpha\text{-ketoglutarate} + NADH \longrightarrow \text{glutamate} + NAD^+$$

Carp white muscle has a very high capacity to utilize amino acids for energetic purposes under aerobic conditions (Creach and Serfaty, 1974). Thus, given an active aerobic catabolism of amino acids there must be at any given time a pool of partially oxidized products. A wide spectrum of glutamate transaminase activity has been demonstrated in fish muscle (Siebert et al, 1964) and it is probable that the small increase in a number of amino acids during anaerobic metabolism is due to transamination of pre-existing α-ketoacids with glutamate. The glutamate dehydrogenase reaction could not only serve to maintain low NH_4^+ levels during anaerobic metabolism but may also confer an energetic advantage to the tissue since it would provide an additional method of oxidizing NADH.

Proposed Scheme of Nitrogen Metabolism

On the basis of the hypoxia and the swimming experiments it is possible to construct a fairly comprehensive metabolic scheme for nitrogen metabolism during anaerobic work in carp white muscle (Figure 4). The energy required for work is ultimately derived from the hydrolysis of ATP to ADP and P_i (reaction a). When ATP levels cannot be maintained by the energy production pathways the content of ADP increases, and as the ADP level increases ATP and AMP are formed by the adenylate kinase reaction simply because of a mass action effect (reaction b). As the work load on the tissue exceeds its aerobic capabilities GTP levels drop (reaction c and d), AMP deaminase is activated (reaction e) and the adenylate pool is decreased. This is possible since AMP, the substrate of the AMP deaminase reaction, is made available by adenylate kinase. NH_4^+ released from AMP is fixed into glutamate by glutamate dehydrogenase (reaction f) and is subsequently transferred to a variety of α-keto acids to form amino acids (reaction g). The question then remains as to how the adenylate pool is replenished in the recovery period following fatigue.

Lowenstein (1972) has proposed that the 5' AMP deaminase reaction is one step in a reaction span that is termed the purine nucleotide cycle (Figure 5). According to Lowenstein, IMP further reacts with GTP and aspartate to form adenylosuccinate. The adenylosuccinate in turn is converted to AMP plus fumarate. It has been shown in homogenates of mammalian skeletal muscle that the cycle functions in concert with glycolysis (Tornheim and Lowenstein, 1974). The theory, however, predicts only a transient increase in IMP with general maintenance of the adenylate pool. Clearly the cycle per se does not operate during activity in carp white muscle since there is an accumulation of IMP. Moreover, the cycle is not simply operating at a new steady state (i.e. at

altered levels of the adenylates and IMP) since during anaerobic metabolism in this tissue there is no alteration in the free amino acid pool. If the cycle were to become active only during recovery of the adenylate pool after exercise, the discrepancy between these results and those of Tornheim and Lowenstein (1974) would be apparent rather than real.

Since the enzymes of the purine nucleotide cycle apparently are present in white muscle (Fields, personal communication), it is probable that they supply a pathway for the regeneration of the adenylate pool from IMP during recovery following anaerobic work. Thus, in white muscle the reaction pathway shown in Figure 5 is a "cycle" only in a formal sense, because the two arms of the cycle are functionally separated in time. One arm, catalyzed by AMP deaminase, is formally a catabolic pathway leading to AMP hydrolysis; the other arm is formally an anabolic pathway leading to AMP formation during recovery. The control properties of AMP deaminase as well as adenylosuccinate synthetase, which catalyzes the formation of adenylosuccinate, are entirely consistent with this model. Thus, during white muscle work, AMP deaminase would be deinhibited due to dropping concentrations of GTP, and at the same time, GDP concentrations are presumably increased. GDP is a potent inhibitor of adenylosuccinate synthetase (Muirhead and Bishop, 1974) and this effect, coupled with reduced availability of one of its substrates (GTP), readily explains how this arm of the purine nucleotide cycle in white muscle is held at a reduced rate at the same time as AMP deaminase is being strongly deinhibited. The final aspect to the picture is the role of amino acids during the recovery period. The nitrogen that is stored in the amino acid pool during anaerobic work can be reincorporated into the adenylate pool by a series of transaminations. In this respect the fumarate produced by the purine nucleotide cycle would play an

integral role in providing oxaloacetate for the formation of aspartate with a variety of amino acids. This metabolic scheme makes good biological sense, however it remains to be tested.

Krebs Cycle Pool Size

There is no increase in four of the Krebs cycle intermediates even when white muscle is maximally active. It is thus apparent that this tissue has very little capacity if any to augment the size of its Krebs cycle pool in concert with increased energy demands. This is very much different from mammalian heart (Shafer and Williamson, 1973) or skeletal muscle (Edington et al, 1973) in which the concentration of Krebs cycle intermediates may be highly elevated during strenuous work. In heart burning glucose augmentation of Krebs cycle intermediates is fully accounted for by aspartate depletion. Aspartate is transaminated with α-ketoglutarate to form oxaloacetate and glutamate. The glutamate then undergoes a second transamination with pyruvate to form alanine and regenerate α-ketoglutarate. Thus aspartate carbon appears as Krebs cycle intermediates and aspartate nitrogen appears as alanine (Shafer and Williamson, 1973). The same phenomenon may occur to a limited extent in working white muscle of fish since glycogen is the fuel source. This accounts for the small but significant decrease in aspartate and the tendency for alanine to increase (see Chapter V for an extended discussion of this point).

Figure 4. Depletion of the adenylate pool in white muscle during anaerobic work.

(a) $ATP \longrightarrow ADP + P_i$

(b) $2\ ADP \longrightarrow ATP + AMP$

(c) $GTP + ADP \longrightarrow GDP + ATP$

(d) succinyl CoA $\longrightarrow$ succinate

GDP $\rightarrow$ GTP

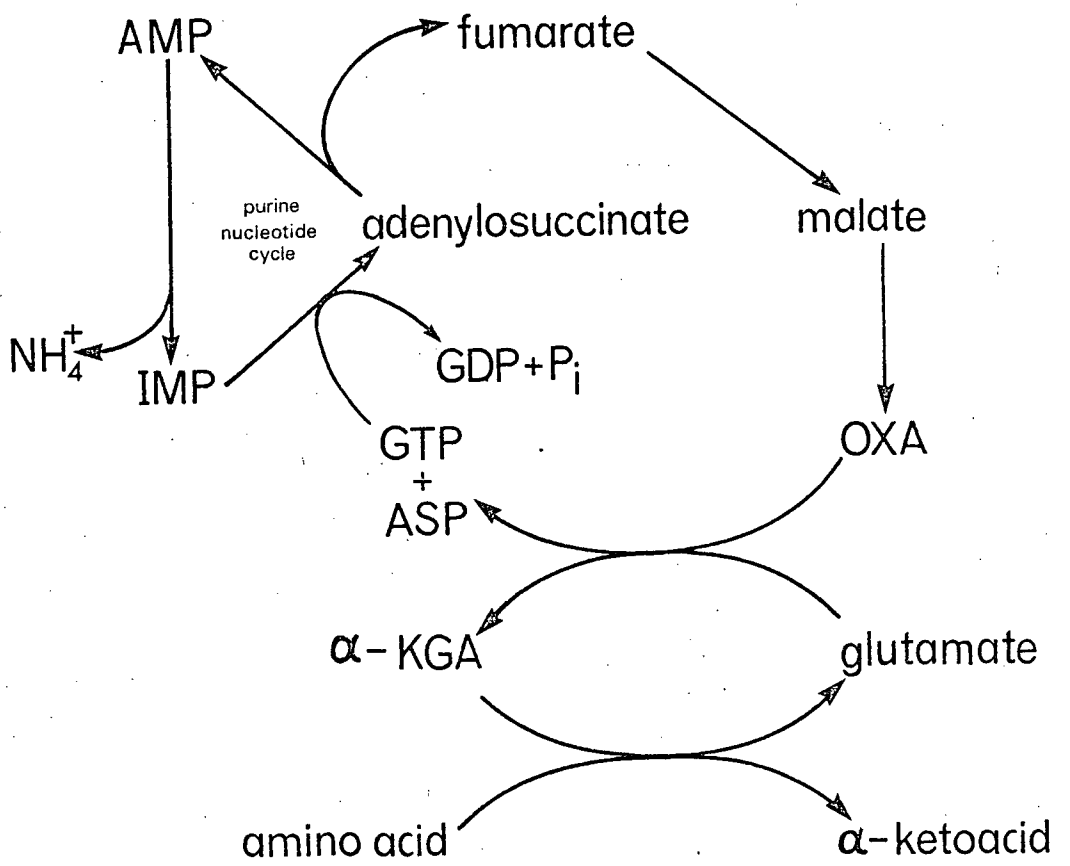
(e) $AMP \longrightarrow IMP + NH_4^+$

(f) $NH_4^+ + NADH + \alpha\text{-ketoglutarate} \longrightarrow glutamate + NAD^+$

(g) glutamate $\longrightarrow$ α-ketoglutarate

α-ketoacid $\rightarrow$ amino acid

Figure 5. Regeneration of the adenylate pool in white muscle during recovery from anaerobic work. Abbreviations not indicated in text: ASP, aspartate; α-KGA, α-ketoglutarate; OXA, oxaloacetate.


AMP
fumarate
purine
nucleotide
cycle
adenylosuccinate
malate
NH_4^+
IMP
GDP+P_i
GTP
+
ASP
OXA
α-KGA
glutamate
amino acid
α-ketoacid

CHAPTER V

CONCLUDING REMARKS: RED-WHITE MUSCLE DIFFERENCES AND THE FUNCTION OF THE PURINE NUCLEOTIDE CYCLE

The skeletal muscle of vertebrates is composed of characteristically different tissue types. At the extreme ends of the spectrum are the fibers which make up the red and white muscles. These two fiber types may be distinguished by numerous criteria such as the content of mitochondria, myoglobin, haemoglobin and oxidative enzymes, blood flow and O_2 consumption rate (see Chapter 1). It is generally accepted that red muscle functions largely aerobically utilizing fats or carbohydrates as its fuel source whereas white muscle has an extraordinary anaerobic component to its metabolism based upon glycogen utilization. In this respect the mammalian heart is very similar to red muscle (Keul et al, 1972). Meaningful studies at the metabolite level of mammalian red or white muscle are essentially impossible since the two fiber types exist in mixed bundles; however, in many fish species red and white muscle occur as discrete easily separable tissue masses. In the present study I have taken advantage of the unique distribution of muscle fibers in fish to elucidate the control of energy metabolism in white muscle alone. Furthermore, in considering the results within the framework of red-white muscle differences it has been possible to interpret some heretofore inexplicable findings in the literature.

Regulation of the Adenylate Pool Size

During strenuous activity by white muscle, when anaerobic metabolism is highly activated, there is an overall reduction in the adenylate pool content. This occurs because ATP concentration cannot be maintained over a certain minimum. However, when heart or red muscle is forced to work there is no decrease in the total level of adenylates since ATP content is maintained once a new steady state is attained (Neely et al, 1972; Gerez and Kirsten,

1965). But when either of these two tissues is subjected to hypoxia there is a decrease in the adenylate pool (Imai et al, 1964; Deuticke and Gerlach, 1966; Neely et al, 1973; Chaudry et al, 1974) just as occurs in white muscle. In skeletal muscle this is accomplished by activation of 5' AMP deaminase and the decrease in the adenylates is in 1:1 stoichiometry with either IMP increase or the sum of IMP and its degradation products (Imai et al, 1964; Deuticke and Gerlach, 1966). It is proposed that following recovery from anaerobic work the adenylate pool is restored by a reaction span known as the purine nucleotide cycle (see Chapter IV, Figure 5II, 2). In heart, however, the adenylate pool is reduced by 5' nucleotidase which catalyzes the conversion of AMP to adenosine plus ribose-5-phosphate (Rubio et al, 1973). Regardless, in all three tissues the phenomenon is the same: thus, when the anaerobic component of metabolism is activated relative to the aerobic component there occurs a reduction in the size of the adenylate pool. But heart contains the enzymes of the purine nucleotide cycle (Lowenstein, 1972; Muirhead and Bishop, 1974) and moreover red muscle is rarely subjected to hypoxia in the normal course of events. The question then arises as to what other functions the purine nucleotide cycle has in the cell. A clue to the question comes from a recent study by Winder et al, (1974) in which it is shown that adenylosuccinase, the final enzyme in the purine nucleotide cycle, is tightly correlated with Krebs cycle activity and not glycolysis. However, before pursuing this relationship further it is necessary to describe a generally unappreciated aspect of Krebs cycle function.

Strategies of Krebs Cycle Activation

It is intuitively obvious that in the transition fromaa resting to a working state involving an increased rate of oxygen consumption there must be

a concomitant activation of the Krebs cycle. It is important to realize that the Krebs cycle can be activated in but two fundamental ways: (a) by increasing the turnover rate or the rate of "spinning", with no change in pool size of intermediates, or (b) by increasing the steady state level of Krebs cycle intermediates as well as increasing "spinning" rate. In the first instance, there is no change in the maximum catalytic potential of the cycle; in the second, there is an increase in catalytic potential of the cycle that is proportional to the augmentation of cycle intermediates. Change in Krebs cycle "spinning" rate is apparently achieved through tight metabolite regulation of key cycle enzymes. This aspect of Krebs cycle control is now fairly well described (Atkinson, 1968b; LaNoue and Williamson, 1971; LaNoue et al, 1972) and will not be emphasized here. Suffice to indicate, however, that in any given metabolic state, merely increasing acetylCoA availability can increase the "spinning" rate of the Krebs cycle at the initially low, basal levels of cycle intermediates. The main reason why this mechanism in itself is usually inadequate is a shortage of oxaloacetate. Basal levels of oxaloacetate are very low, on this there is widespread agreement. Actual values are difficult to estimate, because of compartmentation and of instability during extraction. The best available estimates, however, indicate that oxaloacetate concentrations may be as low as 1-5 μM (Shafer and Williamson, 1973; Garland and Randle, 1964; Williamson, 1965), and this low oxaloacetate availability severely limits the rate at which Krebs cycle spinning can be increased. In fact, at any given metabolic state, it is a relative unavailability of oxaloacetate that probably sets the basal rate of Krebs cycle spinning (LaNoue and Williamson, 1971; LaNoue et al, 1972) and therefore, it is generally agreed, large Krebs cycle activation requires

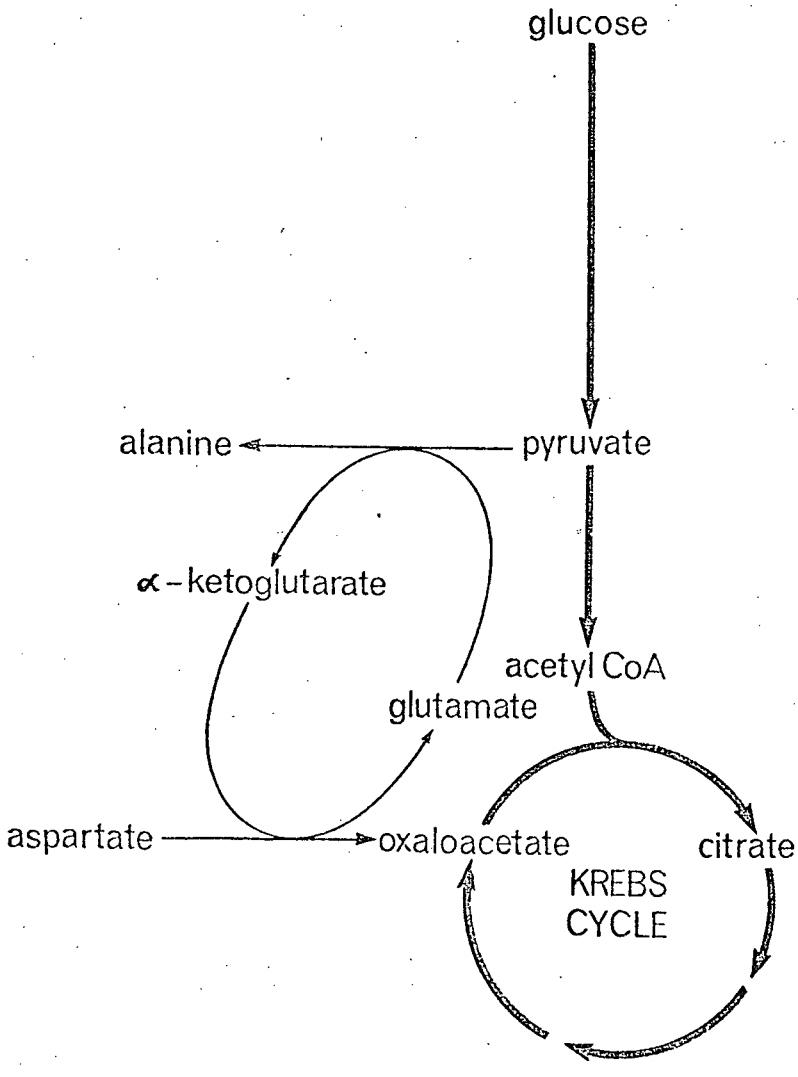
augmenting the cycle intermediates. How is this augmentation achieved?

Krebs Cycle Augmentation During Utilization of Carbohydrate

Mechanisms for augmenting the Krebs cycle intermediates are dependent upon whether carbohydrate or fat is being utilized as the fuel source. Let us first examine what occurs during the catabolism of carbohydrate as this process has been more fully studied. When glucose-derived pyruvate is being shuttled into the mitochondria to form acetylCoA, two problems temporarily arise: firstly, there is a requirement for more oxaloacetate to handle the newly formed acetylCoA, and secondly, a redox imbalance is created in the glycolytic path.

Both problems (the requirement for oxaloacetate and the redox imbalance) are solved by aspartate transaminase catalyzed mobilization of aspartate. This situation is particularly well documented in rat heart burning glucose (Shafer and Williamson, 1973), where the augmentation of Krebs cycle intermediates is fully accounted for by aspartate depletion. During the transition or activation period, aspartate-derived oxaloacetate is reduced to malate in the cytosol, a process that accounts for a large fraction of the required NAD for sustaining glycolysis (the other fraction coming from α-glycerophosphate and lactate dehydrogenases). The malate then moves into the mitochondria where it is reconverted to oxaloacetate, for sparking citrate synthase (Figure 6). Two points to emphasize here are (a) that aspartate carbon appears as Krebs cycle intermediates, and (b) that aspartate nitrogen appears as alanine because the aspartate transaminase reaction is coupled to alanine transaminase through the cosubstrates glutamate and α-ketoglutarate. The latter two are tumbled between aspartate and alanine transaminase in this situation and total alanine accumulation equals augmenta-

Figure 6. Augmentation of the Krebs cycle intermediates during utilization of carbohydrate as an energy source.


glucose
alanine
pyruvate
∝-ketoglutarate
acetyl CoA
glutamate
aspartate
oxaloacetate
citrate
KREBS
CYCLE

tion of Krebs cycle intermediates. Precisely the same mechanism occurs in the activation of metabolism in red muscle (Ruderman and Berger, 1974) and to a limited extent in white muscle. Under these conditions pyruvate derived from glucose is the unquestionable amino acceptor (Ruderman and Berger, 1974). Thus, by this simple process, the Krebs cycle is set at a new and higher catalytic potential for sustaining a prolonged work load.

The limited capacity of white muscle to augment the size of the Krebs cycle pool is reflected by the content of aspartate which in this tissue is only about 0.25 μmoles/gm. In heart, however, aspartate levels are much higher as is the capacity to increase oxaloacetate content (Neely et al, 1972). The aspartate content of red muscle, per se, is not known but it has been shown that during activity Krebs cycle intermediates in this tissue may increase to levels even higher than those found in heart (Edington et al, 1973).

In working white muscle there is a decrease in the adenylate pool with a concomitant increase in free NH_4^+. But in working heart or red muscle, there are no measurable changes in the total adenylate pool, although transitory changes in ATP, ADP, and AMP concentrations can occur (Neely et al, 1972; Shafer and Williamson, 1973). As far as the data currently indicate, no NH_4^+ is produced by working heart (Shafer and Williamson, 1973) or red muscle (Gerez and Kirston, 1965); the only form of nitrogenous "waste" product that accumulates is alanine and it is the only significant form of nitrogen carrier being released from muscle during aerobic glucose metabolism. Glutamine, an equally important nitrogen carrier under some conditions, is not released from heart or red muscle burning glucose unless the system is supplied with exogenous amino acids (Odessey et al, 1974). (In the latter case, glutamine

is formed by mechanisms discussed below.)

Glycolytic Inhibition During Fat and Amino Acid Catabolism

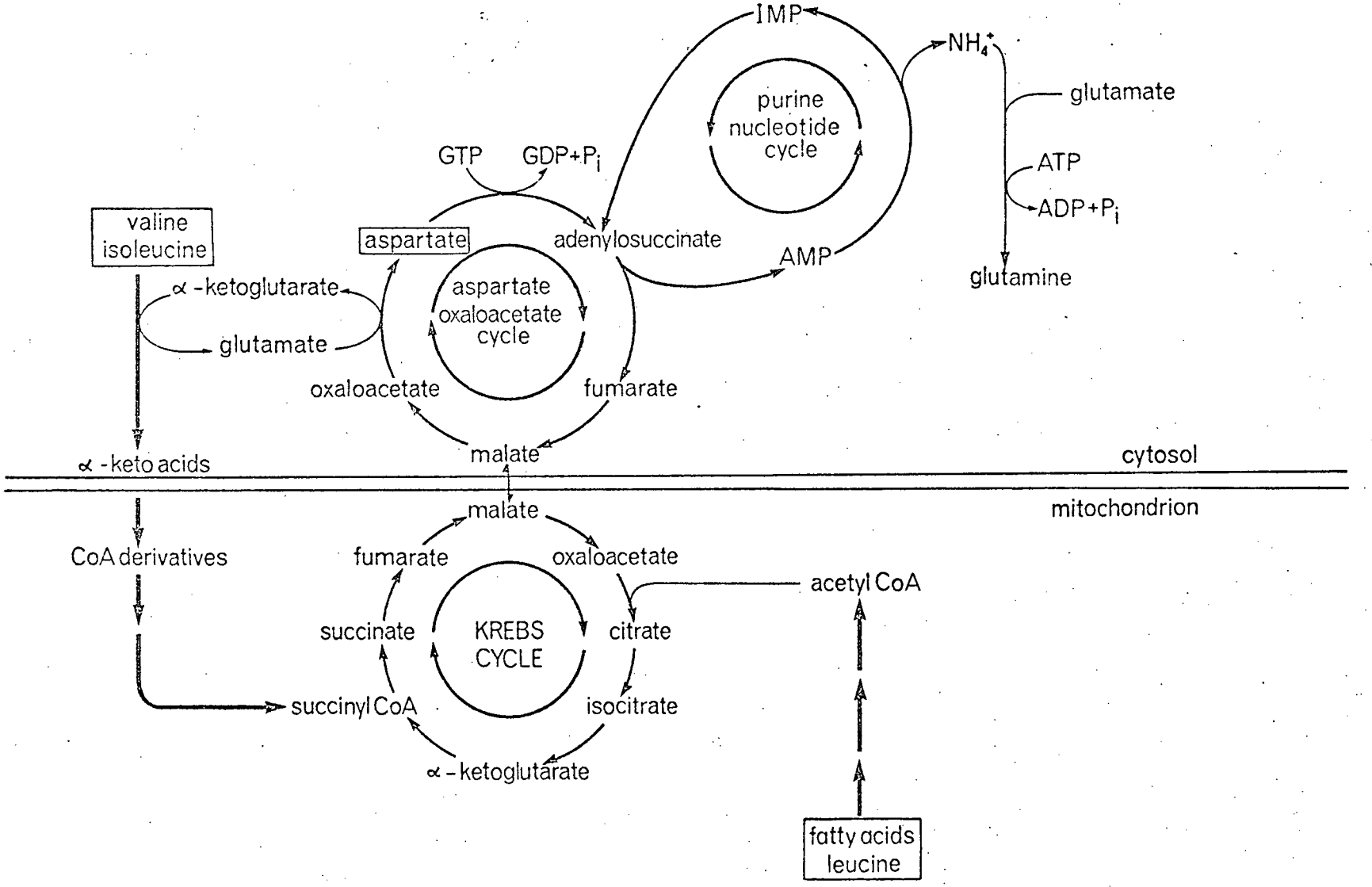
If a glucose-perfused heart is transferred to acetate as the chief exogenous carbon source, glucose utilization drops to nearly zero (Randle et al, 1970), and the same appears true during metabolism of palmitic acid (Randle et al, 1964) and of keto acids formed from valine, isoleucine, and leucine (Johnson and Connelly, 1972). These are crucial observations for they indicate that when fats or amino acids are oxidized, carbohydrate (the only major anaerobic fuel) is being "spared" by feedback inhibitory loops from mitochondrial metabolism to key steps in glycolysis. Although further details concerning these inhibitory interactions undoubtedly will be elucidated, it is already known that creatine phosphate, ATP, and citrate are all potential inhibitors of both phosphofructokinase and pyruvate kinase (Storey and Hochachka, 1974), and these singly or in combination are thought to dampen glycolysis during active fat oxidation. In addition, the α-keto acids formed from valine, isoleucine, and leucine are believed to inhibit glucose utilization by competing with pyruvate for pyruvate dehydrogenase (Johnson and Connelly, 1972). Whatever the mechanism for the inhibitory interaction between mitochondrial metabolism and glycolysis, the important point to bear in mind is that when fat is utilized, glucose becomes largely unavailable as a source of pyruvate. Thus, Krebs cycle priming by aspartate transamination to oxaloacetate, coupled to pyruvate transamination to alanine, as described above (Figure 6, 1), is a reaction span that may quickly run out of a key substrate (pyruvate) under conditions favouring fat metabolism. Not surprisingly, the amount of alanine formed in the heart under these conditions does not account for the augmentation of Krebs cycle intermediates (Randle et al,

1970). Whereas O_2 uptake increases by nearly 10-fold, alanine release from skeletal muscle increases by less than 2-fold (Felig and Wahren, 1971). Sometimes aspartate is depleted, but its depletion does not account for newly formed Krebs cycle intermediates (Randle et al, 1970). At other times, aspartate in fact may be accumulated during transition to active fat oxidation (Neely et al, 1972). What, then, is the source of Krebs cycle intermediates during activation of fatty acid oxidation?

Krebs Cycle Augmentation During Mobilization of Fat

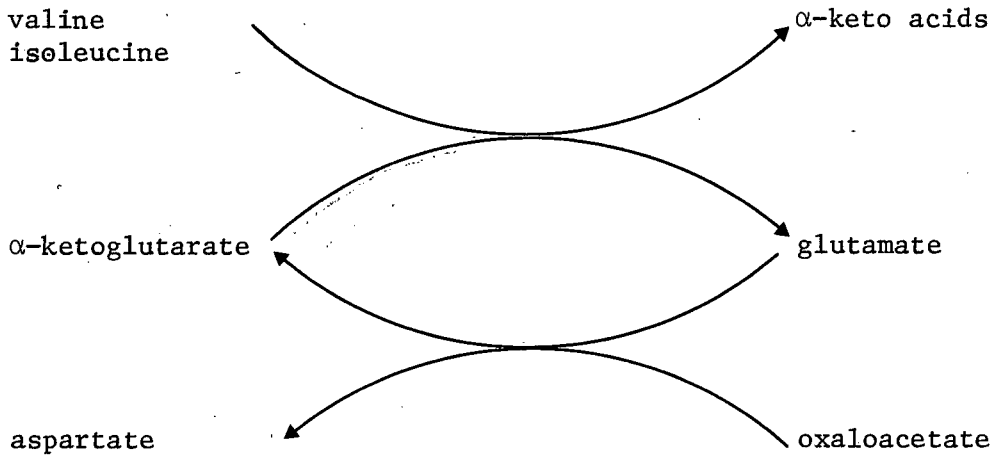
A key insight into the above question comes from a consideration of the interaction between fat and amino acid catabolism. In contrast to the inhibitory effects on glycolysis brought about by fatty acid or amino acid catabolism, the interactions between fatty acid and amino acid catabolism appear to be of a synergistic nature. Thus, the oxidation of amino acids, particularly valine, isoleucine, and leucine, is potently enhanced by fatty acids such as octanoate (Buse et al, 1972). During periods of transition from low to high rates of fatty acid oxidation, the catabolism of valine and isoleucine feeds carbon into the Krebs cycle at the level of succinylCoA, while the carbon of leucine feeds into the Krebs cycle as acetylCoA. It appears that it is these amino acids, not aspartate, that prime the Krebs cycle during activation of fat metabolism. The first two (valine and isoleucine) increase the catalytic potential of the Krebs cycle by increasing the availability of oxaloacetate and generally augmenting the pool size of cycle intermediates, while leucine, along with fatty acids, increases the amount of oxidizable 2-carbon substrate (acetylCoA), and thus leads directly to an increase in Krebs cycle turnover rate (Figure 7) (2). This model explains (a) why leucine perfusion of mammalian muscle leads to a 5-fold drop in

Figure 7. Augmentation of the Krebs cycle intermediates during utilization of fat as an energy source.


IMP
NH4+
glutamate
purine nucleotide cycle
GTP
GDP+Pi
ATP
ADP+Pi
valine
isoleucine
aspartate
adenylosuccinate
AMP
glutamine
α-ketoglutarate
aspartate oxaloacetate cycle
glutamate
oxaloacetate
fumarate
malate
α-keto acids
cytosol
mitochondrion
malate
CoA derivatives
fumarate
oxaloacetate
acetyl CoA
succinate
KREBS CYCLE
citrate
succinyl CoA
isocitrate
α-ketoglutarate
fatty acids
leucine

valine released into the efferent circulation by that muscle (Ruderman and Berger, 1974), and (b) why exercise leads to a measurable uptake of valine and isoleucine (Felig and Wahren, 1971).

Whereas the carbon of valine, isoleucine, and leucine enters the Krebs cycle, the nitrogen appears first in aspartate, but ultimately in glutamine and to a lesser extent in alanine. Unlike muscle burning glucose (which releases alanine as the nitrogen carrier), muscle burning fat and/or amino acids, releases both glutamine and alanine, and of the two, glutamine appears the predominant form of "waste" nitrogen, removing from muscle 2-4 times as much nitrogen/mole of amino acid as does alanine. If muscle is perfused with leucine or valine, glutamine, as well as smaller amounts of alanine, are again released into the efferent flow (Ruderman and Berger, 1974). These data, therefore, are consistent with the following reaction scheme (see also Figure 6; 1):



In this view, glutamate and α-ketoglutarate tumble between aspartate transaminase and transaminases for valine, isoleucine, and leucine (Figure 7).

As a means for regenerating α-ketoglutarate required for mobilizing these amino acids, aspartate transaminase is favoured over alanine transaminase because of limiting availability of pyruvate; however, any alanine which is released from fat burning muscle is formed by alanine transaminase (Ruderman and Berger, 1974), the pyruvate for the reaction presumably arising from a residual glycolytic activity that can in fact be increased with exogenous glucose (Odessey et al, 1974).

The Aspartate-Oxaloacetate Cycle

Accordingly, cytosolic aspartate transaminase function during activation of fat catabolism is favoured in the direction of aspartate production; just the opposite, of course, occurs at this enzyme locus during aerobic glucose catabolism. The aspartate formed this way may accumulate to a new steady state level (Neely et al, 1972) or it may be depleted (Randle et al, 1970), but for a full appreciation of its fate and function we must first inquire as to the origin of glutamine. On this question, recent evidence (Hills, 1972; Ruderman and Berger, 1974) strongly indicates that glutamine formation in muscle burning various amino acids (including valine and leucine) is catalyzed by glutamine synthetase:

$$\text{glutamate} + \text{ATP} + NH_4^+ \longrightarrow \text{glutamine} + \text{ADP} + P_i$$

Clearly, a source of NH_4^+ is required for this reaction. As glutamate dehydrogenase is not present in heart or red muscle, it is widely accepted that the primary pathway for the controlled release of amino nitrogen in muscle tissue is the purine nucleotide cycle (Lowenstein, 1972).

Therefore, it is proposed that in red muscle or heart, the reaction steps of the purine nucleotide cycle function as a cycle during mobilization of amino acids, particularly valine and isoleucine. The aspartate initiating the

purine nucleotide cycle in fact can be regenerated from the fumarate formed by it, since fumarate can be converted to oxaloacetate. This scheme, termed the aspartate-oxaloacetate cycle, provides a source of oxaloacetate for the aspartate transaminase and in effect is a cyclic, catalytic mechanism (initiated by aspartate and reforming aspartate), that primes (a) the flow of nitrogen through the purine nucleotide cycle to glutamine, and (b) the flow of carbon from isoleucine and valine into the pool of Krebs cycle intermediates. For this reason, I predict that during muscle activation, depletion of valine and isoleucine should be inversely proportional to glutamine formation and release from muscle. For this reason, too, the augmentation of Krebs cycle intermediates should be inversely proportional to valine and isoleucine depletion.

In this view, whether or not aspartate is depleted, accumulated, or unchanged during transition to active fat oxidation appears to depend upon the availability of isoleucine and valine. Clearly some of the fumarate formed by the aspartate-oxaloacetate cycle could feed into, and be retained within, the pool of Krebs cycle intermediates; that amount should appear as an aspartate depletion. If isoleucine and valine reserves are adequate, however, aspartate may actually accumulate. In a balanced situation, clearly aspartate would neither be depleted nor accumulated. All three alternatives in fact have been observed (Neely et al, 1972; Randle et al, 1970), though never previously explained.

Thus, it appears that when red muscle and/or heart burn fat, activation of the Krebs cycle requires the simultaneous mobilization of amino acids, particularly valine and isoleucine. At least during the activation period, the "cogs" of the Krebs cycle appear to mesh with those of the aspartate-

oxaloacetate cycle which primes the flow of amino acid carbon into the Krebs cycle and the flow of amino nitrogen through the purine nucleotide cycle to glutamine (Figure 7). Several advantages accrue from this kind of metabolic organization. Firstly, the only major anaerobic source of energy (carbohydrate) is maximally "spared" during aerobic metabolism. Secondly, if one compares the energy yielded during valine (or isoleucine) priming versus that gained by the aspartate priming mechanism, about 10 times as much utilizable energy (in the form of ATP equivalents) is obtained (Krebs, 1964). Thirdly, this mechanism allows amino acids to be used in muscle both for priming the Krebs cycle as well as directly for energy production without muscle requiring a urea-cycle for handling "waste" nitrogen. Finally, for reasons that are still unclear, but probably involving a greater absolute augmentation of cycle intermediates, the maximum degree of Krebs cycle activation obtainable appears higher during fat oxidation than it is during glucose oxidation (Neely et al, 1972). In conclusion, it is interesting to note that heart muscle is capable of burning a variety of substrates, fatty acids being preferred in vivo (Neely and Morgan, 1974); red muscle fibers appear to be rather similar to the heart in this respect, whereas, as we have seen, white muscle displays a strong and unique dependence upon carbohydrate.

Summary

Much of the data in relation to energy metabolism in mammalian skeletal muscle is difficult to interpret due to the heterogeneity of this tissue. In the present study this problem has been circumvented by the utilization of a species of fish in which red and white muscle exist as discrete easily separable tissue masses. During transition to work in white muscle, as well as during hypoxic stress in red muscle and heart, there occurs a reduction in the total content of the adenylate pool. In skeletal muscle this is accom-

plished by activation of 5' AMP deaminase, the first enzyme in a reaction span known as the purine nucleotide cycle. Following recovery from anaerobic work the second arm of the purine nucleotide cycle is turned on, serving to regenerate AMP and hence the adenylate pool. However, the main function of the purine nucleotide cycle in red muscle and heart is related to aerobic metabolism.

When muscle undergoes a transition from a resting to a working state, there is an increase in the size of the Krebs cycle pool. The simplest method for this augmentation presents itself when carbohydrates are utilized as a fuel source; under these conditions, aspartate conversion to oxaloacetate, catalyzed by aspartate transaminase, serves to spark the Krebs cycle. Because aspartate transaminase is coupled to alanine transaminase, the increase in Krebs cycle pool size is quantitatively accounted for by alanine accumulation. When muscle burns fat, however, this augmentation mechanism is reduced in importance due to inhibition of glycolysis and to reduced availability of pyruvate. Under these conditions, the catabolism of valine and isoleucine is favoured and these appear to be the predominant sources of carbon for augmenting Krebs cycle pool size. Although the carbon of these branched chain amino acids appears as Krebs cycle intermediates, the nitrogen appears first in aspartate, but then is released as NH_4^+ by the purine nucleotide cycle; the NH_4^+ then serves as substrate for glutamine synthetase, in which circumstance glutamine becomes the primary means for removal of amino nitrogen from muscle. Thus, when muscle burns fat the purine nucleotide cycle functions to channel amino acids into the Krebs cycle.

CHAPTER VI

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APPENDIX I

BLOOD LACTATE LEVELS IN FREE SWIMMING TROUT BEFORE AND AFTER STRENUOUS EXERCISE RESULTING IN FATIGUE

INTRODUCTION

The myotomal musculature of fish is largely composed of two systems commonly referred to as the red and white fibers. The two fiber types may be distinguished in numerous ways including haemoglobin, myoglobin and mitochondrial content, vascular supply and enzymatic properties. On the basis of these characteristics it is generally accepted that red muscle has a metabolism that is aerobically based burning fats and/or carbohydrates whereas white muscle functions largely anaerobically utilizing glycogen with the concomitant production of lactate. During slow swimming the propulsive force is derived entirely from the red musculature. But at the highest swimming velocities the white muscle becomes maximally active and together with the red muscle supplies the power for locomotion. At some point in the transition from low to high swimming speeds, there is recruitment of the white fibers (see Introduciton to thesis for references).

It is well recognized that some fish can maintain swimming speeds just below their critical velocity for extended periods of time (Brett, 1964). During excursions of this nature new steady state levels must be attained and in conditions involving white muscle activity lactate cannot be allowed to accumulate in that tissue and hence must be eliminated. Numerous workers have found that blood from rainbow trout sampled by cardiac puncture on restrained fish after periods of moderate activity contained elevated levels of lactate (Black, 1957; Black et al, 1960; Black et al, 1966; Miller et al, 1959). In this study the lactate content of blood taken serially by indwelling catheters from individual rainbow trout (Salmo gairdneri) during swimming at well defined velocities is recorded. It is reported that under the present conditions, there is no marked increase in the concentration of blood lactate during the

swimming period at any time prior to fatigue; however, lactate concentration rises rapidly following fatigue.

The fate of lactate produced in the white muscle of fish remains an open question. In mammalian systems which have been better studied it is known that 80-90% of the blood lactate is oxidized to CO_2 and water (Drury and Wick, 1956); the site of oxidation being heart (Keul et al, 1972), skeletal muscle (Jorfeldt, 1970), liver (Rowell et al, 1972) and kidney (Levy, 1962). In resting conditions approximately 15% of the lactate is converted to glucose (Reichard et al, 1963) in the liver (Rowell et al, 1966); however, during activity this process (i.e. the Cori cycle) may be quantitatively more important (Keul et al, 1972). Black, on the basis of his studies with fish, was forced to conclude that in these animals the Cori cycle is of little importance (Black et al, 1966). Moreover, following anaerobic stress glycogen does not return to prestress levels even after 24 hours (Black et al, 1962; Heath and Pritchard, 1965), therefore it is probable that in fish, as with mammals, most of the blood lactate is oxidized to CO_2. Bilinski and Jonas (1972) have shown that the capacity to channel lactate through the citric acid cycle per unit weight of tissue decreases in the following order: gill, kidney, red muscle, liver, heart, white muscle. An attempt has been made to quantitate the in vivo significance of gill as a site of lactate utilization by sampling blood before and after its passage through this tissue. The results suggest that lactate is removed from the blood by the gills during the recovery period following strenuous activity.

MATERIALS AND METHODS

Animals

Female trout (Salmo gairdneri) (40-53 cm) were purchased from a commercial supplier (Trout Lodge, Ephrato, Wash., U. S. A.) and transported to U. B. C. by tank truck, where they were held in large cylindrical tanks (8000 litres). Constant inflow of fresh dechlorinated water (6-7^{o}C) was maintained at all times. Fish used for experiments were physically trained for periods of at least two weeks prior to use. Training was accomplished by transferring the fish to 2000 litre circular tanks in which the water was kept in motion by water jets driven by pumps. The water velocity varied from nearly 0 at the centre of the tank to 35-40 cm/sec at the circumference. The fish tended to swim constantly in the high water velocity zone. All fish were fed Clark's Trout Pellets six times weekly.

Cannulation Techniques

Dorsal aortic cannulation was accomplished by the method of Smith and Bell (1964) (MS222 anaesthesia) using a 50 cm length of PE60 tubing terminated at the proximal end with a 12 mm section of 21a Huber point needle. Ventral cannulation was accomplished using a cannula similar to the dorsal one except that the needle was 2 cm long and bent at 60^{o} 6 mm from the tip. This cannula was inserted into the ventral aorta through the tongue at the level of the third gill arch. The cannula was sutured to the tongue and extended out of the mouth. After partial recovery on the operating table (constant respiratory frequency) the fish was transferred to a water tunnel (Brett, 1964), of 126.5 cm^{2} cross-sectional area and 35 litre volume, to recover for a minimum of 18 hrs at a water velocity of 9 cm/sec.

Experimental Design

The fish were exercised in a series of 60 min stepwise increasing velocity increments, until fatigue occurred. Each velocity increment was about 0.25 lengths/sec. Blood samples, prior to fatigue, from four individual fish, were taken at min 60 of the test period. Fatigue was defined as the inability of the fish to remove itself from the electrified grid (10-20 v AC) at the downstream end of the tunnel. The water was maintained at 6-7°C and 100% air saturation. Critical velocity was calculated using the empirical formula of Brett (1964) such that the last velocity that the fish successfully maintained was added to the velocity at which the fish fatigued multiplied by the proportion of the 60 minute period that it was able to sustain this final speed. The mean critical velocity for the animals of this experiment was 1.6 lengths/sec.

Analytical Techniques

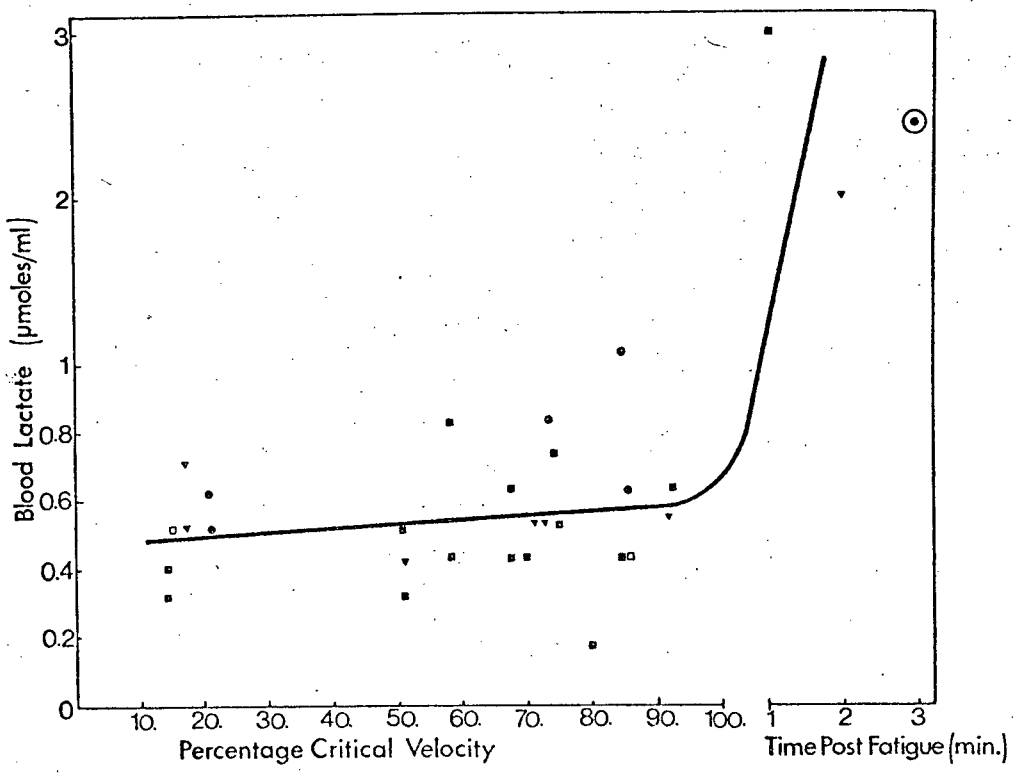
Blood samples (0.3-1.0 ml) were taken from the ends of the cannulae with a 1 ml Hamilton syringe. In some cases both arterial and venous samples were taken from the same animal. When this occurred the venous sample was obtained 1 minute before the arterial sample. Upon removal of the blood it was immediately diluted 1.0:3.5 v/v with cold 8% $HClO_4$. The sample was centrifuged to remove protein and the supernatant was neutralized with 3 M K_2CO_3 containing 0.5 M triethanolamine. $KClO_4$ was removed by centrifugation and an aliquot of the supernatant was analyzed for lactate enzymatically. Assays were carried out on a Unicam SP 1800 dual beam spectophotometer connected to a strip chart recorder. Arterial and venous blood lactate content were compared with the Wilcoxon test for paired observations and a probability of less than 0.05 was considered to be significant.

RESULTS AND DISCUSSION

There is no change in lactate concentration between arterial and venous blood when samples are taken prior to fatigue. In order to avoid duplicate samples from the same animal, only arterial blood lactate levels of individual swimming trout before and immediately after fatigue are shown in Figure 8. Clearly, there is no increase in blood lactate at any sustained swimming speed, even though, in many cases, the exercise level approaches the critical velocity and is maintained at that level for 60 minutes. On the basis of heart rate and ventilation frequency, following activity, on the animals of this experiment (Kiceniuk, personal communication) and other evidence cited in Chapter I, it appears that there must be white muscle involvement at least at the highest sustainable velocities. Webb (1971) was of the opinion that in trout, the white muscle comes into play at about 80% of the critical velocity, yet there is no increase in blood lactate at speeds up to 93% of the critical velocity. Since, in the present study blood was sampled only after a steady state level had been attained it must be concluded that the rate of elimination of lactate from white muscle is equal to the rate of utilization elsewhere. It is thought that blood flow to the liver is reduced during exercise (Satchell, 1971), thus it is unlikely that this tissue is a major site of lactate deposition. As indicated above, the gills are not a major site of lactate utilization during the exercise period. It is possible that lactate produced in the white muscle is further oxidized in the red muscle as Wittenberger and Diaciuc (1965) have suggested occurs in the carp. It would be of interest to ascertain if there are differences between trout and carp in their capacity to eliminate lactate produced in white muscle.

Following fatigue there is a rapid increase in blood lactate concen-

Figure 8. Blood lactate levels of individual swimming trout at specified swimming speed and following fatigue. Multiple points at a given percentage critical velocity are representative of repeat runs on different days. All blood samples taken from dorsal aorta except one represented by ⊛ which is from ventral aorta. The curve is a regression line drawn through all points prior to fatigue.


Blood Lactate (μmoles/ml)
3
2
1
0.8
0.6
0.4
0.2
0
10. 20. 30. 40. 50. 60. 70. 80. 90. 100.
Percentage Critical Velocity
1 2 3
Time Post Fatigue (min.)

tration to about 2.5 μmoles/ml (Figure 8; Table VIII). This represents a 4- to 5-fold increase over the level at the highest sustained speed. Elevated levels of blood lactate immediately following strenuous exercise have been repeatedly shown (Black et al, 1966; Stevens and Black, 1966; Hammond and Hickman, 1966). Furthermore, although the data over the recovery period are limited they fit the general pattern often described and discussed, of a rapid increase in blood lactate concentration immediately following anaerobic work, reaching a maximum in 2-4 hours and then slowly returning to normal (Black et al, 1962; Black et al, 1966; Hammond and Hickman, 1966). The fact that blood lactate increases to such a degree following activity indicates that although lactate is eliminated from white muscle during activity a large amount is also retained. The question remains as to how much lactate is eliminated relative to how much is allowed to accumulate during sustained swimming.

The post fatigue levels of blood lactate reported here are in agreement with the findings of others in absolute value and in the manner in which content increases and then decreases. However, it had been previously claimed that blood lactate levels in trout exercised at moderate speeds are 2-3 times higher than in unexercised fish (Black, 1957; Black et al, 1966; Miller et al, 1959). It is quite clear from Figure 8 that blood lactate does not increase markedly even at high sustained velocities. The dissimilarity between the present data and the data of others may be due to methodological procedures. In all previous studies blood was obtained by cardiac puncture whereas in the present work blood was sampled by indwelling catheters. The two techniques appear to yield similar results when blood is sampled after the exercise period; however, blood taken from fish prior to fatigue by cardiac puncture contains elevated levels of lactate. It is possible that in manipulating the

fish for cardiac puncture there is an increased cardiac output which flushes an increased amount of lactate out of the white muscle. Another unaccounted for parameter in sampling at moderate swimming speeds is the activity of the animal per se during the sampling period. Thus, blood lactate increases following cessation of exercise involving white muscle activity but does not increase when swimming is allowed to continue. Randall (personal communication) has suggested that following cessation of activity there may be a localized hyperaemia in the white musculature which causes a massive flushing out of lactate. This could explain not only the findings at intermediate velocities but also the general phenomenon of a rapid increase in blood lactate concentration following strenuous exercise. Whatever the answer may be, it is clear that this problem warrants resolution.

Table VIII lists the animals in which both arterial and venous blood samples were taken following fatigue. Of the eleven measurements made, eight showed a negative arterial-venous difference which on the average is about 0.4 μmoles/ml. It therefore appears that in the recovery period following strenuous exercise there is a net uptake of lactate from the blood during its passage through the gills. A 1000 gm trout has about 50 ml of blood and a circulation time of approximately 2 min (Randall, 1970). Thus the gills of a trout this size could take up on the average about 10 μmoles lactate/min. After entering the gill lactate must either be excreted or further metabolized. During exercise fish excrete a minimal amount of lactate (Karuppannan, 1972), but there is no reason to believe that following exercise this level increases. It is probable that any lactate taken up by the gills is reconverted to pyruvate, which may be further utilized in a variety of ways. Gill has the capacity to oxidize lactate totally to CO_2 and water (Bilinski and Jonas, 1972)

Table VIII. Arterial and venous blood lactate concentrations of rainbow trout following exercise to fatigue.

Fish	Time following fatigue (min)	Venous (ventral aorta)	Arterial (dorsal aorta)	A-V difference
1	1080	1.1*	1.0	-0.1
2	120	10.3	9.8	-0.5
3	1	3.3	2.8	-0.5
	30	5.4	5.6	+0.2
	60	8.4	7.1	-1.3
	660	1.5	1.8	+0.3
4	1	1.8	0.6	-1.2
	60	3.1	3.0	-0.1
	90	4.1	4.9	+0.8
	120	6.4	5.6	-0.8
	135	6.8	5.3	-1.5
Mean				-0.4

*Lactate concentration in μmoles/ml blood.

and the *in vivo* data of Rao (1968) indicate that the oxygen consumption of gill of a 1000 gm trout is great enough to completely oxidize 10 μmoles lactate/min. Furthermore, although studies on the intermediary metabolism of the fish gill are notably lacking, it is known that the crustacean gill has an extremely high gluconeogenic capacity (Thabrew *et al*, 1971). If the fish gill is at all similar to the analagous crustacean tissue, a 1000 gm trout could easily direct a large fraction of the lactate into glucose. In this respect it is interesting to note that fish gill has particularly high glycogen deposits which lie in close proximity to an abundant mitochondrial system (Conte, 1969). It may be that glycogen serves as the metabolic fuel for this tissue. It is also possible that there is a functional electron shuttle system between white muscle and gill; such that lactate formed in muscle is simply converted to pyruvate in the gill and subsequently returns to the muscle. In this case the gill would be functioning to oxidize NADH produced from the lactate dehydrogenase reaction. During a bout of strenuous activity a 1000 gm trout may accumulate 30,000 μmoles of lactate in its white muscle (Black *et al*, 1962; Stevens and Black, 1966; Hammond and Hickman, 1966). Since it then takes from 12-24 hours for blood lactate to return to normal it is possible that the gill plays a heretofore unrecognized role in the metabolism of this metabolite during the recovery period.

SUMMARY

Rainbow trout (Salmo gairdneri) were exercised in a series of 60 minute stepwise increasing velocity increments. There is no increase in blood lactate concentration at any time during the exercise period; even though, some of the animals were exercised at 93% of their critical velocity on a sustained basis. The data indicate that under these conditions the rate of production of lactate by white muscle is equal to its rate of utilization elsewhere. Immediately following fatigue blood lactate level rapidly increases. During the recovery period there appears to be a net uptake of lactate by the gills.

APPENDIX II

ENZYME NOMENCLATURE

adenylate kinase (E.C. 2.7.4.3)

adenylosuccinase (E.C. 4.3.2.2)

adenylosuccinate synthetase (E.C. 6.3.4.4)

alanine transaminase (E.C. 2.6.1.2)

AMP deaminase (E.C. 3.5.4.6)

aspartate transaminase (E.C. 2.6.1.1)

citrate lyase (E.C. 4.1.3.8)

citrate synthase (E.C. 4.1.3.7)

fructose-1,6-diphosphatase (E.C. 3.1.3.11)

glutamate dehydrogenase (E.C. 1.4.1.2)

glutamate-oxaloacetate transaminase (E.C. 2.6.1.1)

glutamate-pyruvate transaminase (E.C. 2.6.1.2)

glutamine synthetase (E.C. 6.3.1.2)

glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49)

α-glycerophosphate dehydrogenase (E.C. 1.1.1.8)

glycogen phosphorylase (E.C. 2.4.1.1)

hexokinase (E.C. 2.7.1.2)

lactate dehydrogenase (E.C. 1.1.1.27)

malate dehydrogenase (E.C. 1.1.1.38)

nucleoside phosphorylase (E.C. 3.2.2.1)

5' nucleotidase (E.C. 3.1.3.5)

phosphofructokinase (E.C. 2.7.1.11)

phosphoglucoisomerase (E.C. 5.3.1.9)

pyruvate dehydrogenase (E.C. 1.2.4.1)

pyruvate kinase (E.C. 2.7.1.40)

succinic thiokinase (E.C. 6.2.1.5)

tetrahydrofolic acid formylase (E.C. 3.5.1.10)

xanthic oxidase (E.C. 1.2.3.2)