METABOLIC REGULATION IN SKELETAL MUSCLE DURING EXERCISE: A FISH-MAMMAL COMPARISON

By

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We accept this thesis as conforming to the required standard

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ABSTRACT

The aim of the present investigation was to examine the control of anaerobic glycogenolysis in working and fatigued skeletal muscle. The two animals chosen for the study were a teleost fish and the laboratory rat. The rationale behind using a comparative approach to investigate fundamental questions on metabolic control resides in the different abilities of each animal to perform exercise, and to their markedly different myofibrillar organization.

In the process of defining the hierarchical recruitment of fuel and pathway selection in rainbow trout fast-twitch white skeletal muscle, it was clear that the near-maximal myosin ATPase activity was supported solely by PCr hydrolysis. It was not until the rate and force of contraction decreased that the relative contribution of anaerobic glycogenolysis became increasingly important. Despite glycogenolysis possessing a lower maximal ATP generating potential than PCr hydrolysis, it has the advantage of being less constrained by time, and is recruited to extend muscle performance, but at submaximal workloads. Demonstration of the same temporal pattern of activation was not attempted for rat skeletal muscle because of complex fiber heterogeneity.

The etiology of fatigue after 10 and 30 minutes of burst swimming in trout was due to the near depletion of glycogen in white muscle. Inhibition of anaerobic glycogenolysis was not correlated with limitations to either the availability of ADP or NAD⁺, or inhibition of
phosphofructokinase (PFK-1). Similarly, the onset of fatigue and inhibition of glycogenolysis in three different skeletal muscles (gastrocnemius, plantaris and soleus) of the rat after 30 min of endurance treadmill running (25 meters/min), was not related to ADP availability, but associated with the near-depletion of muscle glycogen. As the endogenous stores of glycogen became limiting, hexokinase (Hk) appeared to be activated in trout white muscle after 10 min, and in the three rat skeletal muscles after 30 min, indicating an increase in uptake and phosphorylation of blood-borne glucose.

In rats running at a high speed for 2 minutes, glycogenolysis was maintained through the coordination of glycogen phosphorylase and PFK-1. Muscle performance in these rats was maintained despite large percentage swings in cytosolic redox, the ATP/ADP ratio and phosphorylation potential. A common belief in the literature is that inhibition of glycogenolysis during short-term strenuous exercise is brought about by the pH dependent ATP inhibition of PFK-catalysis. Evidence was provided indicating PFK-1 is operational in skeletal muscle at about pH 6.6 for both the fish and the rat. Fish partially solved the problem of PFK-1 inhibition by lowering ATP, whereas the rat appeared to rely on the synergistic action of a number of positive modulators. A detailed kinetic analysis of purified rabbit muscle PFK-1 revealed that any modulator that increases the ratio of unprotonated to protonated form of the enzyme, could supply the muscle cell with a means of maintaining glycogenolytic flux despite falling pH.
A number of striking differences were apparent between the regulation of glycogenolysis in fish and rat skeletal muscle. The first major difference was the direction of change in cytosolic redox or the NAD$^+/\text{NADH}$ ratio. In fish white muscle the ratio increased, and the cytosol became more oxidized with exercise, whereas the opposite occurred in rat fast-twitch skeletal muscle. The difference was a consequence of lactate retention in fish white muscle. On the basis of crossover analysis, pyruvate kinase (PK) appeared to be activated in trout white muscle at both fatigue states. However, this was misleading, and also considered a consequence of rising pyruvate and due to lactate retention via the mass action effect at the LDH equilibrium. Obviously, the change in redox and the apparent crossover at PK are linked, and a literature survey revealed that during short-term maximal work, a mammalian skeletal muscle may indeed behave as fish white muscle. The other contrasting feature of this comparative analysis was the demonstration that ATP in trout white muscle can fall by 80% at exhaustion. No such large percentage reductions in ATP occurred in either of the rat fast-twitch skeletal muscles, or indeed have been reported in skeletal muscle of any other exercising animal. In all cases the total nucleotide pool remained constant.

A general conclusion to be drawn from this study is that muscle fatigue should be viewed as a multi-component process in response to limiting glycogen, and not leveled at any one particular step of the glycogenolytic pathway.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>Gly</td>
<td>Glycogen</td>
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<tr>
<td>Glu</td>
<td>Glucose</td>
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<tr>
<td>G 1-P</td>
<td>Glucose 1-phosphate</td>
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<td>G 6-P</td>
<td>Glucose 6-phosphate</td>
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<tr>
<td>F 6-P</td>
<td>Fructose 6-phosphate</td>
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<tr>
<td>F 1,6-BP</td>
<td>Fructose 1,6-bisphosphate</td>
</tr>
<tr>
<td>DHAP</td>
<td>Dihydroxyacetone phosphate</td>
</tr>
<tr>
<td>GAP</td>
<td>Glyceraldehyde 3-phosphate</td>
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<tr>
<td>1,3-DPG</td>
<td>1,3-diphosphoglycerate</td>
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<tr>
<td>3-PGA</td>
<td>3-phosphoglycerate</td>
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<tr>
<td>2-PGA</td>
<td>2-phosphoglycerate</td>
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<tr>
<td>PEP</td>
<td>Phosphoenolpyruvate</td>
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<td>Pyruvate</td>
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<td>Lact</td>
<td>Lactate</td>
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<td>umol/g wet wt</td>
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<td>nmol/g wet wt</td>
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CHAPTER I: GENERAL INTRODUCTION

A fundamental goal of the study of tissue metabolism is to elucidate the regulatory mechanisms that adjust metabolic rates to ATP turnover. Skeletal muscle provides an ideal system to investigate such control mechanisms because of its capacity to increase its metabolic rate by many orders of magnitude during rest to work transitions (Cori, 1956). Any systems analysis of metabolic control in skeletal muscle must bear in mind the following: (1) What factors govern the selection of the appropriate fuel at the appropriate time to support a given workload? (2) Which enzymes are important in control of flux, and how are they regulated? (3) How are the various metabolic pathways synchronized with each other in the cell, and what is the role of compartmentation, and (4) What factors limit flux and muscle performance?

At the outset, the aim of the present thesis dissertation was to critically re-examine the regulation of anaerobic glycogenolysis in different muscle types of fish and the rat during short-term high intensity exercise, and following endurance exercise to fatigue and exhaustion. In an effort to present a general introduction into the hierarchical control mechanisms involved in the modulation of glycogenolysis, I feel it first appropriate to briefly review relevant aspects of muscle contraction and the role of ATP.
THE CONTRACTILE CYCLE

Central to our understanding of muscle biochemistry is that each time the myosin S1 head undergoes a pull and release cycle with actin, one molecule of ATP is hydrolysed to ADP, Pi and H⁺ (Perry 1979, Eisenberg and Hill 1985). Each contraction-relaxation cycle in skeletal muscle is regulated by transient changes in the concentration of free cytosolic Ca²⁺. According to the steric model of thin filament regulation, Ca²⁺ binds to and elicits a conformational change in troponin, a regulatory protein, which is located at regular intervals (40 nM) along the length of the tropomyosin-actin complex. The Ca²⁺ mediated conformational change in troponin leads to a 10 degree rotational shift in the long beadlike tropomyosin from the periphery towards the groove center of the actin double helix. This shift in tropomyosin exposes the actin binding sites to myosin ATPase, and muscle tension is developed as the thick myosin and thin actin filaments slide past each other by several hundreds of nanometers (For a review see Ebashi 1980, Huxley 1985). During relaxation, the active sequestering of free Ca²⁺ (10⁻⁵ to 10⁻⁷ M) by the sarcoplasmic reticulum precludes myosin ATPase activity by altering the steric properties of the troponin-tropomyosin actin complex. In muscle, the myosin ATPase acts as an energy transducer by coupling the chemical reaction of ATP hydrolysis to the generation of force. We can define the power of skeletal muscle as the rate at which the fiber can apply force over a distance, which for the most part
depends upon the rate at which ATP can be turned over by the Ca\(^{++}\) activated F-actin myosin ATPase (termed myofibrillar or myosin ATPase). This reaction may be written as:

\[
\text{ATP}^{4-} + \text{H}_2\text{O} \rightarrow \text{ADP}^{3-} + \text{P}_i^{2-} + \text{H}^+ + \text{work}
\]

The work term denotes the coupling of ATP hydrolysis to the myofibrillar ATPase. In the myofibrillar compartment, the coupled reaction is displaced by about 7 orders of magnitude from thermodynamic equilibrium. The thermodynamic equilibrium constant for the hydrolysis of ATP is around \(2.8 \times 10^{-5}\) M at pH 7.2, 38\(^o\)C, 1.0 mM Mg\(^{++}\), ionic strength 0.25 (Veech 1980).

**SHORT-TERM GENERATION OF ATP**

Energy metabolism and its regulation is highly dependent on oxygen availability. Aerobic metabolism provides energy at a highly efficient rate from mitochondrial oxidation of the blood borne fuels free fatty acids, glucose and lactate (Hollozy and Booth, 1976, Wahren 1979, Hollozy and Coyle 1984). However, with increasing workload, a point is reached where a disparity between oxygen utilization and ATP turnover develops, and ATP generation is largely accomplished by activation of the anaerobic processes of phosphocreatine (PCr) hydrolysis and glycogenolysis (Margaria
1972; Hochachka et al., 1983). One consequence of recruiting anaerobic pathways is that muscle work is constrained by time and inversely related to ATP turnover with supra-maximal efforts lasting only a few seconds (Wilkie, 1980; Gollnick, 1982).

(1) PHOSPHOCREATINE HYDROLYSIS

The first mechanism to be discussed, PCr hydrolysis, is brought about by the direct transfer of phosphate from phosphocreatine to ADP via the near-equilibrium reaction catalysed by creatine kinase (Lohmann, 1934).

$$\text{PCr}^{2-} + \text{ADP}^{3-} + \text{H}^+ \rightleftharpoons \text{ATP}^{4-} + \text{Cr}$$

It is generally well accepted that during maximal ATP turnover in muscle the creatine kinase reaction is driven by the rate at which ADP and H+ ions are released from the myosin S1 head during contraction (Kuby et al., 1954; Meyer et al., 1985). On the basis of the high maximal velocity of creatine kinase in skeletal muscle (Kuby et al., 1954), PCr hydrolysis could theoretically support a maximal myofibrillar ATPase activity of 500 u moles ATP/g wet wt/min (Bendall, 1961) for a period of about 16 sec before depletion of PCr. This calculation assumes an endogenous PCr store of 30 u moles/g muscle (Burt et al., 1976, Meyer et al., 1982). During such high flux rates another high activity enzyme, myokinase, may contribute to ATP generation but requires substantially elevated ADP concentrations (Km of 0.33 mM, Noda, 1973) as would be the case when PCr is nearly depleted (Cain and Davies, 1962). Recently,
using saturation-transfer $^{31}$P NMR, the creatine kinase reaction has been shown to operate close to equilibrium in both frog and cat skeletal muscle (Kushmerick et al., 1980 Gadian et al., 1981).

(11) ANAEROBIC GLYCOGENOLYSIS

The second mechanism of ATP generation in the absence of oxygen in skeletal muscle is through the conversion of glycogen to lactate with two substrate phosphorylations of ADP occurring at phosphoglycerate kinase (PGK) and pyruvate kinase (PK).

$$\text{Glucosyl unit } + 3\text{P}_i^{2-} + 3\text{ADP}^{3-} + \text{H}^+ \rightarrow 2\text{Lactate}^{4-} + 3\text{ATP}^{4-} + 3\text{H}_2\text{O}$$

The process of anaerobic glycogenolysis generates about one-twelfth of the ATP that can be obtained from the complete oxidation of the glucosyl unit from glycogen to $\text{CO}_2$ and $\text{H}_2\text{O}$. Why the conversion of glycogen to lactate is preferred over the complete oxidation of either glycogen or exogenous glucose to support high flux rates is thought to be due to the higher ATP yield per unit time (McGilvery, 1975). The higher ATP yield per unit time is likely due to localization of glycogen and glycogenolytic enzymes close to the myofibrillar ATPase, and the kinetic structure of the pathway.

Glycogenolysis is a spontaneous reaction in a thermodynamic sense, proceeding with a relatively large decrease in free energy of $-57 \text{ kcal/mol}$, pH7.0, 25 °C (Krebs and Kornberg, 1957). Later in
1964, Bucher and Russman focused in on the free energy changes of the twelve sequential reactions of the pathway, and compared the thermodynamic equilibrium constant of each reaction determined \textit{in vitro} to its respective mass action ratio measured \textit{in vivo} (concentration ratio of products over reactants). These workers pioneered much of our current understanding on metabolic control by characterizing each enzyme as catalyzing either a near- or non-equilibrium reaction. In general, enzymes that catalyze near-equilibrium or readily reversible reactions ($\Delta G^\circ \approx \Delta G$) are present in muscle with activities 10-to 100-fold greater than the maximum glycogenolytic flux (Bucher and Russman, 1964; Scrutton and Utter, 1968). Because they catalyze readily reversible reactions these enzymes are considered poor places to control unidirectional flux along a multienzyme pathway. In contrast, those enzymes that catalyze reactions displaced far from thermodynamic equilibrium are generally irreversible and characterized by low maximal activities to glycogenolytic flux ratios (Rolleston, 1972). This class of enzymes are important in providing directionality to pathway flux, and serve to integrate flux with the ATP requirements of the cell by being sensitive to a number of external allosteric effectors and covalent modification (Sols 1981). For all practical purposes, the irreversible reactions of glycolysis and glycogenolysis are catalyzed by hexokinase (Hk), glycogen phosphorylase, phosphofructokinase-1 (PFK-1) and PK (Hess, 1962; Williamson, 1965; Scrutton and Utter, 1968). It is not fortuitous therefore that these regulatory enzymes are strategically
positioned at the beginning, in the middle and at the end of the pathway, and that all are subject to varying degrees of multimodulation. Before looking at the regulation of the glycolytic and glycogenolytic pathway in more detail, it is worth mentioning that the application of thermodynamic principles provides information about whether a reaction can proceed spontaneously but not about the rate at which it proceeds. Regulation of metabolism therefore is essentially regulation of activity of enzymes and related membrane transport systems (Randle and Tubbs, 1979).

**CONTROL OF GLYCOGENOLYSIS IN SKELETAL MUSCLE**

Historically, the early work of Carl F. Cori in 1933 and later in 1956 showing that the rate of accumulation of hexose monophosphates exceeded the rate of lactate formation in tetanically stimulated rat gastrocnemius in situ and isolated frog sartorius muscle, provided the first unequivocal demonstration of a rate controlling step at the level of PFK-1. Additional studies about 5 years later extended Cori's observation by implicating glycogen phosphorylase as well (Ozand and Narahara, 1964; Danforth, 1965). This classic series of investigations together with the thermodynamic treatment of Bucher and Russmann (1964) provided the experimental basis for much of our conceptual framework on metabolic control in skeletal muscle.

Having briefly discussed the strategic positioning of the
various regulatory enzymes of glycogenolysis, the pathway can be subdivided and analysed in four broad sections: (1) Conversion of glycogen to glucose 6-phosphate (G 6-P), (2) Conversion of G 6-P to fructose 1,6-bisphosphate (F 1,6-BP), (3) Conversion of F 1,6-BP to pyruvate, and finally, (4) Conversion of pyruvate to lactate. The following discussion will examine control of glycogenolysis in more detail by reviewing the regulation of three key enzymes: glycogen phosphorylase, PFK-1 and PK.

**GLYCOGEN PHOSPHORYLASE**

In skeletal muscle, the rate of glycogen breakdown primarily is under neural and hormonal control (Kavinsky and Meyer, 1977., Cohen 1983). The two best understood mechanisms involve phosphorylation of the inactive b form of glycogen phosphorylase to the active a form by the enzyme, phosphorylase kinase. The first control mechanism involves neural mediation by Ca\(^{++}\) activation. The Ca\(^{++}\) binds to calmodulin, a subunit of the inactive phosphorylase kinase, and as all the binding sites become filled, the entire molecule shifts to the active conformation leading to the subsequent conversion of glycogen to glucose 1-phosphate in the presence of P\(_i\) (Soderling and Park, 1974, Cohen 1983). The second mechanism of control is mediated by catacholamine activated cyclic AMP which in turn leads to the phosphorylation of phosphorylase kinase b to a (Cohen 1983). A third mechanism that has been
extensively studied \textit{in vitro}, but not well understood \textit{in vivo}, involves non-covalent activation of phosphorylase \textit{b} by either AMP or IMP (Fischer et al., 1971, Griffiths et al., 1976). AMP is regarded the more physiological effector since its apparent activation constant is 50 \text{uM} compared to 2 \text{mM} for IMP (Rahim et al., 1978). However, the most important of the three mechanisms involved in the activation of glycogen phosphorylase during the initial stages of excitation-contraction coupling is the release of Ca$^{++}$ from the sarcoplasmic reticulum. This explains why the activity level of phosphorylase \textit{a} can rise from 5 to 90\% with a short half-time of about 0.7 sec (Helmreich et al., 1965).

Since glycogen consists of molecules of different sizes, it is impossible to determine the ratio $[\text{Glycogen}_{n-1}/\text{Glycogen}_n]$ so we write the apparent equilibrium constant as $[\text{Gl-P/P}_i]$. At equilibrium, the ratio is 0.28 at pH 6.8, 25$^\circ$C (Fischer et al., 1971). In resting muscle, the mass action ratio may be at least two orders of magnitude lower than the equilibrium constant, assuming that a G 1-P concentration of 0.003 \text{mM} and a free P$_i$ of 1.0 \text{mM}. As glycogen is converted to G 1-P, the G 1-P enters the pathway of glycolysis at the level of G 6-P. This reaction is catalyzed by the near-equilibrium enzyme, phosphoglucomutase (PGM), which requires priming by glucose 1,6-bisphosphate (G 1,6-BP). G 1,6-BP is formed by phosphorylation of G 1-P in the presence of ATP and phosphoglucookinase (Bietner, 1979). Both G 6-P and G 1,6-BP are potent inhibitors of hexokinase and together with P$_i$ provide the
most likely means by which the cell selects glycogen over glucose during high muscle work rates (Lueck and Fromm, 1974).

**PHOSPHOFRUCTOKINASE**

PFK-1 catalyzes the phosphorylation of fructose 6-phosphate (F 6-P) to F 1,6-BP. This reaction is the first irreversible step of the glycolytic pathway below the G 6-P crossroad. The kinetic behavior of PFK-1 *in vitro* and *in vivo* is highly complex (Uyeda, 1979). Using the skeletal muscle enzyme, Passonneau and Lowry (1962) demonstrated that ATP acts as both inhibitor and substrate, that F 6-P acts as both activator and substrate, that both products F 1,6BP and ADP act as activators, that H⁺ ion acts as a potent inhibitor, and that AMP, Pᵢ and ammonia (NH₄⁺) all act as activators of PFK-1 below pH 7.2. Furthermore, adding to this long list of effectors, two other metabolites, fructose 2,6-bisphosphate (F 2,6-BP) and G 1,6-BP, have recently been found to be the most potent activators of PFK-1 so far discovered (Bietner, 1979; Hers and VanShafitien, 1982). The reported inhibition of muscle PFK-1 by PCr has proven to be due to a contaminant of PCr (Fitch et al., 1979).

The question of how PFK-1 operates in working muscle has challenged biochemists for over three decades. At physiological concentrations of F 6-P, it is known that high concentrations of ATP cause a marked inhibition at pH 7.0 (Ui, 1966), a condition that has also been observed at high enzyme concentrations (Bosca et al.,
1985). As the pH decreases, the ATP sensitivity and F 6-P cooperativity increases. This inhibition of PFK-1 is due to the enhanced pH-dependent binding of ATP at an allosteric site (Passoneau and Lowry, 1962; Uyeda, 1979), and can be counteracted by the various positive effectors present in the cell (Trivedi and Danforth, 1966). Bock and Frieden (1976 a,b) have provided evidence to suggest that this interaction arises from F 6-P binding to unprotonated forms of the muscle enzyme, while ATP binds preferentially to the protonated forms at two ionizable groups (Pettigrew and Frieden, 1979). This mechanism of interaction has also been demonstrated with PFK-1 from ascites tumor cells (Sols, 1981). It can be stated with a fair degree of confidence that although PFK-1 as a control point of glycogenolysis is recognized beyond dispute, the mechanism of its action remains unclear.

**PYRUVATE KINASE**

PK catalyzes the transfer of the phosphate group from phosphoenolpyruvate (PEP) to ADP with the subsequent formation of pyruvate and ATP. One important regulatory function of this enzyme is to maintain unidirectional flux towards pyruvate formation. In contrast to the liver enzyme, muscle PK typically reveals a hyperbolic saturation curve in response to increasing PEP (Ainsworth and MacFarlane, 1973). On the basis of this response the muscle enzyme has been assumed for a long time less important to flux control than the other non-equilibrium enzymes, glycogen
phosphorylase and PFK-1. However, on careful re-examination of the kinetic properties of muscle PK, a pronounced sigmoidicity was apparent at physiological pH when the initial velocities were determined as a function of either ADP or Mg^{++} (Phillips and Ainsworth, 1977). Furthermore these studies revealed a very important link between the activity of PFK-1 and PK by demonstrating that the product of the PFK-1 reaction, F 1,6-BP, strongly activates the muscle enzyme.

In summary, PK 'turns off' whenever the ATP concentration is high in the absence of positive effectors, and 'turns on' by feedforward activation through the combined action of PEP and F 1,6-BP availability. Since all the preceding enzymes of glycolysis up to PFK-1 operate close to thermodynamic equilibrium in skeletal muscle (Veech et al., 1979; Connett, 1985), the activity of PK serves two vital functions: (i) provides directionality to pathway flux, and (ii) regulates the levels of glycolytic intermediates at each of the respective steps up to PFK-1.

**NAD^+/NADH CYCLES IN GLYCOGENOLYSIS**

A knowledge of the redox state of the cytoplasmic free NAD^+/NADH couple is essential for an understanding of the regulation of glycogenolysis in skeletal muscle (Edington, 1970). During high flux rates, the NADH formed at the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) step is rapidly re-oxidized further down the pathway by lactate dehydrogenase concomitant
with the reduction of pyruvate to lactate (McGilvery, 1983). Since the total amount of nicotinamide adenine dinucleotide present in a cell is constant at about 0.8 umoles/g wet wt tissue, regulatory mechanisms must exist to maintain the NAD$^+/\text{NADH}$ ratio within certain limits if glycogenolysis is to continue (Cleland, 1967). In the rested state, the cytoplasmic NAD$^+/\text{NADH}$ ratio in mammalian skeletal muscle was calculated to be about 300 (Edington et al., 1973). In contrast, this ratio in the mitochondrial compartment is believed to be two orders of magnitude lower (Williamson et al., 1967).

**ROLE OF ADP, ATP/ADP RATIO AND PHOSPHORYLATION POTENTIAL IN REGULATING GLYCOGENOLYSIS**

The primary signals in muscle that respond to the demands of increasing work are thought to be changes in free Ca$^{++}$ and free cytosolic ADP (Jacobus et al., 1982; Chance et al., 1985). The transient increase in free ADP will occur whenever the rate of ATP hydrolysis slightly exceeds the rate of ATP generation. The rise in free ADP causes a decrease in both the ATP/ADP ratio and phosphorylation potential (ATP/ADP, P$_i$ ratio). A widely held belief is that the arrangement of the ATP generating mechanisms in a cell operate to maintain a high ATP/ADP ratio or phosphorylation potential so that the hydrolysis of ATP remains favourable. In the context of this general discussion on coupling anaerobic processes to myosin ATPase, the three parameters (free ADP, ATP/ADP or
ATP/ADP, $P_i$ ratios) either directly or indirectly play a critical role in regulating the activities of the kinase reactions of both PCr hydrolysis and glycogenolysis during high muscle work rates (McGilvery, 1975). That free ADP in the cytosol of muscle has been calculated to be as low as 20 to 40 uM (~5% of the total ADP content in muscle; Veech et al., 1979), further attests to the important catalytic role of this metabolite in control of metabolism.

**LIMITS OF ANAEROBIC ATP GENERATION**

Operationally, fatigue can be defined as the inability of an organism or muscle to maintain a pre-determined exercise intensity or myosin ATPase activity (Edwards, 1975; Faulkner, 1983). The term exhaustion may be used synonymously with fatigue but for the purpose of this thesis, emphasis is placed on the term exhaustion to mean the inability of an animal to maintain any exercise intensity with the subsequent loss of postural support and orientation.

When a muscle is contracting at maximal speed of shortening, the limited store of PCr must obviously be a prime candidate for the development of fatigue (Vergara et al., 1977). If the speed of shortening is reduced, the onset of fatigue and exhaustion will be delayed, and in this case may be brought about by glycogen depletion or by a number of interrelated factors that are primarily governed by the intensity and duration of contraction (Hermansen,
Traditionally, the onset of fatigue in skeletal muscle has been associated with one of the products of ATP hydrolysis, $H^+$ ions (Bolitho-Donaldson and Hermansen, 1978; Nassar-Gentina et al., 1978; Sahlin, 1978; Sahlin et al., 1981). The $H^+$ ion has a myriad of functions in a muscle cell, and an increase in its concentration (intracellular pH down to 6.4) has been postulated to bring about the onset of fatigue by affecting the kinetic and regulatory properties of PFK-1 (Hollozy et al., 1978; Hermansen, 1981), the $Ca^{++}$ sequestering and releasing properties of the sarcoplasmic reticulum (Nakamura and Schwarts, 1972; Fitts et al., 1982), and even the myofibrillar ATPase itself (Portzehl et al., 1969; Fabiato and Fabiato, 1978; Dawson et al., 1980). The neuromuscular junction has also been implicated with a decrease in force development (Bigland-Ritchie et al., 1982, Faulkner, 1983). Superimposed on these potentially limiting effects of $H^+$ ion on the rate of anaerobic glycogenolysis and muscle performance are the effects of concomitant changes in either the ATP/ADP ratio, the phosphorylation potential or the NAD$^+$/NADH redox couple.

**SPECIFIC AIMS AND STUDY OUTLINE**

The central theme of the present thesis is regulation of the anaerobic ATP-generating pathways in vertebrate skeletal muscle during exercise and fatigue, with particular emphasis on glycogenolysis. The animals of choice were a teleost fish and laboratory rat. The rationale behind using two different animal
models to investigate the same fundamental questions on metabolic control resides in the different abilities of each to perform exercise, and to their markedly different myofibrillar organization (Ariano et al., 1973; Webb, 1978; Johnston, 1981; Armstrong and Laughlin, 1985). In contrast to the laboratory rat, fish are highly specialized for fast-start performances (Webb, 1978), a specialization that is predictable on the basis of their myotomal organization. The major bulk of the body musculature of fish primarily is composed of fast-twitch glycolytic (FG) or white fibers, with red or slow oxidative (SO) appearing as a separate thin triangular strip running longitudinally beneath the lateral line (Johnston, 1977, 1981). In general, the red muscle mass constitutes about 10% of the total body musculature, but this percentage may vary widely depending upon the activity pattern and lifestyle of the species (Greer-Walker and Pull, 1975). Between the red and white muscle layers there is an intermediate zone which is often referred to as fast-oxidative glycolytic or FOG fiber type, after the terminology of Peter et al., 1972 (Johnston, 1981). The myofibrillar organization of higher vertebrates is much more complex and heterogeneous in nature with each skeletal muscle being composed of widely varying percentages of the three basic fiber types (FG, FOG & SO) [Peter et al., 1972]. In the case of the rat, even though 50% of the total musculature of the hindlimb is composed of fast-twitch fibers (FG & FOG), detailed analysis of its cross-sectional area reveals that muscles with a higher percentage of SO fibers are generally located in the deeper regions,
whereas those muscles with a higher percentage of fast-twitch fibers are located at the periphery of the leg (see Armstrong and Laughlin, 1985).

The three basic fiber types have been classified on the basis of their different structural, biochemical and physiological properties. In general, the FG fibers are typically larger (up to 100Å in sarcomere diameter); have a high Ca\(^{++}\)/Mg\(^{++}\) activated actomyosin ATPase; a high anaerobic glycogenolytic and low aerobic capacity; low myoglobin, capillary and mitochondrial density; a high buffering capacity, and easily fatiguable (Peter et al., 1972; Baldwin et al., 1973; Hollozy and Booth 1976; Johnston 1985). SO fibers on the other hand have a smaller cross-sectional area and display the opposite metabolic profiles as described for FG fibers. Not surprisingly, as discussed above for fish those skeletal muscles with a higher percentage of FG fibers are preferentially recruited for short-term high intensity work, whereas muscles with a higher proportion of SO fibers are recruited during lighter to moderate intensity exercise (Davison et al., 1976, Hollozy and Booth, 1976; Bone et al., 1978, Hollozy and Coyle, 1984). As running speed increases, electromyographic studies on rat hindlimb muscles have shown that there is a progressive shift in activation of motor units from the deep SO muscles to the more peripheral FG muscles (Armstrong and Laughlin, 1985). The same pattern of activation of muscle types has also been demonstrated in fish swimming at increasing speeds (Bone et al., 1978), except that the SO fibers are located at the periphery. The different spatial
organization of muscle types in the rat hindlimb and fish probably reflect the different weight bearing requirements of quadrapedal terrestrial locomotion verses the near neutral buoyancy of fish in their aquatic environment.

Because of the obvious functional differences between the three basic muscle fiber types, and the different spatial patterns of myofibrillar organization of the two animals, it was conceivable that identification of fiber-specific regulatory mechanisms of glycogenolysis may be more apparent in fish white skeletal muscle than in the comparable fast-twitch skeletal muscles of the rat. The regulatory parameters that will be examined in this study include the free cytosolic ADP concentration, the ATP/ADP ratio, the phosphorylation potential, the redox potential and the interrelated effects of decreasing intracellular pH. Since the \( \text{NAD}^+ / \text{NADH} \) and ATP/ADP ratios are functionally linked through the combined GAPDH-PGK-LDH equilibria, the study sets out to establish the relative importance of each parameter in controlling glycogenolysis, and more important, their respective roles in limiting muscle performance. Moreover, coupling between the anaerobic processes and the myosin ATPase in muscle is coordinated through the ADP or ATP requiring kinases of PCr hydrolysis and glycogenolysis. A commonly held view in the field of exercise biochemistry and physiology is that the onset of fatigue during short-term high intensity exercise is brought about by the inhibitory effect of pH on PFK-1 (Hollozy et al., 1978; Hermansen, 1981). The present author believes that such statements are not
well founded and in addition to examining control of PFK-1 in fish skeletal muscle at fatigue, and in rat skeletal muscle after two different exercise intensities, a rigorous kinetic study will attempt to show how the enzyme can achieve significant catalytic rates in working muscle despite falling pH.

The work will be presented in three separate sections. The first section will consider the regulation of PCr hydrolysis and anaerobic glycogenolysis in fast-twitch white muscle of rainbow trout (Salmo gairdneri) after a 10 sec sprint, a 10 min burst swim at approximately 120% VO₂ max to fatigue, and following a 30 min endurance swim to exhaustion. The second section will examine anaerobic glycogenolysis in three different muscle types of the rat after 2 minutes of high intensity treadmill running, and following a 30 min endurance run to fatigue. The third section will deal with a re-examination of the kinetic and regulatory properties of purified rabbit muscle PFK-1. The fourth and final section will give a general account of the major findings of this interdisciplinary investigation between the fish and rat, and will attempt to summarize the similarities and differences between each in regulating, and in the case of fatigue, limiting glycogenolysis in skeletal muscle accompanying exercise.
CHAPTER 2: MATERIALS AND METHODS
MATERIALS AND METHODS: SECTION 1

FISH

Rainbow trout (Salmo gairdneri) of both sexes were obtained from the Sun-Valley Trout Farm, Mission, B.C. Canada. Fish were fed ad libitum and maintained in outdoor tanks with a continuous supply of fresh, aerated, dechlorinated tap water.

EXERCISE PROTOCOL AND MUSCLE DISSECTION

The exercise protocols used for the study included a 10 second supra-maximal sprint, and two prolonged burst swimming events of about 10 and 30 min duration. To avoid confusion, it was necessary to modify the terminology of Hoar and Randall (1978) and define a high intensity burst swim lasting less than 20 sec as a sprint. Two different stocks of fish were used: the first winter stock (water temperature 4 - 6°C, weighing 60 to 70 g and 18 to 20 cm in length) was used for the sprint protocol; while the second summer stock (water temperature 10°C, weighing 200 to 250 g, and 27 to 30 cm in length) was used for the two prolonged swimming protocols.

Fish were exercised in a Brett-type swim tunnel (Brett, 1964). Prior to each experiment, fish were characterized into groups on the basis of steady state swimming performance using stepwise increases in water velocity of 10 min duration (increments of 0.2 body lengths/sec), and continued until a steady state swimming
velocity could no longer be maintained (Brett, 1964; Jones and Randall, 1978). This method is useful for selecting fish of similar physiological state and swimming ability. With this exercise procedure, the maximum steady-state swimming velocity achieved prior to fatigue is known as the critical velocity or $U_{\text{crit}}$ (Brett, 1964). At swimming speeds below $U_{\text{crit}}$, metabolism has been shown to be predominately aerobic with a small anaerobic component appearing at about 80% $U_{\text{crit}}$ (Bone et al., 1978). Even though no simple relationship between oxygen uptake and $U_{\text{crit}}$ has been described for fish, it is reasonable to assume that $U_{\text{crit}}$ is analogous to the VO$_2$ max index used to evaluate mammalian exercise performance.

All fish were transferred by net from black holding boxes to the swim tube and those fish that struggled were discarded. In the case of the sprint protocol, the water velocity was immediately increased to the maximal 90 cm/sec, which corresponded to about 5 body lengths/sec. It was previously determined one week earlier that the time to fatigue was on the average 18 sec. Fish were netted after 10 sec of sprinting, and the white epaxial muscle immediately excised from a site posterior to the dorsal fin. Samples were immediately freeze-clamped in liquid nitrogen (-200°C) and stored at -70°C until required. The average time between capture of fish and freeze-clamping muscle was about 6 sec. Those fish subjected to burst swimming at about 120% $U_{\text{crit}}$ lasted on average 10 min before fatigue set in and their swimming position could no longer
be maintained. The longer burst swimming group were started at the highest maximum water velocity with the speed being continuously decreased as each fish could no longer maintain position in the swim tunnel. After a few minutes, however, the water velocity was increased again to make certain that the anaerobic ATP generating pathways were being recruited at all times. By continually oscillating the speed control in this way, fish were completely exhausted at both high and low swimming velocities. The procedure was halted when fish could no longer swim. The average time to complete exhaustion was 30 min. White epaxial muscle was dissected out immediately in an identical manner as that described above for sprint fish.

MUSCLE HOMOGENIZATION, EXTRACTION AND NEUTRALIZATION

White epaxial muscle was powdered under liquid nitrogen using a pre-cooled mortar and pestle. Tendons and fragments of connective tissue were dissected free and discarded. About 500 mg of powdered tissue was transferred to a pre-cooled pre-weighed vial containing 1.0 ml of ice-cold 0.6 N perchloric acid (PCA) and then accurately re-weighed. A further 1.0 ml PCA was added and the powder homogenized at intermediate to high speed for 15 sec at 0°C using an Ultra-Turrax T18 homogenizer coupled to 10 mm shaft and generator. The homogenization procedure was repeated and the sides of the vial washed down with a further 0.5 ml PCA. The suspension was stirred under low speed and 100 ul was removed in
duplicate for glycogen determination. The remaining homogenate was then transferred to two 1.5 ml eppendorf polypropylene test-tubes, and centrifuged for 2 min at 13,000 r.p.m. and 4°C in a microcentrifuge. A known volume (approximately 2.0 ml) of supernatant was removed and immediately transferred to a pre-cooled test-tube containing a pre-determined volume of saturated Tris base \([\text{tris(hydroxymethyl)-aminomethane}]\) to bring the pH of the extract to 7.0. The neutralized PCA extract was frozen immediately in liquid nitrogen and kept at -70°C until required. All biochemical analysis was carried out within two to three weeks. The homogenization and neutralization procedure described is rapid and takes about 6 minutes to complete. In a parallel set of validation studies the highly acid-labile phosphocreatine was shown to undergo not more than 5% hydrolysis with this technique.

**GLYCOLYTIC INTERMEDIATES**

The total content of all glycolytic intermediates were measured in a Pye-Unicam SP8-100 UV-VIS spectrophotometer at 340 nm using the routine NADH or NAD coupled enzymatic procedures described in Bergmeyer (1983). Each assay was validated with the appropriate standard(s).

**NUCLEOTIDES AND PHOSPHAGENS**

The nucleotides ATP, ADP, AMP and IMP, and PCr and creatine (Cr) concentrations were analytically determined by High Performance Liquid Chromatography (HPLC). The procedure was
carried out on a Spectrophysics 8000 B HPLC coupled to a Spectroflow 773 Absorbance Detector. The nucleotides were passed through a Brownlee anion-exchange column at a flow rate of 2.0 ml/min and eluted in 30 min using a linear gradient of dihydrogen potassium phosphate (KH₂PO₄ MCB PXI-566-3). The initial gradient comprised 50 mM KH₂PO₄·HCl pH 2.31 at 25⁰ and 600 mM KH₂PO₄·HCl, pH 2.63 at 25°C and the nucleotides were detected at 254 nm. The column temperature was 55⁰C. PCr, Cr and AMP were eluted at the same flow rate of 2 ml/min in 12 min using an isocratic 50 mM KH₂PO₄·HCl buffer at pH 3.1, and detected at 210 nm. 20 ul of neutralized sample was required for each set of determinations. Following every set of six chromatographic runs, a single 20 ul injection of known standards was passed through the system to check retention times of each metabolite. Internal standards were also used to validate the procedure for muscle analysis.

**ORTHOPHOSPHATE DETERMINATION**

Orthophosphate (Pᵢ) was determined by the colorometric method of Black and Jones (1983). This method has been subsequently validated with ³¹P NMR on neutralized muscle extracts from resting and exercising fish (Parkhouse and Dobson, unpublished data).
**INTRACELLULAR pH**

Intracellular pH of muscle was estimated by using the homogenate technique described by Costill et al., 1982) with the following modifications: Frozen muscle powder ground under liquid nitrogen and kept at -70°C was homogenized at 0°C in a salt solution (1 : 9 w/v) containing 145 mM KCl, 10 mM NaCl and 5 mM iodoacetate, pH 7.0 at 10°C. The pH of the muscle homogenate was measured at 10°C (body temperature of fish) with a Radiometer Micro-pH electrode coupled to a PHM-71 Acid-Base Analyser.

For intracellular pH of rat gastrocnemius and plantaris the identical procedure was used except the measurement temperature was 38°C.

**CALCULATION OF FREE CYTOSOLIC ADP**

Free cytosolic ADP was calculated using the creatine kinase equilibrium as described in detail by Veech et al., (1979). The calculation is based on the assumption that the reaction is maintained near-equilibrium, an assumption that appears to be valid in working muscle (Veech et al., 1979; Kushmerick et al., 1980; Gadian et al., 1981; Shoubridge et al., 1982; Meyer and Kushmerick, 1985). The equilibrium constant for the creatine kinase reaction is \(1.66 \times 10^{+9} \text{ M at pH 7.0, free Mg}^{++} 1 \text{ mM, ionic strength of 0.25 and temperature 38°C. It should be emphasized that the metabolite concentrations used in the calculation are those determined...} \)
analytically and include all ionized and complexed species. All tissue metabolites are expressed in μ moles/g cell water in order to make the metabolite ratios directly comparable to the equilibrium constant measured in vitro. The percentage of tissue weight that is water was determined in a parallel set of studies and found to be 80%, a value that is similar to rat skeletal muscle (Veech et al., 1979). The free absolute concentration of ADP in the cytosol of fish white muscle in vivo may differ slightly from the calculated estimates in view of the fact that the apparent thermodynamic equilibrium constant for the creatine kinase reaction used was determined at 38°C. The relative change in free ADP occurring with exercise should however be valid. The equilibrium constant could not be temperature corrected for 10°C because the constant has not been well defined at any temperature other than 38°C, and therefore precludes an accurate calculation of ΔH⁰ (Veech, personal communication).

CALCULATING FREE CYTOSOLIC NAD⁺/NADH RATIO

The cytoplasmic NAD⁺/NADH ratio was calculated using the lactate dehydrogenase equilibrium (Williamson et al., 1967., Veech et al., 1969) according to the following expression:

\[
\text{NAD}^+ / \text{NADH} = \frac{[\text{Pyruvate}]}{[\text{lactate}]} \times \frac{[H^+]}{\text{Keq}}
\]

where lactate and pyruvate concentrations denote the total
measured contents. The equilibrium constant (Keq) was calculated to be $1.1 \times 10^{-12} \text{M}^{-1}$ at 10°C, pH 7.0 and at an ionic strength of 0.25 assuming a $\Delta H^\circ$ of +14 kcal/mol. This calculated equilibrium constant is very similar to that determined directly at 16°C (Hakala et al., 1956). This method is based on the following two assumptions: (a) that the LDH reaction is maintained near-equilibrium in working muscle, and (b) that the myofibrillar compartment has an ionic strength of 0.25 and free Mg$^{++}$ of 1.0 mM.

CROSSOVER THEOREM: APPLICABILITY TO IDENTIFY CONTROL SITES OF GLYCOGENOLYSIS

The crossover theorem was developed by Chance and Williams (1956) for use in identifying control points of the phosphorylating mitochondrial electron transport chain. Later in the mid-sixties it was applied to glycolysis (see Williamson, 1970). For graphical representation, individual reactions of the glycolytic pathway are listed in order of succession on the abcissa, and the relative changes of their metabolite concentrations (expressed as a percentage of control values) are plotted on the ordinate. The zero line represents the steady state, where no changes in levels of intermediates with time take place. Crossovers between pairs of neighboring metabolites may occur from minus to plus or from plus to minus relative to the zero line. Crossovers indicate sites of interaction or control points along the pathway in response to a change in flux. A detailed account of the theoretical basis for the
crossover theorem can be found in Williamson (1970) and Heinrich et al., (1977).

MATERIALS AND METHODS: SECTION 2

RATS

Adult male Sprague-Dawley rats weighing approximately 300 to 320g were obtained from Acme Biomedical Supplies, Calgary, Canada. Rats were individually housed and maintained in a temperature (25°C) and light (12hr dark/12 hr light cycle) controlled room. They were fed ad libitum on purina rat chow and allowed free access to water. All experiments were carried out between 9.00 a.m. and 12.00 noon to avoid any diurnal variation in muscle glycogen (Conlee et al., 1976; Garetto and Armstrong, 1983).

TRAINING AND EXERCISE PROTOCOL

Rats were trained by running at 30 m/min on a treadmill for 10 min a day 5 days a week for a period of 4 to 8 weeks prior to the experiment. Rats selected for the short-term 2 min high intensity run were familiarized with the protocol for 7 days before the experiment. The 2 min protocol consisted of running at a treadmill speed of 75 m/min for 20 sec and 56 m/min for the remaining 1 min 40sec. These animals were not fatigued after 2 min. The
endurance protocol consisted of running at a treadmill speed of 25 m/min until fatigue. Fatigue was characterized by the animal's inability to maintain a running speed of 25m/min.

**ANAESTHESIA**

The exercised animals were anaesthetized with an intra-peritoneal injection of a 1.5 ml solution comprising 0.5 ml Na pentobarbital (50 mg/ml) and 1.0 ml curare (10mg/ml). Curare is a neuromuscular blocking agent and was found to be suitable in preventing muscle twitching following exercise. The response time of the anaesthetic without curare was 60 to 90 sec. Use of this procedure for the control group, however, was not successful and produced noticeable irregular twitching and violent kicking resulting in extremely low PCR values (Dobson, Belcastro and Parkhouse, unpublished data). Despite a longer response time of 3 to 5 min, the preferred technique of anaesthesia for control rats was the use of ether soaked cotton wool placed in a sealed Bell jar. This technique has been widely used for detailed biochemical analysis of muscle in the pre-exercise or 'rested' state (Veech et al., 1979).

**MUSCLE DISSECTION**

Following anaesthesia, rats were placed in a supine position and skeletal muscles of the left lower extremity rapidly
exteriorized. The soleus muscle was the first to be excised, taking 30 sec, and immediately freeze clamped in liquid nitrogen with pre-cooled aluminum tongs. The plantaris was excised next, taking about 60 sec, followed by the gastrocnemius (80-90 sec). The same procedure was repeated for the right lower leg with respective dissection times of 1 min 50 sec, 2 min 15 sec and 2 min 45 sec. All times for individual muscles refer to the time elapsed between the fully anaesthetized state and freeze-clamping in liquid nitrogen. Care was taken not to stretch the muscles during the dissection procedure. The tissues were stored for approximately 2 weeks at -70°C prior to analysis. Before the homogenization procedure, individual soleus muscles were pooled from the left leg of three rats for the 2 min running group, while the plantaris was pooled from the left leg of two rats. The gastrocnemius was not pooled. An identical procedure was adopted for the three muscles of the right leg. No significant biochemical differences were noted between muscles pooled from either the left or right lower extremities. A similar grouping procedure was used for the endurance rats except that muscles were paired on the basis of running time to fatigue.

**CALCULATION OF FREE CYTOSOLIC ADP**

Free cytosolic ADP was calculated using the combined glyceraldehyde 3-phosphate dehydrogenase-phosphoglycerate kinase
and lactate dehydrogenase equilibrium expression (GAPDH-PGK) [see Veech et al., 1979]. The same assumptions hold for this method as described for the creatine kinase system. The equilibrium constant for the combined GAPDH-PGK reaction is similar to that described by Veech et al., (1979) as modified by Connett (1985).

**ANALYTICAL METHODS**

All other analytical methods used were identical to those described in detail in SECTION 1 of the MATERIALS AND METHODS and can be found under their respective headings.

**MATERIALS AND METHODS: SECTION 3**

**PHOSPHOFRUCTOKINASE**

Crystalline rabbit muscle PFK-1 (EC 2.7.1.11) was obtained as an ammonium sulphate suspension from Sigma Chemical Company (Type III Lot number: 81F-9590). The specific activity and purity of the enzyme was 110 U/mg protein, which was similar to that used by Bock and Frieden (1976a). One unit of activity is defined as the production of 1.0 u mole of Fructose 1,6BP/min at 25°C and pH 8.0. Nucleotides, buffers, and auxiliary enzymes were obtained from Sigma Chemical with the exception of AMP and rabbit muscle α-glycerophosphate dehydrogenase and triose-phosphate isomerase,
which were purchased from Boehringer Mannheim. All other biochemicals were reagent grade and used as received from the supplier.

PFK-1 was dialized at 4°C against two changes of 50 mM Tris-phosphate (pH 8.0 at 4°C), 10 mM 1,4-dithiothreitol, and 1 mM EDTA-Na. After dialysis, the ratio of the absorbance of the enzyme solution at 280 and 260 nm was >1.65 (Bock and Frieden, 1974). Two batches of the enzyme were used with similar results.

**ASSAY PROCEDURE**

Enzyme activity was measured by the addition of an aliquot of the dialized enzyme sample (diluted approximately 1:4 with buffer) to a reaction cuvette, and the rate of formation of F 1,6-BP was monitored at 25°C in a final volume of 1 ml. The assay mixture contained 50 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES)-KOH, 0.15 mM NADH, 10 mM MgCl₂, 0.10 mM 1,4-dithiothreitol, 0.01 mM EDTA, 1.0 or 5.0 mM ATP, aldolase (0.8 U), α-glycerophosphate dehydrogenase (1.0 U), and triose-phosphate isomerase (5 U), with various concentrations of F 6-P and pH values (7.67, 7.25, 7.0 and 6.8 at 25°C). The coupling enzymes were either desalted by overnight dialysis or by passing through a Sephadex G-25 column. The reaction mixture was thermally incubated for 10 min at 25°C, and the reaction initiated by the addition of PFK-1. The absorbance was monitored at 340 nm with a Unicam SP-1800 dual
beam recording spectrophotometer. One unit of PFK activity corresponds to the oxidation of 2 umol NADH, which is equivalent to the production of 1 umol F 1,6-BP per min under the above assay conditions. Initial velocities were linear functions of time and enzyme concentration.

**DATA ANALYSIS**

In the presence of positive modulators, initial velocities were readily obtained from the slopes of recorded optical densities. However, under some circumstances, reaction rates were linear with time for only about 5 to 10 seconds. This was particularly notable in the presence of citrate where the time-dependent loss of activity is mainly due to the conversion of the active tetrameric enzyme to the inactive dimeric form (Lad et al., 1973). It was also evident, but to a lesser degree, at high ATP levels and low pH, when the F 6-P concentrations were 0.0125 mM or lower, again possibly due to the loss of tetrameric enzyme. In such cases, initial velocities were determined by measuring the tangent to the recorded curve and extrapolating back to the time of addition of enzyme. However, in the absence of citrate, at higher F 6-P concentrations and/or in the presence of positive modulators, no such loss of tetramer based enzyme activity was observed, and reaction velocities were linear for periods up to several minutes. Otherwise, wherever possible, this problem was avoided by using high physiological F 6-P concentrations (0.1 mM).
LIMITATIONS TO RESEARCH

When conducting biochemical measurements on skeletal muscle of exercising animals a number of cautionary points should be realized. The first limitation is to understand that recovery metabolism may occur between the time of cessation of exercise and freeze clamping the muscle. This is not as critical for fish muscle because of short sampling times (sec) but may be significant for each of the respective rat skeletal muscles. This may bias the interpretation of the biochemical data in rat skeletal muscle due to complex fiber heterogeneity and longer sampling times. It must be emphasized that concentrations of metabolites represent total contents, and in the case of highly heterogeneous muscles of the rat, represent a relative contribution from all fiber types. This problem is more pronounced in those muscle types that are well perfused with blood e.g. soleus muscle. The problem is less important in the fast-twitch muscles (gastrocnemius and plantaris). However, one should still be aware that each fast-twitch muscle consists of different percentages of FG and FOG populations.
CHAPTER 3: REGULATION OF ANAEROBIC GENERATING PATHWAYS IN RAINBOW TROUT FAST-TWITCH WHITE MUSCLE DURING EXERCISE
CHAPTER 3: INTRODUCTION

It is well established that the temporal activation of phosphocreatine hydrolysis and anaerobic glycogenolysis in working muscle depends to a very large extent on the intensity and duration of contraction (Hohorst et al., 1962; Danforth, 1965). Less clear are the regulatory mechanisms controlling flux, and those fiber specific related mechanisms leading to fatigue (Wenger and Reed, 1976; Karlsson, 1980). Fish provide a particularly useful metabolic system for investigating such mechanisms during high work rates because of their fast-start performance capacity (Webb, 1978), and their myofibrillar organization. As outlined in the introduction, fish locomotory muscle is composed of two spatially separate muscle masses of different fiber types: white muscle and red (Johnston, 1982). The white muscle mass comprises most of the body musculature with the red muscle appearing as a thin triangular strip running longitudinally beneath the lateral line. In the case of trout, 90% of the body musculature is white and 10% is red (Johnston, 1977, 1981). Thus a major advantage of using fish models to study the biochemistry of exercise is that many of the interpretative problems arising from complex patterns of fiber heterogeneity are minimized.

The present study was undertaken to investigate the contribution and control of phosphocreatine hydrolysis and anaerobic glycogenolysis in generating ATP for a short 10 sec sprint, and for two longer (10 and 30 min) swimming protocols to
fatigue and exhaustion respectively.

**RESULTS: FUELS AND ATP TURNOVER**

(1) **PHOSPHOCREATINE**

PCr decreased from 27.03 to 21.36 μmole/g wet wt muscle following a 10 sec sprint (Table 3.1.) Assuming that 1 mole of ATP is produced per mole PCr hydrolyzed, and knowing the duration of each burst during the 10 sec period, the ATP turnover was estimated to be 188 μmole ATP/g wet wt/min (Table 3.2.) This value may be three times higher if we make some reasonable assumptions about the level of PCr in white muscle at the pre-exercise state prior to the swim (see Discussion). In the 10 and 30 min burst swimming fish, PCr in white muscle decreased by about 90%. In both exercise groups the total PCr plus Cr remained constant (Table 3.3.)

(2) **GLYCOGEN**

In fish subjected to the 10 sec sprint there was no significant change in white muscle glycogen or lactate (Table 3.1.) In direct contrast, the 10 and 30 min swimming fish utilized 95 and 98% of their endogenous glycogen store (Table 3.3.). A large fraction of the glycogen utilized appeared as muscle lactate. Muscle glucose increased 2.5 fold in the 10 min burst swimming fish but no change was observed in white muscle of the 30 min group (Table 3.4.). Assuming that 3 moles of ATP is produced per mole of glucosyl residue from glycogen to lactate, and knowing both the average
number of bursts (67± 4 SEM n=6) and the approximate duration of each burst (0.8 sec), the ATP turnover in white muscle during 10 min of burst swimming was estimated to be 78 u moles/g wet wt/min (Table 3.2.). The number of bursts for the 30 min swimming fish was too variable to allow such an estimate of ATP turnover to be made.

**NUCLEOTIDE AND PHOSPHATE POOL**

The individual nucleotide concentrations underwent no appreciable change in white muscle following the 10 sec sprint (Table 3.1.). However, major changes occurred during the 10 and 30 min exercise protocols (Table 3.3). One of the most striking was the 54 and 80% fall in ATP for the 10 and 30 min trout burst swimming groups respectively. This dramatic decrease in ATP appeared as a stoichiometric increase in IMP, with the total AMP content undergoing a much smaller percentage change (Table 3.3.). In this way, the total nucleotide pool remained essentially constant. The high Pi value for muscle in the pre-exercise state was confirmed by 31P NMR (Parkhouse and Dobson, unpublished data), and was not considered an artifact of the colorometric method used, but more appropriately relate to the fish capture technique (see discussion). The concentration of Pi increased in both groups with exercise (Table 3.3.).
THERMODYNAMIC STRUCTURE OF THE GLYCOGENOLYTIC PATHWAY IN TROUT WHITE MUSCLE

The apparent deviation of the calculated mass action ratios from thermodynamic equilibrium in trout white muscle is presented in Fig. 3.1. It is clear that the mass action ratios of those reactions catalyzed by glycogen phosphorylase, Hk, PFK-1 and PK are more than two orders of magnitude displaced from thermodynamic equilibrium. Furthermore, no marked changes were apparent accompanying exercise except at the combined GAPDH-PGK and PGK catalysed steps (Fig. 3.1.).

GLYCOLYTIC INTERMEDIATES AND POTENTIAL CONTROL SITES

The glycolytic intermediates in white muscle in the pre- and two exercise states are presented in Table 3.4. In trout burst swimming for 10 min significant changes in the concentrations of glycolytic intermediates were observed, and potential control sites along the pathway were identified at Hk, PFK-1 and PK (Fig. 3.2.). A large crossover also appeared at the combined GAPDH-PGK step (Fig. 3.1.). According to this data, in conjunction with the thermodynamic structure of the pathway, it is reasonable to assume that the three most important regulatory enzymes of glycogenolysis in trout white muscle are glycogen phosphorylase, Hk, PFK-1 and perhaps PK. In those fish that underwent 30 min of
swimming, the glycolytic intermediates were extensively depleted, and no potential crossovers were apparent except that a greater magnification of change occurred at the combined GAPDH-PGK step (Table 3.4. and Fig. 3.2.).

**CYTOPLASMIC NAD/NADH RATIO AND MUSCLE pH**

The cytoplasmic NAD+/NADH ratios were calculated for the control and for the 10 and 30 min burst swimming trout, and the results obtained are presented in Table 3.5. The NAD+/NADH ratio increased 2.7 fold from 809 to 2176 in white muscle of the 10 min swimming fish, and increased 2.5 fold for the 30 min group.

Intracellular pH of white muscle was 6.93 in the pre-exercise state and decreased to 6.66 and 6.47 for the 10 and 30 minute burst swimming fish respectively (Table 3.3.).

**CYTOPLASMIC FREE ADP AND PHOSPHORYLATION STATE**

Free ADP could not be calculated using the combined GAPDH-PGK equilibrium expression because as we have already discussed the reaction was shown to deviate markedly from equilibrium with exercise. Alternatively, free cytoplasmic ADP was calculated using the creatine kinase equilibrium and summarized in Table (3.5.). In the pre-exercise state, free ADP in white muscle was 0.069 µ moles/g cell water, which represents about 8% or the total ADP content measured in neutralized PCA extracts (Table
Free ADP increased about 5 fold during exercise to 0.357 and 0.328 u moles/g cell water for the 10 and 30 min burst swimming trout, which represents 27 and 23% of the total ADP content respectively (Table 3.5.).

The phosphorylation potential in white muscle of trout was 4140 in the pre-exercise state and dramatically decreased to 205 and 110 for the 10 and 30 min burst swimming fish. Similarly, the ATP/ADP ratios decreased by over 90% to extremely low values in white muscle accompanying exercise.

**DISCUSSION**

**COUPLING PCr HYDROLYSIS TO MYOSIN ATPase**

A fundamental observation of the study on fish white skeletal muscle is the unequivocal demonstration that PCr hydrolysis precludes anaerobic glycogenolysis during near maximal myofibrillar ATP ase activity (Table 3.1.). A conservative estimate of the ATP turnover supported by PCr hydrolysis was 188 u moles ATP/g wet wt muscle/min (Table 3.2.). This value may be as high as 598 u moles/g wet wt/min, since the PCr concentrations in white muscle reported in Table 3.1. are considered serious underestimates of the pre-exercise state prior to the 10 sec sprint. There are several reasons for believing this: First, the pre-exercise values were determined on fish that had been captured by hand from black holding boxes. One inevitable consequence of this
procedure is that struggling results in a rapid loss of PCr, the rate of which has been shown to be proportional to the number of tail flips (Mommsen, Stanely and Dobson, unpublished data). No struggling occurred in the sprint fish during the transfer by net from the black box to the swim tunnel. Secondly, identical experiments carried out one week earlier on the same fish, but without sacrifice, revealed that the time to fatigue was 18 seconds. That 21 u moles PCr/g wet wt remained in white muscle after 10 sec (Table 3.1.) is entirely consistent with the view that about 40 u moles PCr/g would be present prior to the sprint. Thirdly, based on evidence from $^{31}$P NMR, PCr values for a variety of vertebrate FG skeletal muscles 'at rest' are in the range of about 27 to 30 u moles/g wet wt, which represents about 77% of the total PCr and Cr pool (Burt et al., 1976; Meyer et al., 1982; Kushmerick and Meyer, 1985). This important in vivo information from other vertebrate fast-twitch skeletal muscles is consistent with the predicted value of around 40 u moles/g wet wt for trout since the total PCr and Cr pool is a much higher 58 u moles/g wet wt (Table 3.1.). In addition, assuming the free $P_i$ concentration at rest is between 1 and 3 u moles/g wet wt (Kushmerick and Meyer, 1985), the analytically determined 15 u moles $P_i$/g muscle in trout added to the PCr value of 21 u moles/g also yields a similar estimate of around 40 u moles PCr/g wet wt in white muscle prior to the sprint.
COUPLING ANAEROBIC GLYCOGENOLYSIS TO MYOSIN ATPase

That anaerobic glycogenolysis cannot pace the near-maximum myofibrillar ATPase in trout white muscle was a significant finding of the present study. The ATP turnover supported by anaerobic glycogenolysis over 10 minutes of burst swimming was estimated to be 78 u moles/g wet wt/min (Table 3.2.). This rate of ATP generation agrees well with the value of 90 u moles ATP/g wet wt trout white muscle/min at 10°C calculated on the basis of the maximal enzyme activity of the rate controlling enzyme, PFK-1 (Yamamoto, work in progress). The estimate of 78 u moles ATP/g wet wt/min for trout white muscle is also consistent with the rate of ATP generation from anaerobic glycogenolysis determined for in the isolated frog gastocnemius following intense electrical stimulation (Cori, 1956).

BIOCHEMICAL CHARACTERIZATION OF THE GLYCOGENOLYTIC PATHWAY

There is little doubt that the development of fatigue or exhaustion in trout after 10 and 30 minutes of burst swimming was associated with the depletion of endogenous stores of muscle glycogen. That the onset of fatigue and exhaustion is linked to a near-depletion of muscle glycogen following high intensity
prolonged exercise has been reported for a number of vertebrates, including man (Gollnick et al., 1973; Armstrong et al., 1974; Baldwin et al., 1975; Gollnick, 1982). However, despite a similar etiology for the development of fatigue and exhaustion for the 10 and 30 min swimming trout respectively, a number of important differences were noted in the steady-state levels of their glycolytic intermediates, and the apparent functional integrity of the glycolytic pathway.

Potential control sites along the pathway were identified by crossover analysis at Hk, PFK-1 and PK for the 10 min burst swimming fish (Fig. 3.2.). Because glycogen was largely depleted no crossover appeared at this locus. Even though anaerobic utilization of blood-borne glucose cannot support the same myofibrillar ATPase activity as glycogen because of the low catalytic potential of Hk (Johnston, 1977), the apparent crossover at Hk indicates increased uptake and phosphorylation of glucose at this time. The possible role of glucose utilization will be discussed in the General Discussion Chapter 6. Facilitation of Hk activity may have occurred in response to the decrease in G 6-P (Table 3.4.), or in response to more subtle changes in the potent regulator, G 1,6-BP (Bietner, 1979).

Explaining the apparent crossover at PFK-1 is more challenging. The finding that an increase in F 6-P and a decrease in F 1,6-BP concentration occurred after 10 min indicates that F 6-P is being delivered faster than PFK-1 can catalyze its conversion to products. Often fatigue has been related to the pH dependent ATP inhibition of
PFK-1, but this does not appear to be the case for trout white muscle. The 54% decrease in ATP would be expected to offset the inhibitory effect of pH, particularly in the presence of a number of positive modulators, $P_i$, AMP and $NH_4^+$ (Table 3.3.). This proposal is also supported by the slight increase in the mass action ratio favouring F 1,6-BP formation.

In direct contrast to the 10 min burst swimming fish, no crossover points were detected at either Hk or PFK-1 for the 30 min group because of the large decreases in glycolytic intermediates in white muscle (Fig.3.2. and Table 3.3.). In fact, the sum of all of the glycolytic intermediates from G 6-P to pyruvate decreased by a dramatic 80% compared to the 25% decrease for the 10 min burst swimming fish. This near total depletion of glycolytic intermediates was accompanied by a 80% fall in ATP, a 20 fold reduction in the ATP/ADP ratio, a 38 fold reduction in the cytoplasmic phosphorylation potential and a 2.5 fold increase in the NAD+/NADH ratio (Table 3.5.). Obviously, in the absence of the appropriate compensatory mechanisms, these major metabolic perturbations at exhaustion must have a profound effect on the kinase reactions of PCr hydrolysis and glycogenolysis. Interestingly, the dramatic fall in ATP was stoichiometrically matched with a rise in IMP (Table 3.3.). That IMP accumulates in trout white muscle following exercise provides direct evidence for the activation of AMP deaminase, and operation of the purine nucleotide cycle in this species (Lowenstein, 1972). The has also
been shown for carp white muscle (Driedzic and Hochachka, 1976).

One interesting feature common to both fatigue states was the marked displacement of the combined GAPDH-PGK reaction from thermodynamic equilibrium (Figs. 3.1. and 3.2.). Because the combined GAPDH-PGK reaction is unique among the glycolytic steps by interacting with both the phosphorylation potential and redox couple (Scopes, 1973), reasons for the apparent shift from near-equilibrium with exercise are complex. Notwithstanding, one contributing factor appears to be the effect of change in the ATP/ADP ratio on the PGK equilibrium since the mass action ratio undergoes a major 20 and 41 fold reduction favoring 1,3-diphosphoglycerate formation for both the 10 and 30 minute swimming groups respectively (Fig. 3.1). Rovetto et al., (1975) proposed that inhibition of glycolysis in the ischemic myocardium was due to the effect of H+ ion and limiting supplies of NAD+ on GAPDH. This explanation does not apply for trout white muscle at the two activity states since the NAD+/NADH increased, not decreased (Table 3.5.). The 2 to 3-fold increase in the free cytosolic NAD+/NADH ratio means that NAD+ was being reduced by the GAPDH reaction at a slower rate than NADH was oxidized by LDH. The most likely explanation for this effect may be inhibition of PGK by the low ATP/ADP ratio which may have have an uncoupling effect on the GAPDH and LDH reactions, and in this way effect redox balance. Interestingly, the lactate/pyruvate ratio did not change significantly with exercise which means that the change in redox was largely affected by increasing H+ ion (Table 3.5.).
Lastly, another observation that was common to both activity states was the crossover indicated at the PK locus. However this apparent crossover can be explained as a consequence of the LDH equilibrium. This will be considered in more detail in the General Discussion Chapter 6.

The main findings of this chapter can be summarized as follows:

(i) During supra-maximal work rates, the myosin ATPase was shown to be supported solely by PCr hydrolysis. The ATP turnover was estimated to be between 188 and 598 u moles/g wet wt muscle/min.

(ii) Fatigue after 10 min was related to the near-depletion of glycogen in white muscle. The glycolytic pathway appeared functional at this time with control sites being identified at Hk and PFK-1. PFK-1 did not appear to be inhibited by low muscle pH (pH 6.66) and was not considered causal to the onset of fatigue. The preferential fuel at this time was exogenous glucose.

(iii) Total exhaustion after 30 min of burst swimming was similarly related to glycogen depletion, but differed from the 10 min by showing a dramatic 80% reduction in the sum of glycolytic intermediates from G 6-P to pyruvate. No control sites were identified along the pathway.

(iv) The ATP concentration dramatically decreased in white muscle by 54 and 80% following the 10 and 30 min burst swims to
fatigue and exhaustion respectively. The total nucleotide pool remained constant through the operation of the purine nucleotide cycle.

(v) In both fatigue states, the free cytosolic NAD\(^+\)/NADH ratio and ADP increased. These regulatory parameters were considered not to be limiting to glycogenolytic flux.

(vi) The free cytosolic ATP/ADP ratio and phosphorylation potential decreased by over 70 to 80%. This was due to the increase in free ADP and the decrease in ATP. Associated with these changes was a marked displacement of the PGK, and the combined GAPDH-PGK reactions from thermodynamic equilibrium.

(vii) The apparent crossover at PK was misleading and considered to be a consequence of the LDH equilibrium.
### TABLE 3.1. CHANGES IN FUEL AND NUCLEOTIDE CONCENTRATIONS IN TROUT FAST-TWITCH WHITE MUSCLE FOLLOWING A 10 SECOND SPRINT

<table>
<thead>
<tr>
<th>Activity</th>
<th>PCr</th>
<th>Cr</th>
<th>Total</th>
<th>Glycogen</th>
<th>Lactate</th>
<th>ATP</th>
<th>ADP</th>
<th>AMP</th>
<th>IMP</th>
<th>Total</th>
<th>P&lt;</th>
<th>NH₄⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-exercise</td>
<td>27.03</td>
<td>31.89</td>
<td>58.92</td>
<td>20.40</td>
<td>1.79</td>
<td>4.99</td>
<td>0.745</td>
<td>0.045</td>
<td>0.145</td>
<td>5.925</td>
<td>15.22</td>
<td>0.943</td>
</tr>
<tr>
<td>(4)</td>
<td>±1.29</td>
<td>±0.92</td>
<td>±2.09</td>
<td>±0.92</td>
<td>±0.43</td>
<td>±.26</td>
<td>±.081</td>
<td>±.009</td>
<td>±.024</td>
<td>±.425</td>
<td>±0.56</td>
<td>±.162</td>
</tr>
<tr>
<td>10 Sec Sprint</td>
<td>21.36</td>
<td>38.62</td>
<td>59.98</td>
<td>22.12</td>
<td>2.20</td>
<td>4.15</td>
<td>0.656</td>
<td>0.025</td>
<td>0.116</td>
<td>4.947</td>
<td>19.90</td>
<td>0.806</td>
</tr>
<tr>
<td>(6)</td>
<td>±0.76</td>
<td>±0.58</td>
<td>±0.73</td>
<td>±3.72</td>
<td>±0.37</td>
<td>±.85</td>
<td>±.034</td>
<td>±.003</td>
<td>±.029</td>
<td>±.377</td>
<td>±1.76</td>
<td>±1.124</td>
</tr>
<tr>
<td>(S) (S) (NS) (NS) (NS) (NS) (NS) (NS) (NS) (NS) (S) (NS)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Metabolite concentrations (µ moles/gm wet wt muscle) are given as the mean±SEM with the number of fish sampled in parentheses. Statistical significance (P) between the sprint and control group was evaluated using the two-tailed Student’s t-test. P<0.05 (S) Significant (NS) Not significant.
TABLE 3.2. ATP TURNOVER IN TROUT FAST-TWITCH WHITE MUSCLE ACCOMPANYING HIGH INTENSITY SWIMMING

<table>
<thead>
<tr>
<th>EVENT</th>
<th>ESTIMATED % VO_2 MAX</th>
<th>TIME TO FATIGUE</th>
<th>MAJOR FUEL UTILIZED</th>
<th>ATP TURNOVER u moles/g wet wt/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 sec Sprint (6)</td>
<td>150</td>
<td>18 ± 1.2</td>
<td>PHOSPHOCREATINE</td>
<td>188 - 598</td>
</tr>
<tr>
<td>10 min Burst  (6)</td>
<td>120</td>
<td>10 ± 2.1</td>
<td>GLYCOGEN</td>
<td>78</td>
</tr>
</tbody>
</table>

ATP turnover was calculated assuming 1 mole of ATP produced per mole of PCR hydrolysed, and 3 moles ATP produced per mole of glucosyl residues from glycogen. The number of fish sampled are in parentheses. Estimates of percentage maximal oxygen consumption were calculated on the basis of a critical velocity around 3 (see Materials and Methods).
TABLE 3.3  CHANGES IN FUEL, NUCLEOTIDE CONCENTRATION AND pH IN TROUT FAST-TWITCH WHITE MUSCLE FOLLOWING A 10 AND 30 MINUTE BURST SWIM TO FATIGUE AND EXHAUSTION

<table>
<thead>
<tr>
<th>Activity</th>
<th>PCr</th>
<th>Cr</th>
<th>Total</th>
<th>Glycogen</th>
<th>Lactate</th>
<th>ATP</th>
<th>ADP</th>
<th>AMP</th>
<th>IMP</th>
<th>Total</th>
<th>P&lt;</th>
<th>NH&lt;sub&gt;4&lt;/sub&gt;</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-exercise</td>
<td>19.83</td>
<td>31.50</td>
<td>51.33</td>
<td>22.40</td>
<td>5.76</td>
<td>7.334</td>
<td>0.738</td>
<td>0.063</td>
<td>0.304</td>
<td>8.439</td>
<td>25.72</td>
<td>1.030</td>
<td>6.93</td>
</tr>
<tr>
<td>(6)</td>
<td>+0.92</td>
<td>+0.87</td>
<td>+1.37</td>
<td>+1.68</td>
<td>+.49</td>
<td>+.291</td>
<td>+.034</td>
<td>+.017</td>
<td>+.080</td>
<td>+.355</td>
<td>+1.93</td>
<td>+1.40</td>
<td>+.02</td>
</tr>
<tr>
<td>10 Min Burst</td>
<td>1.56</td>
<td>49.62</td>
<td>51.18</td>
<td>1.33</td>
<td>35.88</td>
<td>3.350</td>
<td>1.072</td>
<td>0.271</td>
<td>4.087</td>
<td>8.773</td>
<td>46.75</td>
<td>3.581</td>
<td>6.66</td>
</tr>
<tr>
<td>(6)</td>
<td>+.59</td>
<td>+0.48</td>
<td>+2.29</td>
<td>+.61</td>
<td>+1.30</td>
<td>+.913</td>
<td>+.044</td>
<td>+.072</td>
<td>+.895</td>
<td>+.245</td>
<td>+2.81</td>
<td>+.508</td>
<td>+.02</td>
</tr>
<tr>
<td>(S) (S) (NS)</td>
<td>(S)</td>
<td>(S)</td>
<td>(S)</td>
<td>(S)</td>
<td>(S)</td>
<td>(S)</td>
<td>(S)</td>
<td>(S) (S)</td>
<td>(S) (S)</td>
<td>(NS)</td>
<td>(S)</td>
<td>(S) (S)</td>
<td>(S)</td>
</tr>
<tr>
<td>30 Minute Endurance</td>
<td>1.51</td>
<td>51.39</td>
<td>52.90</td>
<td>0.38</td>
<td>33.39</td>
<td>1.346</td>
<td>1.123</td>
<td>0.244</td>
<td>6.074</td>
<td>8.704</td>
<td>45.23</td>
<td>5.517</td>
<td>6.48</td>
</tr>
<tr>
<td>(6)</td>
<td>+.85</td>
<td>+1.76</td>
<td>+1.35</td>
<td>+.08</td>
<td>+1.58</td>
<td>+.138</td>
<td>+.068</td>
<td>+.032</td>
<td>+.464</td>
<td>+.626</td>
<td>+1.83</td>
<td>+.319</td>
<td>+.04</td>
</tr>
<tr>
<td>(S) (S) (NS)</td>
<td>(S)</td>
<td>(S)</td>
<td>(S)</td>
<td>(S)</td>
<td>(S)</td>
<td>(S)</td>
<td>(S)</td>
<td>(S) (S)</td>
<td>(S) (S)</td>
<td>(NS)</td>
<td>(S)</td>
<td>(S) (S)</td>
<td>(S)</td>
</tr>
</tbody>
</table>

Metabolite concentrations (u moles/g wet wt muscle) are given as the mean±SEM with the number of fish sampled in parentheses. Statistical significance (P) between the exercise and control fish was evaluated using the two-tailed Student's t-test. P<0.05 (S) Significant (NS) Not significant.
### TABLE 3.4. GLYCOLYTIC INTERMEDIATES IN TROUT FAST-TWITCH WHITE MUSCLE FOLLOWING 10 AND 30 MINUTES OF BURST SWIMMING TO FATIGUE AND EXHAUSTION

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Pre-exercise</th>
<th>10 Min</th>
<th>30 Min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>0.800±0.060</td>
<td>2.101±1.51 (S)</td>
<td>0.890±1.10 (NS)</td>
</tr>
<tr>
<td>Glucose 1-phosphate</td>
<td>0.175±0.039</td>
<td>0.076±0.009 (S)</td>
<td>0.025±0.006 (S)</td>
</tr>
<tr>
<td>Glucose 6-phosphate</td>
<td>0.455±0.077</td>
<td>0.279±0.118 (NS)</td>
<td>0.036±0.014 (S)</td>
</tr>
<tr>
<td>Fructose 6-phosphate</td>
<td>0.073±0.009</td>
<td>0.157±0.011 (S)</td>
<td>0.025±0.019 (S)</td>
</tr>
<tr>
<td>Fructose 1,6-bisphosphate</td>
<td>2.074±0.251</td>
<td>1.145±0.043 (S)</td>
<td>0.074±0.010 (S)</td>
</tr>
<tr>
<td>Dihydroxyacetone phosphate</td>
<td>1.001±0.009</td>
<td>0.559±0.073 (S)</td>
<td>0.330±0.030 (S)</td>
</tr>
<tr>
<td>Glyceraldehyde 3-phosphate</td>
<td>0.061±0.021</td>
<td>0.100±0.021 (NS)</td>
<td>0.118±0.021 (NS)</td>
</tr>
<tr>
<td>1,3-diphosphoglycerate</td>
<td>0.097±0.021</td>
<td>0.157±0.029 (NS)</td>
<td>0.127±0.018 (NS)</td>
</tr>
<tr>
<td>3-phosphoglycerate</td>
<td>0.661±0.096</td>
<td>0.597±0.137 (NS)</td>
<td>0.052±0.013 (S)</td>
</tr>
<tr>
<td>2-phosphoglycerate</td>
<td>0.062±0.007</td>
<td>0.048±0.007 (NS)</td>
<td>0.023±0.002 (S)</td>
</tr>
<tr>
<td>Phosphoenolpyruvate</td>
<td>0.062±0.007</td>
<td>0.052±0.007 (NS)</td>
<td>0.011±0.002 (S)</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.043±0.012</td>
<td>0.397±0.008 (S)</td>
<td>0.194±0.037 (S)</td>
</tr>
<tr>
<td>Lactate</td>
<td>5.76±0.49</td>
<td>35.9±1.3 (S)</td>
<td>33.4±1.6 (S)</td>
</tr>
</tbody>
</table>

Metabolite concentrations (μ moles/g wet wt muscle) are given as the mean±SEM (n = 6). Statistical significance (P) between the exercise and control group was evaluated using the two-tailed Student's t-test. P<0.05 (S) Significant (NS) Not significant.
### TABLE 3.5. CYTOSOLIC FREE ADP, ATP/ADP RATIO, PHOSPHORYLATION POTENTIAL AND REDOX STATE IN TROUT FAST-TWITCH WHITE MUSCLE FOLLOWING 10 AND 30 MINUTES OF BURST SWIMMING TO FATIGUE AND EXHAUSTION

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pre-exercise</th>
<th>10 Min</th>
<th>30 Min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free ADP</td>
<td>0.069±.001</td>
<td>0.357±.069 (S)</td>
<td>0.328±.070 (S)</td>
</tr>
<tr>
<td>% Total ADP</td>
<td>7.7± .41</td>
<td>26.5± 4.7 (S)</td>
<td>22.6± 3.8 (S)</td>
</tr>
<tr>
<td>ATP/ADP</td>
<td>133± 7.0</td>
<td>12± 4 . (S)</td>
<td>5.0± 1 . (S)</td>
</tr>
<tr>
<td>ATP/ADP P&lt; sub&gt;1&lt;/sub&gt;</td>
<td>4140± 489</td>
<td>205± 80 (S)</td>
<td>110± 18 . (S)</td>
</tr>
<tr>
<td>NAD+/NADH</td>
<td>809± 208</td>
<td>2176± 464 (S)</td>
<td>2053± 507 (S)</td>
</tr>
<tr>
<td>Lactate/Pyruvate</td>
<td>192± 53</td>
<td>129± 41 (S)</td>
<td>164± 27 (S)</td>
</tr>
</tbody>
</table>

The cytoplasmic free ADP concentration was calculated from the measured total tissue contents of the creatine kinase equilibrium (see Materials and Methods). All concentrations are expressed as u moles/g cell H<sub>2</sub>O (n = 6). The ATP/ADP ratio and phosphorylation potential (ATP/ADP P<sub>1</sub>) were calculated using free ADP. Statistical significance (P) between each exercise group and control fish was evaluated using the two-tailed Student's t-test. P<0.05 (S) Significant (NS) Not significant.
Fig. 3.1. Graphical representation of displacement of individual glycolytic reactions from thermodynamic equilibrium in trout fast-twitch white skeletal muscle. Thermodynamic equilibrium constants were determined at pH 7.0 and unit activity of water at 25°C. Unless otherwise specified equilibrium constants are from Burton (1957). (a) Noltmann (1972) (b) Bohme et al., (1975) (c) Connett (1985) (d) Veech et al., (1979). M.A.R. is the mass action ratio.
Fig. 3.2. Crossover plot of glycolytic intermediates in trout fast-twitch white skeletal muscle after 10 and 30 minutes of burst swimming to fatigue. Changes in glycolytic intermediates are expressed as a percentage of pre-exercise values.
CHAPTER 4: REGULATION OF ANAEROBIC GLYCOGENOLYSIS IN THREE TYPES OF RAT SKELETAL MUSCLE DURING EXERCISE
CHAPTER 4: INTRODUCTION

Having examined some aspects of regulation of anaerobic glycogenolysis in trout fast-twitch white muscle during both short- and long-term high intensity swimming to fatigue and exhaustion, an obvious extension was to investigate whether similar metabolic changes occurred in fast-twitch skeletal muscles of the rat during a similar set of exercise protocols. To a first approximation, the two muscle types most similar to trout white muscle in terms of biochemical and physiological properties are the two ankle extensors, the gastrocnemius and plantaris. The gastrocnemius is a mixed fast-twitch muscle comprising 58% FG, 38% FOG & 4% SO fiber types, while the plantaris is comprised of 53% FOG, 41% FG & 6% SO fibers (Ariano et al., 1973). A third muscle, the soleus, a SO fiber type, was selected to highlight any fiber specific mechanisms in the control of glycogenolysis that may occur during exercise and fatigue. Despite a great number of studies on the different biochemical strategies of the various muscle types of the rat to different running intensities and duration (Baldwin et al., 1973, Terjung et al., 1974; Baldwin et al., 1975; Fitts et al., 1982), no single study has focused in particular on the control of glycogenolysis at these different work intensities. The aim of this study was to investigate metabolic regulation of glycogenolysis in 3 different muscle types of the rat during a 2 min high intensity run, and following an endurance run to fatigue.
RESULTS

GLYCOGEN, PHOSPHOCREATINE AND ATP TURNOVER

Glycogen underwent approximately a 50% reduction in concentration in all three types of skeletal muscles following the 2 min high intensity run, and a dramatic 90 to 95% depletion following the endurance run to fatigue (Tables 4.1, 4.2 & 4.3). Assuming that 3 moles of ATP is produced per mole of glucosyl from glycogen to lactate, the ATP turnover was estimated for each muscle type and the results obtained presented in Table 4.4.

The concentration of PCr decreased by 50% in the gastrocnemius and plantaris, and by 30% in the soleus accompanying the 2 min run. Further percentage reductions in PCr were observed following the endurance run to fatigue with a 60 to 65% fall in the gastrocnemius and plantaris, and a modest 25% fall in the soleus (Tables 4.1, 4.2 & 4.3). The concentration of \( P_i \) increased significantly in the gastrocnemius and plantaris during both exercise protocols, but not in the soleus. As expected, the sum of both PCr & \( P_i \), and PCr & Cr concentrations for each muscle type remained constant with exercise.

NUCLEOTIDES

The concentration of ATP in the gastrocnemius and plantaris decreased only by 10 to 30%, with no significant change occurring in the soleus accompanying both running protocols. The total ADP
content in each muscle similarly did not change appreciably with exercise. AMP changed only slightly in all three muscle types with exercise. Because of the variable levels of IMP in each muscle at the pre-exercise state, larger percentage increases were observed in the gastrocnemius than in plantaris after the 2 min run. Greater increases were apparent following the endurance run with a 30 fold increase in IMP in the gastrocnemius and a 5 fold increase in the plantaris. No significant change in IMP concentration occurred in the soleus with exercise. The total adenine nucleotide pool remained constant in all three muscles. NH$_4^+$ concentration underwent a 2 to 4 fold increase in the soleus and gastrocnemius muscles respectively after the 2 min high intensity run but no significant change occurred in the plantaris. Endurance running to fatigue was associated with higher NH$_4^+$ levels in the gastrocnemius (a 7 fold increase) and plantaris (a 2 fold increase), but not in the soleus (Tables 4.1., 4.2. & 4.3).

**THERMODYNAMIC STRUCTURE OF THE GLYCOGENOLYTIC PATHWAY IN RAT SKELETAL MUSCLE**

Graphical representation of individual reactions of glycolysis showing their relative displacement from thermodynamic equilibrium in rat gastrocnemius is shown in Fig.4.1. The mass action ratios of those reactions catalyzed by HK and PFK-1 are displaced by over five orders of magnitude from thermodynamic equilibrium, while those catalyzed by glycogen phosphorylase and
PK are displaced by about two orders of magnitude. This thermodynamic structure of glycolysis did not appreciably change with exercise (Fig. 4.1.). Similar thermodynamic profiles were observed for the plantaris and soleus muscles, and have not been included.

**GLYCOLYTIC INTERMEDIATES AND POTENTIAL CONTROL SITES**

The glycolytic intermediates of rat gastrocnemius, plantaris and soleus muscles at the three activity states are presented in Tables 4.5., 4.6. & 4.7. During the 2 min high intensity, and endurance run to fatigue, major changes were observed in many of the glycolytic intermediates and potential control sites were identified at glycogen phosphorylase and PFK-1 in all three muscle skeletal muscles following the 2 min run, but only at HK following the endurance run to fatigue (Figs. 4.2., 4.3. 4.4).

**CYTOPLASMIC NAD/NADH RATIO AND MUSCLE pH**

The cytoplasmic NAD+/NADH ratio for rat gastrocnemius and plantaris is presented in Tables 4.8. &4.9. The NAD+/NADH ratio decreased by about 50 to 70% in both muscles accompanying the 2 min high intensity run. This pattern of change was not significantly altered following the endurance run to fatigue. That is, despite the plantaris becoming more reduced and statistically significant relative to the control state, when compared with the
gastrocnemius at either of the two exercise states significance was lost due to the large standard errors ($P < 0.05$) [Table 4.9]. The decrease in the NAD$^+/NADH$ ratio was accompanied by a five fold increase in the lactate/pyruvate ratio in both gastrocnemius and plantaris muscles following each of the exercise protocols. A smaller 2-fold increase in the lactate/pyruvate ratio was observed in the soleus after the 2 min run, and a 3 to 4-fold increase after the endurance run (Table 4.10.)

Intracellular pH decreased from 6.92 to about 6.6 in both the gastrocnemius and plantaris skeletal muscles following the 2 min high intensity run, and to about 6.7 following the endurance run to fatigue (Tables 4.1., 4.2. & 4.3). Intracellular pH was not measured in the soleus.

**CYTOPLASMIC FREE ADP AND PHOSPHORYLATION STATE**

Free cytoplasmic ADP was calculated using the GAPDH-PGK-LDH equilibrium. In the pre-exercise state, free ADP was 0.060, 0.041 and 0.037 μ moles/g cell water for the gastrocnemius, plantaris and soleus muscles respectively (Tables 4.8., 4.9. & 4.10.) These values represent about 5, 4 and 4% of the total ADP content measured (Tables 4.1., 4.2. & 4.3.). During the 2 min high intensity run, free cytoplasmic ADP increased about 3 fold in each of the three muscles, and between 4 to 6-fold following the endurance run to fatigue. In the latter group, these concentrations represent 29, 14 and 25% of the total ADP content measured. Free
ADP was not estimated using the CPK equilibrium because intracellular pH was not measured in the soleus.

The cytoplasmic ATP/ADP ratios, and phosphorylation potentials were calculated using the free ADP from the combined GAPDH-PGK expression. The values obtained are summarized in Tables (4.8., 4.9. & 4.10.). In the pre-exercise state, the ATP/ADP ratio was 137, 175 and 127 for the gastrocnemius, plantaris and soleus muscles respectively. In all muscles, the ATP/ADP ratio decreased by about 70% following the 2 min high intensity run, and by over 80% following the endurance run. Similarly, the phosphorylation potential (ATP/ADP P_i) dramatically decreased in each muscle following exercise but larger percentage drops occurred in the gastrocnemius and plantaris (Tables 4.8., 4.9. & 4.10.).

**DISCUSSION**

**COUPLING GLYCOGENOLYSIS TO MYOSIN ATPase**

Estimates of the ATP turnover supported by glycogenolysis in rat gastrocnemius, plantaris and soleus following 2 min of high intensity running were calculated to be 30, 28, and 16 u moles/g wet wt muscle/min respectively (Table 4.4.). These values are conservative estimates and assume that the generation of ATP by glycogenolysis was totally anaerobic. If however there was a small but significant aerobic component to glycogen breakdown, the
estimates for each respective muscle would be considerably higher. This difference between the anaerobic and aerobic catabolism of glycogen is due to the higher ATP yield on a molar basis accompanying complete oxidation (McGilvery, 1983). If we make the assumption that 10% of the glycogen utilized was completely oxidized, the ATP generated per unit time would be 64, 61 and 34 u moles ATP/g wet wt/min for the gastrocnemius, plantaris and soleus respectively (Table 4.4.). This range of ATP turnovers calculated for the different rat skeletal muscles during short-term high intensity running is well within the theoretical maximum values predicted on the basis of in vitro maximal velocities of PFK-1. The maximum velocity of PFK-1 for rat plantaris is about 100 u moles F 1,6BP formed/gm wet wt muscle/min (unit) at 30°C, and about 23 units for the soleus (Baldwin et al., 1982). Because of similar percentages of fast-twitch fibers, the PFK-1 activity for the gastrocnemius was assumed similar to plantaris. After making the appropriate temperature correction assuming a Q10 of 1.8, the maximum rate of ATP generation from glycogenolysis would be around 492 u moles ATP/g/min for plantaris and gastrocnemius, and around 113 u moles ATP/g/min for the soleus at 38°C.
VALIDITY OF USING ELECTRICAL STIMULATION STUDIES IN INTERPRETING DIFFERENT MUSCLE RECRUITMENT PATTERNS DURING EXERCISE

Using the perfused rat hindquarter preparation, Spriet et al., (1985) demonstrated similar percentage decreases in glycogen content from the gastrocnemius and plantaris following 5 min of high frequency stimulation, but found no significant change occurring in the soleus. The soleus has long been considered an important postural muscle in the rat (see Armstrong and Laughlin, 1985), but the contrasting findings of the present study, and those of others (Gardiner et al., 1982) demonstrate unequivocally that the soleus is recruited to a very large extent during high speed locomotion. The marked discrepancy between these results and those obtained using the isolated rat hindlimb preparation is believed due to the different muscle recruitment patterns and running gaits accompanying quadrupedal terrestrial locomotion. A point of emphasis is that care should be taken when extrapolating biochemical and physiological information from in situ electrical stimulation studies to whole animal exercise performance.

REGULATION OF GLYCOGENOLYSIS DURING 2 MIN OF HIGH INTENSITY RUNNING

Using crossover analysis to identify the potential control sites along the pathway, it was apparent that glycogen phosphorylase and
PFK-1 are pivotal in coordinating glycogenolytic flux with the ATP requirements of the myofibrillar ATPase (Figs. 4.2., 4.3., & 4.4). One way that these enzymes achieve such a high degree of control over fuel selection is via the branchpoint regulation of G 6-P (Lueck and Fromm, 1974). That the concentration of G 6-P significantly increased in gastrocnemius, plantaris and soleus during the 2 min run (Tables 4.5., 4.6. & 4.7.), attests to the importance of this key metabolite to inhibit Hk, and select glycogen as principal fuel. This increase in G 6-P has also been confirmed in single rat skeletal muscle fibers accompanying electrical stimulation (Hintz et al., 1982). Furthermore, accumulation of glucose in all three muscles without an apparent crossover during the 2 min run also indicates inhibition of Hk at this time (Figs 4.2., 4.3. & 4.4. and Tables 4.5., 4.6. & 4.7.). Notwithstanding, an increase in G 1,6-BP may also be important in regulating the activity of Hk (Bietner, 1979).

PFK-1 not only regulates the concentration of G 6-P, but on the basis of crossover analysis showing that F 6-P rises and F 1,6-BP decreases in all three muscles accompanying an increase in glycogenolytic flux, suggests that indeed this is a major rate controlling step of the pathway. According to the original formulation of the crossover theorem of Chance and Williams (1956), an increase in substrate concentration and a decrease in product at one site along a multi-enzyme pathway indicates inhibition at this locus. However, this theorem as discussed in the Materials and Methods, Section 1, was originally proposed for the sequence of carriers in the mitochondrial electron transport chain.
where the total concentration of each substrate-product pair is conserved at all times. This property does not hold for the glycolytic pathway, particularly during high flux rates where control is leveled at multiple sites located at strategic points along the pathway. Thus a substrate-product pair at any one locus along the glycolytic pathway may vary in concentration at any time depending on the activity state of muscle. From this study it is clear that inhibition is not occurring at the PFK-1 locus, and that activation in this case is characterized by F 6-P rising in parallel with flux. This proposal supports the early work of Bucher and Russman (1964), later to be confirmed by Wilson et al., (1967) and Edington et al., (1973). An important question to consider is how does F 6-P increase during high flux rates? The most obvious reason is that glycogen phosphorylase is regulated to deliver F 6-P faster than PFK-1 can convert it to product. This makes a great deal of sense because an increase in F 6-P not only activates but more importantly stabilizes PFK-1 against pH-dependent ATP inhibition (Passoneau and Lowry, 1962; Mansour, 1972). The finding that F 6-P increases is also supported on theoretical grounds in light of glycogen phosphorylase having a greater maximal activity compared to PFK-1 (Harris et al., 1976; Baldwin et al., 1982). Thus facilitation of PFK-1 activity is probably brought about by feedforward activation by F 6-P, together with the complex interaction between the long list of positive modulators (Sols, 1981; Bosca et al., 1985).

Superimposed on this basic system of metabolic control by
glycogen phosphorylase and PFK-1, are the ADP-requiring enzymes of PGK and PK. In order that glycogenolysis and myosin ATPase are tightly coupled in skeletal muscle during high work rates, both the activities of PGK and PK must be paced with the rate of product formation of ADP from ATP hydrolysis. The activity of PGK may further be controlled and coordinated by the non-equilibrium PK enzyme through regulation of metabolite levels which would have an impact further back along the pathway via the series of near-equilibrium catalyzed reactions.

During the 2 min high intensity run the free cytosolic ADP concentration increased about 3 fold in each of the rat skeletal muscles (Tables 4.5., 4.6. & 4.7.). An important consequence of free ADP rising is that both the cytosolic ATP/ADP ratio and phosphorylation potential decrease. Following the 2 min run, the ATP/ADP ratio decreased by about 70 to 80% in the three muscles. Similar percentage decreases were seen in the phosphorylation potential. These ratios are important not only in the regulation of mitochondrial phosphorylation (Slater, 1976), but provide a direct index between ATP hydrolysis and the ATP generating mechanisms of glycogenolysis in the cytosol. This study demonstrates that glycogenolytic flux in skeletal muscle can be sustained in the face of wide variations of both the ATP/ADP ratio and phosphorylation potential. However, even though the rats were not fatigued after 2 minutes, all indications on the basis of these low potentials suggest that fatigue was fast approaching. This prediction was borne out in a parallel set of validation studies carried out on
individuals from the same general stock running the same treadmill protocol. This experiment showed that a marked reduction in performance or fatigue occurred between 2 min 15 and 2 min 30 sec.

Accompanying the fall in ATP in the gastrocnemius and plantaris, was a stoichiometric rise in IMP and NH₄⁺. During exercise this irreversible reaction in skeletal muscle is catalyzed by the allosteric enzyme, AMP deaminase (Lowenstein, 1972). The maximal activity of AMP deaminase in rat skeletal muscle is about 200 μmol/g wet wt/min at pH 7.0 and 38°C (Goodman and Lowenstein, 1977). The reaction is believed to be activated by the dual effect of ADP and AMP availability via the myokinase equilibrium, and by the increase in H⁺ ions generated from the hydrolysis of ATP at the myosin S1 head (Sahlin, 1978; Dudley and Terjung, 1985). That no significant change occurred in the total AMP content with exercise in either the gastrocnemius and plantaris was not surprising, since like ADP, a large fraction of the total AMP is bound, and it is the free concentration that is catalytically important (Veech et al., 1979). Using the free ADP estimates shown in Table 4.8. & 4.9., the concentration of AMP can be calculated using the myokinase equilibrium according to the following expression: \[ \text{AMP}_{\text{free}} = \frac{[\text{ADP}]^2}{\text{ATP}} \times \text{Keq}, \] where the Keq is 1.05 at 38°C, 1 mM free Mg²⁺ at an ionic strength of 0.25 (see Dudley and Terjung, 1985). Using this method, the free AMP increased from 0.42 to 4.0 n mol/g cell water in the gastrocnemius, and from 0.22 to 2.6 n mol/g cell water in the plantaris accompanying the 2 min
run. The 10 fold increase in the concentration of free AMP following short-term high intensity running, together with the increase in $H^+$ ion (a pH of about 6.62, Tables 4.1. & 4.2.) attests to the importance of both these factors in activating AMP deaminase at this time. If the myokinase reaction is indeed in equilibrium, as was assumed in the above calculations, then the freely available AMP accounts for only 0.5 and 1.4% of the total content of AMP in plantaris and gastrocnemius respectively at the pre-exercise state. These values increase to 6 and 14% of the total AMP respectively following the 2 min run. Thus it appears that virtually all the AMP in the muscle cell is compartmentalized away and not available to either the myokinase or AMP deaminase. However, unlike the well known binding of ADP to actin, there is no readily identifiable structural protein to which AMP binds in skeletal muscle.

In recovery, many studies have shown that the adenine nucleotides in rat skeletal muscle are replenished via reamination of IMP through the action of adenolsuccinate synthetase and adenylsuccinate lyase (Aragon and Lowenstein, 1980). These two reactions together with the AMP deaminase reaction constitute the purine nucleotide cycle (Lowenstein, 1972). Although several functions have been attributed to the purine nucleotide cycle, recent evidence has shown that in rat skeletal muscle the complete cycle does not operate during moderate to intense contractile activity (Meyer and Terjung, 1979, 1980). Many studies have viewed the metabolic importance of the cycle's operation in muscle as (i) maintenance of a high ATP/ADP ratio, (ii) maintenance of
glycogenolytic flux through modulation of PFK-1 by NH₄⁺ ion, and (iii) 'spark' the Krebs cycle by delivering fumarate during steady-state work or in recovery (Lowenstein, 1972, Meyer and Terjung, 1979, Dudley and Terjung, 1985). While not doubting the importance of these functions, the present author's interpretation for the primary function of the purine nucleotide cycle in fast-twitch skeletal muscle is to provide a sink for the loss of adenine nucleotide that occurs when the muscle fails to balance ATP supply to ATP usage during high rates of muscle contraction. It performs this vital function by forming a relatively metabolically inert compound, IMP. It is interesting that in heart muscle where a tight coupling between ATP supply and demand is maintained at all times despite large fluctuations in workload, the purine nucleotide cycle has no functional importance (Taetgmeyer, 1985). Thus the role of the purine nucleotide cycle in fast-twitch skeletal muscle to control glycogenolysis or to provide fumarate to the Krebs cycle should be viewed as secondary consequences of the primary function.

In direct contrast to these changes described for rat fast-twitch skeletal muscle, the ATP remained constant in the soleus (Table 4.3.). This has also been confirmed by electrical stimulation studies of Meyer and Terjung (1979) who showed that on intense stimulation of an isolated rat soleus even after the blood supply had been severely restricted, ATP falls only marginally with very little change in IMP. It appears therefore that unlike fast-twitch muscles, the soleus has a number of protective
mechanisms like the myocardium that ensure the ATP concentration is defended despite high workloads.

As discussed in the general introduction and Chapter 3, control of glycogenolysis is also mediated by the cytosolic redox state. During the 2 min high intensity run, the NAD⁺/NADH ratio decreased in both the gastrocnemius and plantaris (Tables 4.8. & 4.9.). This decrease was accompanied by an increase in the lactate/pyruvate ratio. The disproportionate increase in the lactate/pyruvate ratio compared to the concomitant decrease in redox potential is due to the effect of increasing H⁺ ion on the LDH equilibrium. Similar conclusions have been forwarded for rat skeletal muscle (Aragon and Lowenstein, 1980) and for human muscle following short-term high intensity exercise (Sahlin et al., 1976).

CONTROL OF THE GLYCOGENOLYTIC PATHWAY AFTER ENDURANCE RUNNING TO FATIGUE

It was clear from the present study that the etiology of fatigue after 30 min of endurance running was similar to the 30 min endurance swim of the trout and linked to the near total depletion of muscle glycogen. The concentration of glycogen decreased by 94, 92 and 88% in the gastrocnemius, plantaris and soleus muscles respectively (Tables 4.1., 4.2. & 4.3.). That low glycogen and high lactate content was measured in all three muscles further demonstrates the large dependence of each muscle
type to this exercise intensity, as indeed was the case for the 2 min run.

On the basis of crossover analysis no control points were located at glycogen phosphorylase and PFK-1 in the 3 skeletal muscles examined (Figs. 4.2, & 4.3.), with the possible exception of the soleus (Fig. 4.4.). An apparent crossover may have occurred at the PFK-1 locus in soleus but the small percentage changes in F 6-P and F 1,6-BP relative to control levels make it difficult to draw any firm conclusions. One obvious control site however was apparent at the Hk locus in all three muscle types. Enhanced uptake and phosphorylation of glucose through facilitation of Hk may be linked to either (i) Glycogen replenishment through the 3 step conversion from G 6-P to G 1-P, G 1-P to uridine diphosphate glucose, and finally the transfer of glucosyl units into the residues of the glycogen complex, or (ii) catabolism of glucose to pyruvate which itself can either be converted to lactate or undergo complete oxidation to CO₂ and H₂O.

Accompanying the large reductions in the levels of glycolytic intermediates in the fast-twitch rat skeletal muscles, and to a lesser extent in the soleus, were large increases in free ADP (Tables 4.8., 4.9. & 4.10.). On the basis of these findings it was clear that fatigue was not related to ADP availability. However, the dramatic fall in both the ATP/ADP ratio and phosphorylation potential must obviously have had a significant effect on the control of glycogenolysis. The redox changes accompanying fatigue were also dramatic, with the muscle becoming more reduced. In
other words, NAD$^+$ may have become limiting to the GAPDH reaction and glycolysis at this time.

The main findings of this chapter can be summarized as follows:

(i) During 2 min of high intensity, and endurance running to fatigue, the 50% decrease in muscle glycogen and high lactate levels in the fast- and slow-twitch fibers attests to the importance of recruiting anaerobic glycogenolysis to support myosin ATPase. Unlike trout white muscle, the rapid efflux of lactate from rat skeletal muscle during exercise (Hermansen, 1981) makes it more difficult to assess the exact percentage contribution of anaerobic glycogenolysis to muscle work.

(ii) Coordination of glycogenolytic flux during the 2 min run was achieved through the hierarchical regulation of glycogen phosphorylase and PFK-1. In this way, F 6-P was shown to increase with flux. As a point of emphasis, PFK-1 was totally operational in the fast-twitch muscles despite a muscle pH of 6.6.

(iii) Maintenance of glycogenolytic flux and running performance over the 2 min period was accompanied by a 3 fold increase in free cytosolic ADP, a 70 to 80% fall in the ATP/ADP ratio and phosphorylation potential, and a 50% decrease in the NAD$^+/$/NADH ratio. Unlike fish white muscle the lactate/pyruvate ratios significantly increased. A slight fall in ATP occurred in both fast-twitch muscles and was matched with a stiochiometric rise in
IMP. No change in ATP occurred in the soleus despite its large anaerobic contribution to muscle work.

(iv) The etiology of fatigue following the endurance run was the near-depletion of muscle glycogen in each of the three muscle types. Crossover analysis revealed facilitation of Hk at this time with the concomitant switch in fuel from glycogen to exogenous glucose. The concentration of ATP continued to fall while free ADP continued to rise leading to further reductions in the ATP/ADP ratio and phosphorylation potentials. The NAD+/NADH ratio was low in gastrocnemius and plantaris but not statistically different from the 2 min run.
TABLE 4.1. CHANGES IN FUEL, NUCLEOTIDE CONCENTRATION AND pH IN RAT GASTROCNEMIUS SKELETAL MUSCLE FOLLOWING A 2 MINUTE HIGH INTENSITY, AND ENDURANCE RUN TO FATIGUE

| Activity          | PCr (u moles/g wet wt muscle) | Cr (u moles/g wet wt muscle) | Total (u moles/g wet wt muscle) | Glycogen (u moles/g wet wt muscle) | Lactate (u moles/g wet wt muscle) | ATP (u moles/g wet wt muscle) | ADP (u moles/g wet wt muscle) | AMP (u moles/g wet wt muscle) | IMP (u moles/g wet wt muscle) | Total (u moles/g wet wt muscle) | P< sub i > (u moles/g wet wt muscle) | NH<sub>4</sub> (u moles/g wet wt muscle) | pH |
|-------------------|-------------------------------|------------------------------|---------------------------------|------------------------------------|-----------------------------------|---------------------------------|-------------------------------|--------------------------------|-------------------------------|-------------------------------|---------------------------------|-----------------------------------|-----------------|------|
| Pre-exercise      | 17.71                         | 28.96                        | 46.67                           | 38.56                              | 2.58                              | 6.570                           | 0.915                         | 0.031                          | 0.046                         | 7.56                          | 14.10                           | 0.231                         | 6.92 |
| (5)               | +.42                          | +.17                         | +.34                            | +2.19                              | +.57                              | +.077                           | +.036                         | +.005                          | +.007                          | +.05                          | +.28                             | +.057                         | +.04 |
| 2 Min Run         | 9.83                          | 39.02                        | 48.85                           | 18.39                              | 13.26                             | 5.301                           | 0.864                         | 0.029                          | 0.654                         | 6.85                          | 19.47                           | 1.000                         | 6.63 |
| (5)               | +.65                          | +.81                         | +.55                            | +1.50                              | +1.60                             | +.318                           | +.026                         | +.004                          | +1.196                         | +.34                          | +.092                            | +.253                         | +.04 |
| 30 Minute         | 6.37                          | 42.66                        | 49.03                           | 2.26                               | 9.56                              | 4.710                           | 0.892                         | 0.035                          | 1.368                         | 7.01                          | 26.35                           | 1.680                         | 6.71 |
| Endurance         | +.98                          | +1.3                         | +.99                            | +.19                               | +2.35                             | +.453                           | +.033                         | +.004                          | +.308                         | +.21                          | +1.21                            | +.121                         | +.04 |
| (6)               | (S)                           | (S)                          | (NS)                            | (S)                                | (S)                               | (S) (NS)                        | (NS)                          | (S) (NS)                        | (S) (NS)                        | (S) (NS)                       | (S) (NS)                         | (S) (S)                        | (S) |

Metabolite concentrations (u moles/g wet wt muscle) are given as the mean±SEM with the number of rats sampled in parentheses. Statistical significance (P) between each exercise and control group was evaluated using the two-tailed Student's t-test. P<0.05 (S) Significant (NS) Not significant.
### Table 4.2. Changes in Fuel, Nucleotide Concentration and pH in Rat Plantaris Skeletal Muscle Following a 2 Minute High Intensity, and Endurance Run to Fatigue

<table>
<thead>
<tr>
<th>Activity</th>
<th>Phosphocreatine</th>
<th>Glycogen</th>
<th>Nucleotide and Phosphate Pool</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCr</td>
<td>Cr</td>
<td>Total</td>
<td>Glycogen</td>
</tr>
<tr>
<td>Pre-exercise</td>
<td>15.50</td>
<td>25.74</td>
<td>41.24</td>
<td>32.90</td>
</tr>
<tr>
<td>(6)</td>
<td>±1.49</td>
<td>±1.03</td>
<td>±2.13</td>
<td>±1.19</td>
</tr>
<tr>
<td>2 Min Run</td>
<td>8.39</td>
<td>35.24</td>
<td>43.63</td>
<td>13.99</td>
</tr>
<tr>
<td>(6)</td>
<td>±.99</td>
<td>±1.50</td>
<td>±1.00</td>
<td>±1.21</td>
</tr>
<tr>
<td>(S) (S) (NS) (S)</td>
<td>(NS) (NS) (NS) (NS)</td>
<td>(S) (S)</td>
<td>(NS) (NS) (NS) (NS)</td>
<td>(S)</td>
</tr>
<tr>
<td>30 Minute</td>
<td>5.67</td>
<td>38.57</td>
<td>44.24</td>
<td>2.53</td>
</tr>
<tr>
<td>Endurance</td>
<td>±.90</td>
<td>±1.51</td>
<td>±1.41</td>
<td>±.15</td>
</tr>
<tr>
<td>(5)</td>
<td>(S) (S)</td>
<td>(NS)</td>
<td>(S) (S)</td>
<td>(S) (S)</td>
</tr>
</tbody>
</table>
### TABLE 4.3. CHANGES IN FUEL AND NUCLEOTIDE CONCENTRATIONS IN RAT SOLEUS SKELETAL MUSCLE FOLLOWING A 2 MINUTE HIGH INTENSITY, AND ENDURANCE RUN TO FATIGUE

<table>
<thead>
<tr>
<th>Activity</th>
<th>PHOSPHOCREATINE</th>
<th>GLYCOGEN</th>
<th>NUCLEOTIDE AND PHOSPHATE POOL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCr</td>
<td>Cr</td>
<td>Total</td>
</tr>
<tr>
<td>Pre-exercise (5)</td>
<td>9.29</td>
<td>22.11</td>
<td>31.40</td>
</tr>
<tr>
<td></td>
<td>±.93</td>
<td>±4.36</td>
<td>±1.85</td>
</tr>
<tr>
<td>2 Min Run (6)</td>
<td>6.59</td>
<td>25.94</td>
<td>32.53</td>
</tr>
<tr>
<td></td>
<td>±.75</td>
<td>±1.65</td>
<td>±1.58</td>
</tr>
<tr>
<td></td>
<td>(S)</td>
<td>(NS)</td>
<td>(NS)</td>
</tr>
<tr>
<td>30 Minute Endurance (5)</td>
<td>6.87</td>
<td>24.86</td>
<td>31.73</td>
</tr>
<tr>
<td></td>
<td>±.24</td>
<td>±1.27</td>
<td>±1.20</td>
</tr>
<tr>
<td></td>
<td>(S)</td>
<td>(NS)</td>
<td>(NS)</td>
</tr>
</tbody>
</table>

Metabolite concentrations (umoles/g wet wt muscle) are given as the mean±SEM with the number of rats sampled in parentheses. Statistical significance (P) between each exercise and control group was evaluated using the two-tailed Student's t-test. P<0.05 (S) Significant (NS) Not significant.
## Table 4.4. Estimates of ATP Turnover Supported by Glycogenolysis in Three Different Rat Skeletal Muscles During a 2 Minute High Intensity Run

<table>
<thead>
<tr>
<th>Muscle Type</th>
<th>100% Conversion to Lactate</th>
<th>90% Conversion to Lactate</th>
<th>10% Complete Oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastrocnemius</td>
<td>30</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>Plantaris</td>
<td>28</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>Soleus</td>
<td>16</td>
<td>34</td>
<td></td>
</tr>
</tbody>
</table>

ATP turnover was calculated assuming that 3 moles of ATP are produced per mole glucosyl unit from glycogen to lactate, and that 37 moles of ATP are formed per mole of glucosyl unit from glycogen to CO₂ and H₂O. For details see Discussion.
TABLE 4.5. GLYCOLYTIC INTERMEDIATES IN RAT GASTROCNEUMUS SKELETAL MUSCLE FOLLOWING A 2 MINUTE HIGH INTENSITY, AND 30 MINUTE ENDURANCE RUN TO FATIGUE

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Pre-exercise (5)</th>
<th>2 Min (5)</th>
<th>30 Min (6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>0.467±.052</td>
<td>1.080±.050 (S)</td>
<td>0.938±.146 (S)</td>
</tr>
<tr>
<td>Glucose 1-phosphate</td>
<td>0.044±.009</td>
<td>0.079±.013 (S)</td>
<td>0.029±.003 (NS)</td>
</tr>
<tr>
<td>Glucose 6-phosphate</td>
<td>0.784±.171</td>
<td>1.870±.250 (S)</td>
<td>0.458±.052 (NS)</td>
</tr>
<tr>
<td>Fructose 6-phosphate</td>
<td>0.137±.073</td>
<td>0.367±.062 (S)</td>
<td>0.095±.011 (NS)</td>
</tr>
<tr>
<td>Fructose 1,6-bisphosphate</td>
<td>0.348±.031</td>
<td>0.111±.021 (S)</td>
<td>0.049±.010 (S)</td>
</tr>
<tr>
<td>Dihydroxyacetone phosphate</td>
<td>0.062±.005</td>
<td>0.048±.006 (S)</td>
<td>0.028±.003 (S)</td>
</tr>
<tr>
<td>Glyceraldehyde 3-phosphate</td>
<td>0.028±.005</td>
<td>0.017±.002 (NS)</td>
<td>0.016±.001 (S)</td>
</tr>
<tr>
<td>1,3-diphosphoglycerate</td>
<td>0.030±.006</td>
<td>0.024±.001 (NS)</td>
<td>0.037±.011 (NS)</td>
</tr>
<tr>
<td>3-phosphoglycerate</td>
<td>0.182±.014</td>
<td>0.130±.011 (S)</td>
<td>0.120±.006 (S)</td>
</tr>
<tr>
<td>2-phosphoglycerate</td>
<td>0.013±.001</td>
<td>0.011±.004 (NS)</td>
<td>0.016±.004 (NS)</td>
</tr>
<tr>
<td>Phosphoenolpyruvate</td>
<td>0.048±.004</td>
<td>0.029±.007 (S)</td>
<td>0.029±.005 (S)</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.089±.009</td>
<td>0.091±.009 (NS)</td>
<td>0.063±.006 (S)</td>
</tr>
<tr>
<td>Lactate</td>
<td>2.58±.57</td>
<td>13.26±1.60 (S)</td>
<td>9.56±2.40 (S)</td>
</tr>
</tbody>
</table>

Metabolite concentrations (u moles/g wet wt muscle) are given as the mean±SEM with the number of rats sampled in parentheses. Statistical significance (P) between each exercise and control group was evaluated by using the two-tailed Student's t-test. P <0.05 (S) Significant (NS) Not significant.
<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Pre-exercise (6)</th>
<th>2 Min (6)</th>
<th>30 Min (5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>0.460±.022</td>
<td>0.688±.147 (S)</td>
<td>0.649±.010 (S)</td>
</tr>
<tr>
<td>Glucose 1-phosphate</td>
<td>0.065±.008</td>
<td>0.079±.007 (NS)</td>
<td>0.038±.001 (S)</td>
</tr>
<tr>
<td>Glucose 6-phosphate</td>
<td>0.525±.038</td>
<td>0.678±.047 (S)</td>
<td>0.249±.029 (S)</td>
</tr>
<tr>
<td>Fructose 6-phosphate</td>
<td>0.211±.019</td>
<td>0.300±.024 (S)</td>
<td>0.092±.009 (S)</td>
</tr>
<tr>
<td>Fructose 1,6-bisphosphate</td>
<td>0.296±.033</td>
<td>0.186±.021 (S)</td>
<td>0.063±.006 (S)</td>
</tr>
<tr>
<td>Dihydroxyacetone phosphate</td>
<td>0.074±.003</td>
<td>0.058±.004 (S)</td>
<td>0.028±.001 (S)</td>
</tr>
<tr>
<td>Glyceraldehyde 3-phosphate</td>
<td>0.037±.006</td>
<td>0.023±.004 (NS)</td>
<td>0.026±.003 (NS)</td>
</tr>
<tr>
<td>1,3-diphosphoglycerate</td>
<td>0.029±.006</td>
<td>0.023±.003 (NS)</td>
<td>0.023±.004 (NS)</td>
</tr>
<tr>
<td>3-phosphoglycerate</td>
<td>0.203±.011</td>
<td>0.166±.006 (S)</td>
<td>0.139±.007 (S)</td>
</tr>
<tr>
<td>2-phosphoglycerate</td>
<td>0.028±.004</td>
<td>0.014±.004 (S)</td>
<td>0.005 (S)</td>
</tr>
<tr>
<td>Phosphoenolpyruvate</td>
<td>0.063±.002</td>
<td>0.049±.003 (S)</td>
<td>0.043±.003 (S)</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.133±.014</td>
<td>0.112±.009 (NS)</td>
<td>0.076±.006 (S)</td>
</tr>
<tr>
<td>Lactate</td>
<td>3.22±.28</td>
<td>11.40±1.90 (S)</td>
<td>8.70±1.40 (S)</td>
</tr>
</tbody>
</table>

Metabolite concentrations (u moles/g wet wt muscle) are given as the mean±SEM with the number of rats sampled in parentheses. Statistical significance (P) between each exercise and control group was evaluated using the two-tailed Student's t-test. P<0.05 (S) Significant (NS) Not significant.
### TABLE 4.7. GLYCOLYTIC INTERMEDIATES IN RAT SOLEUS SKELETAL MUSCLE FOLLOWING A 2 MINUTE HIGH INTENSITY, AND 30 MINUTE ENDURANCE RUN TO FATIGUE

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Pre-exercise (5)</th>
<th>2 Min (6)</th>
<th>30 Min (5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>0.971±.089</td>
<td>1.46±.06 (S)</td>
<td>1.71±.19 (NS)</td>
</tr>
<tr>
<td>Glucose 1-phosphate</td>
<td>0.014±.005</td>
<td>0.018±.004 (NS)</td>
<td>0.023±.003 (NS)</td>
</tr>
<tr>
<td>Glucose 6-phosphate</td>
<td>0.431±.071</td>
<td>0.695±.045 (S)</td>
<td>0.229±.026 (S)</td>
</tr>
<tr>
<td>Fructose 6-phosphate</td>
<td>0.080±.017</td>
<td>0.158±.016 (S)</td>
<td>0.097±.017 (NS)</td>
</tr>
<tr>
<td>Fructose 1,6-bisphosphate</td>
<td>0.156±.024</td>
<td>0.132±.012 (NS)</td>
<td>0.058±.005 (S)</td>
</tr>
<tr>
<td>Dihydroxyacetone phosphate</td>
<td>0.056±.003</td>
<td>0.043±.006 (NS)</td>
<td>0.036±.003 (S)</td>
</tr>
<tr>
<td>Glyceraldehyde 3-phosphate</td>
<td>0.036±.006</td>
<td>0.031±.001 (NS)</td>
<td>0.032±.003 (NS)</td>
</tr>
<tr>
<td>1,3-diphosphoglycerate</td>
<td>0.055±.007</td>
<td>0.026±.005 (S)</td>
<td>0.020±.008 (S)</td>
</tr>
<tr>
<td>3-phosphoglycerate</td>
<td>0.165±.010</td>
<td>0.164±.007 (NS)</td>
<td>0.188±.014 (NS)</td>
</tr>
<tr>
<td>2-phosphoglycerate</td>
<td>0.034±.006</td>
<td>0.028±.009 (NS)</td>
<td>0.020±.005 (NS)</td>
</tr>
<tr>
<td>Phosphoenolpyruvate</td>
<td>0.065±.016</td>
<td>0.060±.004 (NS)</td>
<td>0.036±.005 (NS)</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.128±.012</td>
<td>0.117±.009 (NS)</td>
<td>0.081±.009 (S)</td>
</tr>
<tr>
<td>Lactate</td>
<td>3.84±.80</td>
<td>7.26±.51 (S)</td>
<td>7.73±.41 (S)</td>
</tr>
</tbody>
</table>

Metabolite concentrations (µ moles/g wet wt muscle) are given as the mean±SEM with the number of rats sampled in parentheses. Statistical significance (P) between each exercise and control group was evaluated using the two-tailed Student's t-test. P 0.05 < (S) Significant (NS) Not significant.
TABLE 4.8. CYTOSOLIC FREE ADP, ATP/ADP RATIO, PHOSPHORYLATION
POTENTIAL AND REDOX STATE IN RAT GASTROCNEMIUS FOLLOWING A 2
MINUTE HIGH INTENSITY, AND 30 MINUTE ENDURANCE RUN TO FATIGUE

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pre-exercise (5)</th>
<th>2 Min (5)</th>
<th>30 Min (6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free ADP</td>
<td>0.060±.014</td>
<td>0.166±.022 (S)</td>
<td>0.260±.077 (S)</td>
</tr>
<tr>
<td>% Total ADP</td>
<td>5.2±1.2</td>
<td>19.2±1.9 (S)</td>
<td>29.1±7.1 (S)</td>
</tr>
<tr>
<td>ATP/ADP</td>
<td>137 ±29</td>
<td>40 ±6.6 (S)</td>
<td>23 ±6.2 (S)</td>
</tr>
<tr>
<td>ATP/ADP P_i</td>
<td>7773 ±1851</td>
<td>1643±408 (S)</td>
<td>698 ±238 (S)</td>
</tr>
<tr>
<td>NAD+/NADH</td>
<td>488 ±144</td>
<td>154±19 (S)</td>
<td>165 ±46 (S)</td>
</tr>
<tr>
<td>Lactate/Pyruvate</td>
<td>30 ±5.8</td>
<td>154±28 (S)</td>
<td>157 ±42 (S)</td>
</tr>
</tbody>
</table>

The cytoplasmic free ADP concentration was calculated from the measured total contents of the combined GAPDH-PGK-LDH equilibrium (see Materials and Methods). All concentrations are expressed as u moles/g cell H2O. The ATP/ADP ratio and phosphorylation potential (ATP/ADP P_i) were calculated using free ADP. Statistical significance (P) between each exercise and control group was evaluated using the two-tailed Student's t-test. P<0.05 (S) Significant.
TABLE 4.9. CYTOSOLIC FREE ADP, ATP/ADP RATIO, PHOSPHORYLATION POTENTIAL AND REDOX STATE IN RAT PLANTARIS FOLLOWING A 2 MINUTE HIGH INTENSITY, AND 30 MINUTE ENDURANCE RUN TO FATIGUE

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pre-exercise (5)</th>
<th>2 Min (6)</th>
<th>30 Min (5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free ADP</td>
<td>0.041±.005</td>
<td>0.135±.026 (S)</td>
<td>0.160±.028 (S)</td>
</tr>
<tr>
<td>% Total ADP</td>
<td>4.34±0.60</td>
<td>13.43±3.05 (S)</td>
<td>13.95±2.68 (S)</td>
</tr>
<tr>
<td>ATP/ADP</td>
<td>175±21</td>
<td>49±9 (S)</td>
<td>34±11 (S)</td>
</tr>
<tr>
<td>ATP/ADP P_i</td>
<td>9729±1443</td>
<td>2183±678 (S)</td>
<td>1165±326 (S)</td>
</tr>
<tr>
<td>NAD+/NADH</td>
<td>532±108</td>
<td>251±52 (S)</td>
<td>167±22 (S)</td>
</tr>
<tr>
<td>Lactate/Pyruvate</td>
<td>22±4</td>
<td>111±26 (S)</td>
<td>116±16 (S)</td>
</tr>
</tbody>
</table>

The cytoplasmic free ADP concentration was calculated from the measured total contents of the combined GAPDH-PGK-LDH equilibrium (see Material and Methods). All concentrations are expressed as u moles/g cell H₂O. The ATP/ADP ratio and phosphorylation potential (ATP/ADP P_i) were calculated using free ADP. Statistical significance (P) between each exercise and control group was evaluated using the two-tailed Student's t-test. P<0.05 (S) Significant.
### TABLE 4.10. CYTOSOLIC FREE ADP, ATP/ADP RATIO, PHOSPHORYLATION POTENTIAL AND REDOX STATE IN RAT SOLEUS FOLLOWING A 2 MINUTE HIGH INTENSITY, AND 30 MINUTE ENDURANCE RUN TO FATIGUE

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pre-exercise (5)</th>
<th>2 Min (6)</th>
<th>30 Min (5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free ADP</td>
<td>0.037±0.006</td>
<td>0.127±0.026 (S)</td>
<td>0.244±0.068 (S)</td>
</tr>
<tr>
<td>% Total ADP</td>
<td>3.7±0.7</td>
<td>12.0±2.4 (S)</td>
<td>24.7±5.9 (S)</td>
</tr>
<tr>
<td>ATP/ADP</td>
<td>127±23</td>
<td>39 ± 8 (S)</td>
<td>19 ± 4 (S)</td>
</tr>
<tr>
<td>ATP/ADP P&lt;sub&gt;i&lt;/sub&gt;</td>
<td>8121±1707</td>
<td>2472±506 (S)</td>
<td>1149±254 (S)</td>
</tr>
<tr>
<td>NAD&lt;sup&gt;+&lt;/sup&gt;/NADH</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lactate/Pyruvate</td>
<td>29 ± 4</td>
<td>65 ± 9 (S)</td>
<td>102±15 (S)</td>
</tr>
</tbody>
</table>

The cytoplasmic free ADP concentration was calculated from the measured total contents of the combined GAPDH-PGK-LDH equilibrium (see Materials and Methods). All concentrations are expressed as μ moles/g cell H<sub>2</sub>O. The ATP/ADP ratio and phosphorylation potential (ATP/ADP P<sub>i</sub>) were calculated using free ADP. Statistical significance (P) between each exercise and control group was evaluated using the two-tailed Student's t-test. P<0.05 (S) Significant.
Fig. 4.1. Graphical representation of displacement of individual glycolytic reactions from thermodynamic equilibrium in rat gastrocnemius skeletal muscle at three different activity states. Thermodynamic equilibrium constants were determined at pH 7.0 and unit activity of water at 25°C or 38°C (*). Unless otherwise specified values for equilibrium constants were obtained from Burton (1957). (a) Fischer et al., (1971) (b) Noltmann, (1972) (c) Bohme et al., (1975)* (d) Connett, (1985)* (e) Veech et al., (1979)* M.A.R. is the mass action ratio.
Fig. 4.2. Crossover plot of glycolytic intermediates in rat gastrocnemius skeletal muscle following a 2 minute high intensity run, and a 30 minute endurance run to fatigue. Changes in glycolytic intermediates are expressed as a percentage of pre-exercise values. (○) 2 Min run. (●) 30 Min run.
Fig. 4.3. Crossover plot of glycolytic intermediates in rat plantaris skeletal muscle following a 2 minute high intensity run, and a 30 minute endurance run to fatigue. Changes in glycolytic intermediates are expressed as a percentage of pre-exercise values. (○) 2 Min run. (●) 30 Min run.
Fig. 4.4. Crossover plot of glycolytic intermediates in rat soleus skeletal muscle following a 2 minute high intensity run, and a 30 minute endurance run to fatigue. Changes in glycolytic intermediates are expressed as a percentage of pre-exercise values. (O) 2 Min run. (●) 30 Min run.
CHAPTER 5: PHOSPHOFRUCTOKINASE CONTROL IN MUSCLE: NATURE AND REVERSAL OF pH-DEPENDENT ATP INHIBITION
CHAPTER 5: PFK-1 CONTROL IN MUSCLE: NATURE AND REVERSAL OF pH-DEPENDENT ATP INHIBITION

INTRODUCTION

For at least two decades it has been known that PFK-1 is highly sensitive to H+ ions (Ui, 1966; Hofmann, 1976). The basis for this effect is the ATP-induced inhibition of PFK-1 catalysis (Lardy and Parks, 1956). Although ATP is a substrate for the reaction (binds to a high-affinity catalytic site), at higher physiological concentrations of about 5 mM, it also is inhibitory [binds to a low-affinity allosteric site(s)], and this inhibition is more pronounced at a pH below 7.2 (Lardy and Parks, 1956; Uyeda, 1979). According to Frieden et al., (1976), low pH increases the ratio of protonated to unprotonated ionization groups at the ATP binding site(s), which facilitates ATP binding and inhibits PFK-1.

From these types of in vitro kinetic studies on purified PFK-1 and from studies on cell-free muscle homogenates (Wu and Davis, 1981), the view has generally prevailed that glycolytic function is very pH sensitive over the physiological range because of the extreme pH sensitivity of PFK-1 (Hermansen, 1981; Connett et al., 1984). This rationalization is clearly an oversimplification in light of the study of Meyer et al., (1982a) on cat biceps showing that in the initial stages of recovery from fatigue (pH of 6.4), reactivation of glycolysis and muscle work occurred before the pH returned to its control value of around 7.1. The conclusion to be drawn from
these types of studies, and from the work presented in Chapter 3 & 4 on fish and rat skeletal muscle, is that low pH does not necessarily limit glycolysis, and by implication means that mechanisms must exist in muscle to reverse or preclude inhibition of PFK-1 during exercise. Moreover, there are extreme cases in the literature where muscle pH has been reported to fall to 5.9 before there is any curtailment of either muscle work or glycolysis (Bailey and Seymour, 1983).

The aim of this study was to re-investigate the kinetic and regulatory properties of purified rabbit muscle PFK-1 in an attempt to clarify how this enzyme can achieve significant catalytic rates \textit{in vivo} despite falling pH.

\textbf{RESULTS AND DISCUSSION}

\textbf{INTERACTING PH, ATP AND FRUCTOSE 2,6-BP EFFECTS}

The effects of F 6-P on the activity of rabbit muscle PFK-1 in the presence of either 1.0 or 5.0 mM ATP, or 5 mM ATP plus 10 uM F 2,6-BP at various pH values are shown in Figs. 5.1., 5.2. & 5.3. The kinetic and regulatory properties determined under these conditions are summarized in Table 5.1. and Fig. 5.4. As the pH is lowered from 7.67 to 6.8 at 25°C, the apparent Km or $S_{0.5}$ for F 6-P increases fourfold at 1 mM ATP with a 5 to 10% change in Vmax (Fig. 5.1; Table 5.1.). Over this pH range the nature of F 6-P binding to PFK-1 displays typical Michaelis Menten-type kinetics with
corresponding Hill coefficients of around 1.0 (Table 5.1.). However, at the higher physiological concentrations of ATP, the effect of pH on PFK-1 catalysis is markedly different (Fig. 5.2.), that is, decreasing the pH from 7.67 to 6.8 at 25°C causes a dramatic decrease in the affinity for F 6-P and a shift from hyperbolic to increasingly sigmoidal reaction kinetics. Although the estimates of S$_{0.5}$ values are admittedly subject to greater error at low pH, the data indicate a 50-fold increase in the S$_{0.5}$ value at pH 6.8 compared with pH 7.67 (Table 5.1.). In contrast, the Vmax under these conditions is similar to that at 1.0 mM ATP, with only modest changes occurring as a function of pH. An increased regulatory behavior of rabbit muscle PFK-1 at low pH and high ATP is commonly observed for PFK-1 isolated from a variety of other tissues and animal species (Uyeda, 1979; Sols, 1981).

The pH-dependent inhibition of PFK-1 catalysis at 5.0 mM ATP, however can be nearly completely abolished in the presence of 10 μM F 2,6-BP with an accompanying dramatic leftward shift in the F 6-P saturation curve, and lower Hill coefficient (Fig. 5.3.). In other words, in the presence of 10 μM F 2,6-BP and inhibitory concentration of ATP, the rabbit muscle enzyme behaves as if the ATP concentration was low at any given pH. Conversely, at high ATP concentration and at a low pH of 6.8, PFK-1 behaves in the presence of 10 μM F 2,6-BP as if the pH had been increased to about 7.15, or a 0.35 pH unit shift at any given F 2,6-BP concentration (ca Figs. 5.2. & 5.3). A point of emphasis is that the catalytic response of PFK-1 to F 2,6-BP is most pronounced at inhibitory concentrations of ATP.
and low pH. That F 2,6-BP exerts little or no effect at pH values above 7.4 or low ATP is consistent with the view that its role is to offset the pH-dependent ATP inhibition of PFK-1 catalysis.

**MULTIMODULATORY EFFECTS**

The previous experiments established the conditions of interaction between three modulators of PFK-1: H⁺ ions, ATP and F 2,6-BP. Since PFK-1 is influenced by numerous other metabolites and ions (Passoneau and Lowry, 1962; Mansour, 1972; Bloxham and Lardy, 1973; Sols, 1981), it was of interest to assess the relative importance of some of these to release PFK-1 from ATP inhibition over a similar pH range.

It is clear from Fig. 5.5. that at physiological concentration of F 6-P (0.1 mM), the most potent single de-inhibitor (or activator) of rabbit muscle PFK-1 is F 2,6-BP. The second most powerful activator is G 1,6-BP (50 uM) which is about 60% as effective as F 2,6-BP. AMP (0.5 mM), already known to reverse ATP inhibition at low pH in frog muscle (Trivedi and Danforth, 1966), along with NH₄⁺ ion (5.0 mM) or P_i (20 mM) each contribute about 40% the activation potential of F 2,6-BP. When combined, however, G 1,6-BP and AMP together or AMP, NH₄⁺ and P_i together act in synergism and account for about 100% and 80% of the effectiveness of F 2,6-BP, respectively (Fig. 5.5.). That F 2,6-BP is the most powerful activator of PFK-1 over the physiological pH range is in complete agreement with the recent study of Uyeda et al., (1981).
Interestingly, one important development that emerged from the present study was that the apparent $K_a$ of F 2,6-BP was about 100-fold lower than that for G 1,6-BP at pH 7.0; i.e. the apparent $K_a$ of rabbit muscle PFK-1 for F 2,6-BP is 30 nM compared to 4 uM for G 1,6 BP with respective Hill coefficients of 1.1 and 2.1 (Fig. 5.6.).

Citrate is another important modulator of PFK-1 catalysis (Kemp and Foe, 1983). Its inhibitory action is synergistic with ATP, and the mechanism of inhibition is believed to involve a depolymerization of the active tetrameric enzyme into inactive dimers (Lad et al., 1973; Goldhammer and Paradies, 1979). The inactive dimers have been termed 2HE$_2$ by Bock and Frieden (1976 a,b). Because the effect of citrate is clearly dependent on pH and ATP concentration, it was of interest to examine the nature and extent of citrate inhibition in the presence of the positive modulators under physiological conditions. At 5.0 mM ATP and 0.1 mM F 6-P, citrate (0.5 mM) exerts a strong inhibitory effect (50% loss of activity at pH 7.0) when either AMP (0.5 mM), NH$_4^+$ (5.0 mM), or P$_i$ (20 mM) is present, compared with the 25% loss of activity in the presence of F 2,6-BP (10 uM) or G 1,6-BP (50 uM) (Table 5.2.). However, several combinations, F 2,6-BP and AMP or AMP, P$_i$ and NH$_4^+$ together, stabilize PFK-1 against citrate inhibition (< 5% loss of activity). This stabilization of PFK-1 against citrate inhibition by F 2,6-BP, G 1,6-BP, and the other positive modulators adds to the growing list of regulatory functions for these effectors under near physiological conditions.
INTERPRETATION AND PHYSIOLOGICAL SIGNIFICANCE

These new kinetic data impact on our understanding of the mechanisms underlying the regulatory behavior of PFK in vertebrate skeletal muscle. With regard to the mechanism of interaction between H$^+$ ions, ATP and PFK-1 catalysis, an important point to emphasize is that at inhibitory physiological concentrations of ATP (5.0 mM), the ATP dependence of the reaction is most pronounced over the pH range between 6.8 and 7.0. This observation strongly suggests that binding of ATP to the site(s) of regulation on PFK-1 depends on the protonation of imidazole groups which have a pK of about 6.9 at 25°C. This interpretation agrees well with the conclusion of Bock and Frieden (1976 a,b) and Frieden et al., (1976). These workers further proposed that PFK-1 exists in at least two forms, termed E$_4$ and HE$_4$. According to their kinetic model, ATP binds preferentially to HE$_4$, the protonated tetramer; whereas F 6-P, F 1,6-BP and AMP preferentially bind to E$_4$, the unprotonated tetramer. Thus, the effect of change in pH is to alter the ratio of unprotonated/protonated tetrameric forms, with the binding of ATP being favoured at low pH. As a first approximation, it is assumed that adding F 2,6-BP to the reaction cuvette leads to a drop in the apparent pK of the ATP binding site(s) to about 6.6 or even lower. In terms of the ratio of unprotonated to protonated forms of the enzyme, this effect is equivalent to increasing the pH of the medium by about 0.35 pH units (Figs 5.2. & 5.3.). This shift in
equilibrium favoring $E_4$ formation greatly facilitates F 6-P binding while at the same time reducing the allosteric binding of ATP (Frieden et al., 1976), and thereby reducing or reversing the effect of ATP inhibition at low pH. Notwithstanding, a similar interpretation adequately explains the action of G 1,6-BP to reverse the ATP inhibiton of PFK catalysis.

The effect of citrate is rather more complex. The data presented demonstrate that, in the presence of either F 2,6-BP or G 1,6-BP or a combination of F 2,6-BP with AMP or AMP, $P_i$ and NH$_4^+$ together, PFK-1 catalysis is partially or fully stabilized against citrate inhibition. This finding suggests that the presence of positive modulators not only increases the ratio of unprotonated to protonated forms of the enzyme but stabilizes the $E_4$ form by protecting it against citrate inhibition, which may involve a change in the polymerization state (Lad et al., 1973, Kemp and Foe, 1979).

In conclusion, the strong inhibitory effect of physiological concentrations of ATP on PFK-1 catalysis, in metabolic terms, must be considered to be one of the most fundamental kinetic and regulatory properties of the muscle enzyme. This study has highlighted several mechanisms of modulation that could allow PFK-1 to function in a physiological pH range that has long been considered to be inhibitory. In effect, any modulator that increases the ratio of unprotonated to protonated forms of the tetrameric enzyme could supply the muscle cell with a means of controlling the pH dependent ATP inhibition of PFK catalysis. Aside from the controlling effect of $H^+$ per se, the modulators thought to be most
effective in shifting the equilibrium towards the $E_4$ form in muscle include $F\text{ 2,6-BP}$, $G\text{ 1,6-BP}$, $F\text{ 1,6-BP}$, $\text{AMP}$, $\text{NH}_4^+$ and $P_1$. One of the most striking aspects of PFK-1 regulation in white skeletal muscle of fish highlighted in the present study was the decrease in the ATP concentration which in effect minimizes the need for multimodulation by the traditional set of positive effectors. The relevance of these and other metabolic interactions in trout and rat skeletal muscle during exercise will be discussed in the General Discussion, Chapter 6.
TABLE 5.1. Kinetic and regulatory properties of rabbit muscle phosphofructokinase as a function of pH in the presence of 1.0 mM and 5.0 mM ATP, and 5.0 mM ATP with 10 μM fructose 2,6-P$_2$ at 25°C

<table>
<thead>
<tr>
<th>pH</th>
<th>S$_{0.5}$ fructose 6-P (μM)</th>
<th>Hill coefficient, n</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.67</td>
<td>7.25 7.0 6.8 7.67 7.25 7.0 6.8</td>
<td></td>
</tr>
<tr>
<td>1.0 mM ATP</td>
<td>50 71 100 218 1.0 1.0 1.0 1.0</td>
<td></td>
</tr>
<tr>
<td>5.0 mM ATP</td>
<td>72 133 707 4500 1.5 1.85 2.0</td>
<td></td>
</tr>
<tr>
<td>5.0 mM ATP + 10 μM F-2,6-P$_2$</td>
<td>59 63 86 138 2.6 2.6 1.5 1.0</td>
<td></td>
</tr>
</tbody>
</table>

The values presented were calculated from the data shown in Figs. 1, 2 and 3 and as described in Materials and Methods.
TABLE 5.2. Effect of modulator(s) to stabilize rabbit muscle phosphofructokinase against citrate inhibition

<table>
<thead>
<tr>
<th>Effector(s)</th>
<th>Percentage decrease of activity after addition of 0.5 mM citrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP (0.5 mM)</td>
<td>52</td>
</tr>
<tr>
<td>P&lt;sub&gt;1&lt;/sub&gt; (20 mM)</td>
<td>53</td>
</tr>
<tr>
<td>NH&lt;sub&gt;4&lt;/sub&gt;⁺ (5 mM)</td>
<td>53</td>
</tr>
<tr>
<td>F-2,6-P₂ (10 uM)</td>
<td>25</td>
</tr>
<tr>
<td>G-1,6-P₂ (50 uM)</td>
<td>27</td>
</tr>
<tr>
<td>F-2,6-P₂ + AMP</td>
<td>less than 5.0</td>
</tr>
<tr>
<td>AMP, P&lt;sub&gt;1&lt;/sub&gt; and NH&lt;sub&gt;4&lt;/sub&gt;⁺</td>
<td>less than 5.0</td>
</tr>
</tbody>
</table>

Assay conditions: 50 mM Hepes-KOH, pH 7.0 at 25°C and physiological concentrations of ATP (5.0 mM) and F-6-P (0.1 mM)
Fig. 5.1. Effect of fructose 6-phosphate on activity of purified rabbit muscle phosphofructokinase-1 at various pH values in the presence of 1.0 mM ATP. Assay conditions are described in Materials and Methods: Section 3.
Fructose 6-P (mM)
Fig. 5.2. Effect of fructose 6-phosphate on activity of purified rabbit muscle phosphofructokinase at various pH values in the presence of 5.0 mM ATP at 25°C. Assay conditions are described in the Materials and Methods: Section 3.
Fig. 5.3. Effect of fructose 6-phosphate on activity of purified rabbit muscle phosphofructokinase at various pH values in the presence of 5.0 mM ATP with 10 uM fructose 2,6-bisphosphate at 25°C. Assay conditions are described in Materials and Methods: Section 3.
Fructose 6-P (mM)

μmoles x 10^{-3}/min

Fructose 6-P (mM)

5 mM ATP + 10 μM F2,6-P2
Fig. 5.4. Change in apparent $K_m$ or $S_{0.5}$ of purified rabbit muscle phosphofructokinase for fructose 6-phosphate as a function of pH at 25°C. Data plotted from fructose 6-phosphate saturation curves shown in Figs. 5.1., 5.2. & 5.3.
Fig. 5.5. Effect of modulators on the activity of purified rabbit muscle PFK-1 over the physiological pH range. The pH profiles were determined at 25°C in the presence of 0.1 mM F 6-P and 5.0 mM ATP (□),
5.0 mM ATP with either 0.5 mM AMP, 20 mM P_i or 5.0 mM NH_4^+ (●);
5.0 mM ATP with 50 uM glucose 1,6-bisphosphate (▲);
5.0 mM ATP with 0.5 mM AMP, 20 mM P_i and 5.0 mM NH_4^+ (○),
5.0 mM ATP with 10 uM Fructose 2,6-bisphosphate or 50 uM glucose 1,6-bisphosphate and 0.5 mM AMP (■). Assay conditions described in Materials and Methods: Section 3.
Fig. 5.6. Effect of varying fructose 2,6-bisphosphate and glucose 1,6-bisphosphate on activity of purified rabbit muscle PFK-1 at 25°C at physiological concentrations of F 6-P (0.1 mM), ATP (5.0 mM) in 50 mM HEPES-KOH buffer, pH 7.0 at 25°C. Assay conditions are described in Materials and Methods: Section 3.
Fructose 2,6-P$_2$ or Glucose 1,6-P$_2$ (nM)

<table>
<thead>
<tr>
<th></th>
<th>Ka</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>F2,6-P$_2$</td>
<td>30 nM</td>
<td>1.10</td>
</tr>
<tr>
<td>G1,6-P$_2$</td>
<td>4 μM</td>
<td>2.10</td>
</tr>
</tbody>
</table>
CHAPTER 6: GENERAL DISCUSSION
CHAPTER 6: GENERAL DISCUSSION

FUELS AND SHORT-TERM GENERATION OF ATP

The near-maximum ATP turnover in trout white skeletal muscle was estimated to be between 188 and 598 µmoles/g wet wt/min (Table 3.4). This range was in close agreement with the in vitro maximum velocity of myofibrillar ATPase determined for rabbit muscle (Bendall, 1961). PCr hydrolysis was the principal pathway utilized at this time with little or no contribution from anaerobic glycogenolysis. That PCr hydrolysis precludes anaerobic glycogenolysis in trout muscle (Table 3.1.) supports the earlier work on isolated frog skeletal muscle during tetanic stimulation (Danforth, 1965). While not doubting that the same temporal pattern of fuel and pathway activation exists in mammalian skeletal muscle during maximal work rates, published data dealing with this question are rare owing to methodological problems of sampling mammalian skeletal muscle and complex fiber heterogeneity (Karlsson, 1980). The general conclusion to be drawn from these types of studies is that when the myosin ATPase is activated maximally, creatine kinase outcompetes the kinase reactions of glycogenolysis for the ADP being released at the myosin S1 head. That creatine kinase has preferential access to the products of ATP hydrolysis during maximal work rates may relate to the different kinetic properties of the muscle enzyme (Vmax, Km for ADP), and to its juxtaposition with myosin ATPase (Bessman and Carpenter,
It is not until the rate and force of contraction is lowered that the relative contribution of anaerobic glycogenolysis to support the myofibrillar ATPase becomes more important. This general pattern of preferential fuel utilization in skeletal muscle during sub-maximal high intensity exercise was shown for trout swimming at approximately 120% VO$_2$ max for 10 minutes (Chapter 3), and for the rat treadmill running at high speed for 2 minutes (Chapter 4). In the case of trout white skeletal muscle, the ATP turnover supported by anaerobic glycogenolysis was estimated to be 78 u moles/g wet wt/min (Table 3.2.), while that determined for both rat gastrocnemius and plantaris muscles was estimated to be 64 and 61 u moles/g wet wt/min respectively (Table 4.4.). The assumption used for these latter estimates for the rat was that 10% of the glycogen utilized was completely oxidized (see Chapter 4). The ATP turnover estimated for the soleus was lower than that for each of the fast-twitch muscles (Table 4.4.), but since this muscle is predominately comprised of slow-twitch fibers, the aerobic component to glycogen utilization may have been higher than the 10% assumed, and therefore minimizing the differences between the three muscle types examined. Nonetheless one firm conclusion that can be made from this study on the rat was that both fast- and slow-twitch muscles contributed heavily to anaerobic generation of ATP during short-term high intensity running (Tables 4.1., 4.2. & 4.3.). The advantage of recruiting
anaerobic over aerobic metabolic pathways is that these systems can sustain higher myosin ATPase activities over shorter periods of time. Differences in the anaerobic potential of skeletal muscle among different species of the animal kingdom must obviously reflect the differences in locomotory needs associated with complex predator-prey interactions occurring in their natural environment.

**GLYCOGENOLYSIS: THE PATHWAY**

The thermodynamic structure of the glycogenolytic pathway in trout fast-twitch white skeletal muscle, and in the three skeletal muscle types of the rat showed no major differences with exercise and appeared highly conservative (Figs 3.1 & 4.1.), except at the combined GAPDH-PGK and the PGK equilibria in trout white muscle (Fig. 3.1.). This information, together with the published kinetic properties for each of the glycolytic enzymes provides strong support for the proposal that glycogen phosphorylase, Hk, PFK-1 and perhaps PK are the most likely candidates for regulating and coordinating glycogenolytic flux in muscle of both species. Aldolase, however, displayed a mass action ratio quite different from its equilibrium constant in all of the rat skeletal muscles, but to a lesser extent in fish white muscle. This apparent difference can be rationalized in terms of aldolase reacting to a specific beta anomeric or acyclic form of F 1,6-BP which represents a small fraction of the total F 1,6 BP content measured in muscle (Midelfort
et al., 1976). In view of its high catalytic potential, and preferential binding to specific forms of F 1,6-BP, aldolase is generally believed to operate near-equilibrium in mammalian muscle (Reynolds et al., 1971; Connett, 1985).

CONTROL OF GLYCOGENOLYSIS DURING SHORT-TERM HIGH-INTENSITY EXERCISE AND FATIGUE

Even though the exercise protocol for the 10 min burst swimming trout was not directly comparable to the high intensity run for the rat, a number of interesting similarities and differences in the control of glycogenolysis emerged.

On the basis of crossover analysis, control sites were identified at Hk and PFK-1 in fish white (Fig 3.2.), and glycogen phosphorylase and PFK-1 in all three muscle types of the rat (Figs. 4.2, 4.3. & 4.4.).

As discussed in Chapter 3, the apparent crossover at Hk in fish white muscle was obviously linked to the near-depletion of endogenous glycogen. According to the conventional view, the concomitant decrease in G 6-P concentration has a strong de-inhibitory effect on Hk activity with the subsequent increase in uptake and phosphorylation of blood borne glucose (Leuck and Fromm, 1974). Available evidence suggests that this de-inhibition of Hk is brought about by the direct binding of G 6-P to an allosteric or regulatory site on the enzyme (Collowick, 1973).
Indeed, if the apparent $K_i$ of Hk for G 6-P in fish muscle was in the low mM range, then the decrease in G 6-P from 0.455 to 0.279 u moles/g wet wt/ min accompanying 10 minutes of swimming, would indeed confirm its importance in controlling glucose phosphorylation. The role of glucose phosphorylation in trout white muscle following exercise is uncertain but may either assist to replenish glycogen stores or alternatively be fluxed through the glycolytic pathway to provide ATP for recovery metabolism. It should be pointed out that glycogen replenishment in trout white muscle is a slow process and can take up to 24 hrs after strenuous exercise (Black et al., 1962).

In contrast to white muscle of trout, the endogenous stores of glycogen in the three rat skeletal muscles following the 2 min high intensity run did not become limiting (Tables 4.1, 4.2., & 4.3.). This has also been reported for humans following short-term high intensity running to fatigue (Gollnick et al., 1973; Gollnick, 1982). This study indicated that glycogenolytic flux in rat skeletal muscle was maintained and coordinated by the action of glycogen phosphorylase and PFK-1. That no crossover occurred at Hk is indicative of the importance of both these enzymes to regulate G 6-P and suppress glucose phosphorylation at this time.

A striking analogy between trout and rat skeletal muscle was the finding that F 6-P increased and F 1,6-BP decreased following high intensity exercise. That an apparent crossover was evident in the direction described above in trout white muscle, suggested
that PFK-1 was not causal to the fatigue process. Substantiation of this claim also comes from the presence of a number of positive modulators at this time (AMP, P_i & NH_4^+), whose synergistic action, together with an increased F_6-P concentration, would be expected to defend PFK-1 activity in trout white muscle despite falling pH. Moreover, the 55% decrease in ATP concentration itself would be expected to play a major role in minimizing the pH-dependent ATP inhibition of PFK-1 catalysis (Table 3.3.). The situation regarding PFK-1 regulation in rat skeletal muscle was less complicated. The parallel increase in F_6-P with glycogenolytic flux in muscle confirms the earlier observations of Bucher and Russman (1963), Wilson et al., (1967) and Edington et al., (1973). As outlined in detail in Chapter 4, the relative accumulation of reactants and depletion of products at the PFK-1 locus, means that delivery of F_6-P at pH 6.6 was occurring at a faster rate than the enzyme could catalyze its conversion to F_1,6-BP. This proposal was consistent with the maximum catalytic capacities of both glycogen phosphorylase and PFK-1 (Harris et al., 1976; Baldwin et al., 1982). During the 2 min high intensity run, PFK-1 in rat muscle may be considered rate limiting to pathway flux.

On the basis of the work presented on the regulation of rabbit muscle PFK-1 described in Chapter 5, the statement that PFK-1 is completely inhibited at low pH can no longer be made with confidence. H^+ ions should not be thought of as inhibiting PFK-1 per se, but more appropriately, should be viewed as a specific
mechanism of control, since pH-dependent inhibition depends upon the non-catalytic role of ATP binding. That ATP binding can be dramatically modified by the presence of positive modulators either singly or in combination was clearly demonstrated in Fig. 5.5. It was shown that the action of positive modulators is to offset the pH-dependent ATP inhibition of PFK-1 in such a way as to broaden the pH profile into the physiological range that is often reported to be inhibitory to the enzyme. The most potent activators of PFK-1 are the recently discovered F 2,6-BP (Hers and VanShaftigen, 1982), and G 1,6-BP (Hofer and Pette, 1968; Bietner, 1979), but as yet their precise role in skeletal muscle during exercise remains to be clearly established. Notwithstanding, because both modulators have been shown to increase in several working muscle preparations in a way that is consistent with their apparent K_a for activation (Fig 5.6; Hofer and Pette, 1968; Hue et al., 1982; Storey, 1983), it may be assumed that these regulators play an important role in activating or maintaining glycogenolytic flux in working muscle.

PYRUVATE KINASE

Another intriguing aspect of the present comparative analysis of glycogenolytic control in skeletal muscle was the occurrence of an apparent crossover at PK in trout fast-twitch white muscle but not in the rat (Figs. 3.2. and 4.2., 4.3. & 4.4.). In contrast to working
mammalian skeletal muscle, trout retains lactate in white muscle for periods up to 24 hours after strenuous exercise (Black et al., 1962). The phenomenon of lactate retention in fish white muscle is an unresolved question but may be due to an inadequate circulation during high intensity exercise. However, this proposal does not explain why it takes up to 24 hr for trout white muscle to reach pre-exercise lactate levels since blood flow has been shown to increase following strenuous exercise (Neumann et al., 1983). A more plausible explanation of lactate retention in trout white muscle during recovery relates to differences in the properties of the membrane to lactate transport.

As a consequence of the near-equilibrium reaction catalyzed by LDH, the high lactate levels reported in this study for trout white muscle accompanying short-term exercise (Table 3.3.) must be counterbalanced by high pyruvate levels if near-equilibrium is to be maintained. This is particularly important since the other reactants and products of the LDH reaction, the redox potential and H+ ion concentration, undergo smaller percentage increases (Table 3.5.). Thus in this way, increasing pyruvate leads to an apparent but misleading crossover at pyruvate kinase in trout white muscle. An extreme case among the fishes has been reported for skipjack tuna where lactate in white muscle may reach levels as high as 80-100 u moles/g wet wt with a concomitant rise in pyruvate to about 1.0 u mole/g wet wt muscle (Guppy et al., 1979).
NAD/NADH CYCLES

In order that glycogenolytic flux is maintained in working skeletal muscle during exercise an adequate supply of NAD$^+$ is required. As outlined in the introduction, this is accomplished during short-term high intensity exercise by the high activities of GAPDH and LDH. One of the most challenging aspects of this study was to provide an explanation for the contrasting cytosolic redox potentials in skeletal muscle of the fish and the rat accompanying short-term high intensity exercise (Tables 3.5, 4.8, 4.9 & 4.10). The redox state in the cytosol of trout fast-twitch white muscle became more oxidized with exercise, while the cytosol in rat fast-twitch skeletal muscles became more reduced. Since the NAD$^+/\text{NADH}$ ratio is coupled to both the ATP/ADP and ATP/ADP$\cdot$P$_i$ ratios through the combined GAPDH-PGK-LDH equilibria (see Veech et al., 1979), the high NAD$^+/\text{NADH}$ ratio in trout white muscle should be associated with a high ATP/ADP ratio if all the enzymes remain at near equilibrium and tightly coupled. As we see from Table 3.5, this prediction was not borne out, and the increased NAD$^+/\text{NADH}$ ratio in trout white muscle may be linked to an apparent uncoupling between GAPDH-PGK and LDH. This was supported by the large deviation of the mass action ratio from the thermodynamic equilibrium constant at this time (Fig. 3.1). In addition, since the lactate/pyruvate ratio did not significantly change with exercise, increase in the NAD$^+/\text{NADH}$ ratio in trout white skeletal muscle
primarily was due to the increase in $H^+$ ion (See Chapter 3).

In direct contrast, the thermodynamic integrity of the glycolytic pathway in rat skeletal muscle remained unchanged with exercise, with each of the respective dehydrogenases catalyzing near-equilibrium reactions (Fig. 4.1.). That is, the decrease in the cytosolic NAD$^+$/NADH ratio occurred concomitantly with a decrease in the ATP/ADP ratio and phosphorylation potential in the gastrocnemius and plantaris decreased following the 2 min high intensity run. The redox changes reported in this study for rat skeletal muscle support the previous electrical stimulation studies on the rat hindlimb preparation of Aragon and Lowenstein (1980), and human skeletal muscle following strenuous exercise (Sahlin et al., 1976). Similarly, these studies demonstrated that the increased reduction of the cytosol was also accompanied by a rise in the lactate/pyruvate ratio (see Tables 4.8. & 4.9.).

Of particular relevance to this paradox between redox changes in skeletal muscle of the fish and rat are the early fluorometric studies of Jobsis and Duffield (1967) and Jobsis and Stainsby (1967) carried out on skeletal muscle of the frog, toad and dog. These workers found that in response to electrical stimulation (about 5 muscle twitches/sec) NADH in all muscles consistently went oxidized. Even though this change in redox is identical to that described for trout white muscle, the results according to Jobsis and coworkers are not directly applicable since they claim that the fluorescent signal detected was from mitochondrial NADH and not from the cytosol. The exact basis for this discriminating effect
between these two compartments in muscle however has never been fully clarified. A few years later, Edington et al., (1973) examined the changes in the free cytosolic redox state in a rat hindlimb preparation following 30 sec of intense electrical stimulation. The interesting aspect of this study was the finding that accompanying electrical stimulation, the cytosolic redox potential became more oxidized. The difference between this preparation and the perfused rat hindlimb preparation of Aragon and Lowenstein (1980) was that the former was devoid of an adequate blood supply and behaved as an isolated system. Thus the rat hindlimb muscle in the study of Edington et al., (1973) behaved as fish white skeletal muscle. The 6 fold increase in the NAD⁺/NADH ratio reported was due to the retention of lactate in muscle and subsequent increase in pyruvate associated with the LDH equilibrium. The increase in pyruvate would also lead to an apparent crossover at the PKJ locus, as discussed in the previous section for fish. It seems probable that like trout skeletal muscle, this phenomenon may be either a consequence of membrane limitations to lactate transport or an inadequate supply of blood necessary to remove lactate as it is formed during high work rates. Similarly, a 10 fold increase in pyruvate and high lactate levels have been reported in cat gastrocnemius after 30 seconds of tetanus (Wilson et al., 1967). The NAD⁺/NADH ratio in this case increased from 575 ± 8.5 (n = 2) to 748 ± 26 SEM (n = 3), and was accompanied by a 2 fold increase in the lactate/pyruvate ratio. These calculations assume that the intramuscular pH fell from 7.0 to 6.6. A recent study on human
muscle metabolism also confirms that the muscle cytosol can become oxidized in vivo during maximal treadmill exercise (Cheetham et al., 1985). On the basis of the lactate/ pyruvate ratios reported, and assuming the pH of the cytosol decreased from 7.0 to 6.6, the free NAD⁺/NADH ratio was calculated to increase from 524 to 830 following the sprint.

The conclusion to be drawn from the work presented in this thesis on fish and rat, as well as from the above in vitro and in vivo electrical stimulation and human performance studies, is that it appears that the redox state of muscle depends primarily upon the rate of which lactate is retained or effluxed from skeletal muscle. Thus, in tetanically stimulated or maximally working vertebrate muscle, pyruvate concentrations rise in response to the increase in lactate via the LDH equilibrium, and the cytosol initially becomes more oxidized. The increase in pyruvate may be transient and primarily depends on the rate at which lactate is effluxed from the cytosolic compartment of muscle into the blood. That the cytosol may become more oxidized in fish and mammalian skeletal muscle at the onset of heavy exercise can be considered an important regulatory mechanism of glycogenolysis because it means that the increased NAD⁺ availability facilitates flux via mass action effect at the level of the GAPDH reaction.
FREE ADP AND ATP/ADP RATIO AND PHOSPHORYLATION POTENTIAL

Free cytosolic ADP does not become limiting to glycogenolysis during short-term high intensity exercise in either fish or rat skeletal muscle (Tables 3.5., 4.8., & 4.9. & 4.10.). The increase in the concentration of free cytosolic ADP in both species was consistent with the apparent Km values of PGK and PK being in the low mM range (Veech et al., 1979). The levels of free ADP fall in the most sensitive range of each of the respective enzyme's ADP saturation curve. As emphasized throughout this thesis, one consequence of increasing free ADP is that both the ATP/ADP and ATP/ADP P_i ratios fall. For each of the rat skeletal muscles both ratios decreased dramatically to about 30% of their pre-exercise values (Tables 4.8., 4.9. & 4.10.). This effect was further magnified in trout white skeletal muscle because of the 55% decrease in ATP (Table 3.3.).

One conclusion to be drawn from the study on the rat during the 2 min high intensity run, was that anaerobic glycogenolysis and running performance was maintained in spite of the large percentage swings in the cytoplasmic ATP/ADP ratio or phosphorylation potential in both fast- and slow-twitch skeletal muscles.
CHARACTERIZATION OF THE GLYCOGENOLYTIC PATHWAY AND REGULATION FOLLOWING ENDURANCE EXERCISE TO FATIGUE AND EXHAUSTION

The general etiology of fatigue for both the trout and the rat following exercise to complete exhaustion and fatigue respectively, was the near-depletion of intramuscular stores of glycogen (Tables 3.3. and 4.1., 4.2. & 4.3.).

Compared to the 10 min burst swimming trout, those trout selected to swim the endurance protocol to exhaustion displayed no apparent crossovers in white muscle with the exception at the combined GAPDH-PGK equilibrium which has already been discussed in detail in the previous section. As noted in Chapter 3, the sum of all the glycolytic intermediates from G 6-P to pyruvate decreased by a dramatic 80% compared to the 25% decrease in white muscle for the 10 min burst swimming fish. This near total depletion of glycolytic intermediates was accompanied by a 20 fold reduction in the ATP/ADP ratio, a 38 fold reduction in the phosphorylation potential, and a 2.5 fold increase in the NAD+/NADH ratio (Table 3.5.).

Singly, the most striking change of any single parameter in this study was the dramatic 80% fall in ATP in trout white muscle which places this species in a unique position among the vertebrates (Table 3.3.). In a parallel set of studies, we have evidence that the concentration of ATP may fall to levels as low as 0.50 u mol/g wet wt at exhaustion, which represented about 5% of
the total ATP content measured in the pre-exercise state (Dobson, Mommsen and Hochachka, work in progress). The reason why greater percentage reductions in ATP concentration occurred in trout fast-twitch muscle relative to the fast-twitch muscles of the rat, must obviously be linked to differences in the degree of PCr depletion accompanying exercise. As PCr stores become depleted, the creatine kinase reaction can no longer maintain ATP constant, and a mismatch between ATP supply and ATP usage develops. This proposal was supported from the data presented in Table 3.3. and Tables 4.1. & 4.2. showing that the PCr levels in rat fast-twitch muscles decreased by about 75%, compared to a 96% reduction in trout white muscle. That fish can nearly deplete their high energy stores, and drop their ATP levels to low levels, probably relates to their high degree of locomotory specialization designed to achieve fast-start performances in predator-prey interactions, and permitting supra-maximal feats while swimming against strong currents or up waterfalls during long migratory journeys. Another possibility may be the introduction of a psychological component to exercise performance in higher vertebrates, with the rat simply refusing to run 'when the going gets tough'. A recent study on thoroughbred racehorses galloping maximally to fatigue, provides additional support for a psychological component to exercise, because in these animals, muscle ATP was shown to fall by over 50% (Snow et al., 1985). The 'will to win' in these elite performers is undoubtedly a key factor to racing success.

The dramatic percentage reduction in ATP in white muscle
after both 10 and 30 minutes of swimming in trout was stoichiometrically matched with a concomitant rise in IMP. As PCr supply in muscle becomes limiting, the subsequent rise in free cytosolic ADP (Fig. 3.5.) leads to the activation of the myokinase reaction via mass action effect (Noda, 1973). The AMP formed then provides substrate for the AMP deaminase reaction, and in the presence of a low pH (pH optimum for AMP deaminase is 6.1 to 6.5; Setlow and Lownestein, 1967) leads to the subsequent conversion to IMP and NH$_4^+$ (Lowenstein, 1972). The free AMP in trout white muscle in the pre-exercise state was calculated to be 0.5 n moles/gm cell water and increased 58- and 122 fold following the 10 min and 30 min swimming protocols to fatigue and exhaustion respectively. These free estimates represent 0.8, 11 and 25% of the total AMP content measured in trout white skeletal muscle (Table 3.3.). Even though no such dramatic falls in the concentration of ATP were reported for each of the rat skeletal muscles, activation of AMP deaminase however was apparent, and discussed in detail in Chapter 4. One difference however between the trout and rat, was that the total content of AMP in skeletal muscle significantly increased in trout white muscle, but not in either of the fast-twitch skeletal muscles of the rat. An explanation for this difference is not readily forthcoming, and requires further studies on the role of AMP compartmentation in vertebrate skeletal muscle at different activity states. Lastly, it is important to emphasize that the primary function of the purine nucleotide
cycle in vertebrate skeletal muscle must unequivocally be to provide a sink for the dramatic loss of adenine nucleotide at a time when the muscle fails to balance ATP supply to ATP demand during high rates of contraction. This is achieved by an elaborate set of equilibrium and non-equilibrium reactions that conserve the loss of adenine nucleotide in the form of IMP, and thereby maintaining the total nucleotide pool constant. Restoration of ATP levels in muscle after strenuous exercise occurs during recovery (see Driedzic and Hochachka, 1978).

In contrast to trout following 30 min of exhaustive exercise, a potential control site was apparent at the Hk locus for each of the rat skeletal muscles after endurance running for 30 min (Figs. 4.2., 4.3. & 4.4.). That Hk appeared to be facilitated at this time was similar to the case described for trout white muscle after 10 minutes of swimming (Fig. 3.2.). On the basis of the present data it is difficult to decide the preferential route for glucose phosphorylation occurring in each of the muscle types. However, in view of the fact that no other apparent crossovers were identified along the glycolytic pathway, and that the concentration of many of the glycolytic intermediates fell below 50% their control values in the fast-twitch skeletal muscles (Tables 4.5 and 4.6.) and to a lesser extent in the soleus (Table 4.7.), suggests that activation of glycolysis at this time may have been precluded. Thus the role of glucose phosphorylation was probably glycogen replenishment. In support of this proposal, Hollozy and coworkers have demonstrated increases in glucose uptake (up to 10 fold) lasting many hours after
exercise for both skeletal muscles of the frog and rat hindlimb, and concluded on the basis of glycogen accumulation, that the major fate of glucose disposal was glycogen replenishment (Hollozy and Narahara, 1965; Ivy and Hollozy, 1981).

To complete the analysis of glycogenolytic control in rat skeletal muscle, the NAD$^+$/NADH and ATP/ADP ratios, and the phosphorylation potential in each muscle type following 30 min of treadmill running to fatigue changed in similar directions to that described in detail for the 2 min running group except that the values for the ATP/ADP ratio and ATP/ADP $P_i$ were considerably lower (Tables 4.8., 4.9. & 4.10). In summary, it may be speculated that in conjunction with the low ATP/ADP ratio and phosphorylation potential in rat skeletal muscle following the 2 min run, and certainly following the 30 min run to fatigue, the glycogenolytic pathway may become limited by NAD$^+$. 
CHAPTER 7: SUMMARY AND CONCLUSIONS
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It was clear from this study that the highest ATP turnover in skeletal muscle was supported solely by PCr hydrolysis. Despite anaerobic glycogenolysis possessing a lower maximal ATP generating potential than PCr hydrolysis, it has the advantage of not being so constrained by time, and can be recruited to extend muscle performance at sub-maximal workloads from seconds to minutes.

Fatigue after 10 min of burst swimming in trout was related to the near-depletion of glycogen in white muscle. The glycolytic pathway appeared to be functional at this time with control sites being identified at Hk, PFK-1 and PK. PFK-1 did not appear to be inhibited by low muscle pH (6.6), and was considered not causal to the onset of fatigue. In addition to the presence of a number of positive modulators (AMP, $p_i$ & $NH_4^+$), the pH-dependent ATP inhibition of PFK-1 catalysis was partially offset by the lower ATP concentration (a 55% decrease). Furthermore, inhibition of glycogenolysis at fatigue was not due to ADP or NAD$^+$ availability. One consequence of the decrease in ATP and increase in ADP concentrations, was the concomitant fall in both the ATP/ADP ratio and phosphorylation potential (70 to 80%). Total exhaustion after 30 min of burst swimming was similarly related to near-glycogen depletion, but differed from the 10 min group by showing a dramatic 80% compared to a 25% reduction in the sum of glycolytic
intermediates from G 6-P to pyruvate. No control sites were identified along the pathway except at PK. Moreover, ATP concentration in white muscle dramatically decreased by 80%. Despite the large decrease in this adenine nucleotide, the total nucleotide pool remained constant through the activation of myokinase and AMP deaminase with the subsequent formation of IMP and NH₄⁺. Like the 10 min burst swimming group, the free cytoplasmic NAD⁺/NADH ratio and ADP increased in white muscle of trout after 30 min of burst swimming, and was considered not limiting to glycogenolytic flux at this time. Associated with these changes was a marked displacement of the PGK and combined GAPDH-PGK reactions from thermodynamic equilibrium. It was suggested that displacement from equilibrium may be due to the effect of the low ATP/ADP ratio on the PGK reaction. The conclusion to be drawn from this study on rainbow trout white muscle was that inhibition of glycogenolytic flux accompanying fatigue was not due to the inhibition of PFK-1, or to the availability of either ADP or NAD⁺.

The second phase of the study was designed to investigate whether similar patterns of change occurred in three different types of rat skeletal muscle following a similar set of exercise protocols. Even though the 2 min high intensity run was not directly comparable to the 10 min burst swim of trout, a number of interesting similarities and differences emerged, particularly at PFK-1. Coordination of glycogenolytic flux in skeletal muscle of the non-fatigued rat running for 2 min run was achieved through the
Hierarchical control of glycogen phosphorylase and PFK-1. Glycogen was the principal fuel and no apparent crossover occurred at Hk. This was likely due to the inhibitory effect of increasing concentrations of G 6-P on Hk. Accompanying the increase in glycogenolytic flux was an increase in F 6-P and a decrease in F 1,6-BP. A point of emphasis was that PFK-1 was fully operational despite an intracellular pH of 6.6 in rat fast-twitch skeletal muscles. Maintenance of flux, and running performance, was accompanied by about a 3 fold increase in free cytosolic ADP, a 70 to 80% decrease in ATP/ADP ratio and phosphorylation potential, and a 50% decrease in the NAD⁺/NADH ratio. Similar percentage changes were apparent in the slow-twitch soleus muscle. A slight fall in ATP occurred in both fast-twitch skeletal muscles, and was matched with a stoichiometric rise in IMP and NH₄⁺. No change in ATP occurred in the soleus despite a heavy reliance on anaerobic ATP generating processes. In contrast to the 10 min burst swimming fish, the glycogen concentration in each of the rat skeletal muscles remained high (50% decrease). In conjunction with a parallel series of experiments demonstrating that fatigue was indeed fast-approaching (another 15 to 30 sec), it seems highly unlikely that glycogen supplies would become limiting at this time. Other limiting factors must be involved in the fatigue process after 2 min. Of the regulatory parameters examined, the cytoplasmic ATP/ADP, phosphorylation potential, or limiting supplies of NAD⁺ may be likely candidates. In accord with the etiology of fatigue and exhaustion of trout after 30 min of endurance swimming, was the
finding of near-depletion of glycogen in each of the three skeletal muscles of the rat following the endurance run. Crossover analysis revealed facilitation of Hk at this time with the concomitant switch in fuel from glycogen to exogenous glucose. The concentration of ATP continued to fall, while free ADP continued to rise, resulting in large percentage reductions in the ATP/ADP ratio and phosphorylation potential. The NAD+/NADH ratio was low in the gastrocnemius and plantaris but not statistically different from the condition after the 2 min high intensity run.

The third phase of the study was to understand how PFK-1 can achieve high catalytic rates in working muscle despite falling pH. Evidence was presented that strongly suggested that inhibition of PFK-1 activity was not causal to fatigue in trout white muscle after 10 min of burst swimming, and that the enzyme was fully operational at an intramuscular pH of about 6.6 in the two rat fast-twitch skeletal muscles accompanying short-term high intensity running. From a detailed kinetic analysis on purified rabbit muscle PFK-1, it was concluded that any modulator that increases the ratio of unprotonated to protonated forms of the tetrameric enzyme, could supply the muscle cell with the means of controlling the pH-dependent ATP inhibition of PFK catalysis. An important conclusion to be drawn from this study was that H+ ion should not be thought of as inhibiting PFK-1 per se, but more appropriately, should be viewed as a specific mechanism of control. It was shown that positive modulators exert their effect on PFK-1 catalysis in such a way as to broaden the pH profile of the enzyme.
into the physiological range that has often been considered inhibitory.

One of the most challenging aspects of the present study was to provide an explanation for the contrasting cytosolic redox potentials in skeletal muscle of trout and the rat accompanying short-term high intensity exercise. The redox state of the cytosol in trout white muscle became more oxidized, while that in both rat fast-twitch skeletal muscles became more reduced. It was concluded that this opposite direction of change with exercise relates to species-specific differences in the ability of skeletal muscle to retain lactate. When lactate is retained, as in the case of trout white muscle, pyruvate increases due to mass action effect at the LDH equilibrium. Indeed, a literature survey showed at the onset of heavy exercise, mammalian skeletal muscle may behave as a trout white muscle, with the muscle cytosol becoming more oxidized. This finding was considered an important, and often overlooked, mechanism of glycogenolytic control. In effect, it means that during short-term sub-maximal workloads, the increased availability of NAD$^+$ may be considered as a catalytic potentiator of flux through mass action effect at the GAPDH reaction. Another consequence of lactate retention in skeletal muscle, was the apparent crossover identified at PK in fish white skeletal muscle at both fatigue states. This was misleading and caused by rising pyruvate brought about by the same mechanism described above for the redox changes.

Another contrasting feature between the fish and the rat
accompanying exercise was the precipitous fall in ATP concentration in trout fast-twitch skeletal muscle. The reason for this large difference must obviously be linked to differences in the degree of PCr depletion in skeletal muscle. As PCr stores become limiting, the creatine kinase reaction can no longer maintain ATP constant, and a mismatch between ATP supply and ATP usage develops. Evidence was provided that this was indeed the case, with PCr concentrations falling by 75% and over 95% in the rat and fish skeletal muscles respectively. It was concluded that this difference in the degree of PCr depletion may reflect the locomotory specialization of trout to its lifestyle, but species-specific differences in the psychological response to high intensity running performance can not be discounted.

A general conclusion to be drawn from this comparative analysis of glycogenolytic control in trout white, and in three types of rat skeletal muscle, was that fatigue should be viewed as a multi-component process in response to limiting glycogen, and not leveled at any one particular step of the glycogenolytic pathway.
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