THE EFFECTS OF LACTACIDOSIS ON METABOLISM AND IONIC FLUX IN INACTIVE SKELETAL MUSCLE OF THE ISOLATED PERFUSED RAT HINDLIMB

THE EFFECTS OF LACTACIDOSIS ON METABOLISM AND IONIC FLUX IN INACTIVE SKELETAL MUSCLE OF THE ISOLATED PERFUSED RAT HINDLIMB

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ABSTRACT

Lactate removal by inactive skeletal muscle was investigated using an isolated rat hindlimb perfusion model under conditions simulating recovery from maximal exercise. The purpose of this investigation was threefold: 1) to quantify the contributions of the oxidative, glyconeogenic, and triacylglycerol (TG) synthesis pathways to lactate (La) removal, 2) to examine differences in La removal patterns in inactive skeletal muscle of various fiber types, and 3) to investigate the possible mechanisms for La and H^+ removal by inactive muscle. Male Sprague-Dawley rats were perfused for 60 min at rest with either a normal perfusate (NP) (N = 8) or a lactacidotic perfusate (LP) (N = 8). The LP perfusate was characterized by elevated concentrations of La (11.0 mMol), K^+ (7.88 mMol), and hemoglobin (16.7 $g \cdot dl^{-1}$) and a decreased pH (7.15). Arterial and venous perfusate and soleus (SOL), plantaris (PLT), and white gastrocnemius (WG) muscles were analyzed for various metabolite and ion concentrations.

Analysis revealed increased rates of La uptake, glycerol release and CO_2 output in the LP versus the NP group. No difference was observed for O_2 uptake or glucose uptake between the two groups. Tissue analysis revealed no significant change in muscle ATP, CP, glycogen, pyruvate, F-6-P or TG concentration pre versus post perfusion in both LP and NP groups. Significant increases were found in muscle La concentration (pre vs post and LP vs NP), with SOL having the highest concentration followed by PLT and WG. Muscle [F-1,6-diP], F-1,6-diP/F-6-P and pyruvate/F-1,6-diP ratios were elevated following LP perfusion indicating glyconeogenic inhibition. Muscle glucose levels decreased in the NP but not LP group, indicating a possible

shift in substrate utilization in the LP group.

In the LP group, total calculated La uptake by the 3 muscles was 61.0 umole, with 14% accumulating as tissue La post perfusion. Of the remaining 86%, 12-33% could be accounted for by oxidative metabolism, and 5-7% may have been involved in glycerol release. The remaining 60-75% was unaccounted for, but was hypothesized to have been involved in carbon cycling along the glycolytic/glyconeogenic pathway and/or in TG/FFA substrate cycling. No evidence was found of net glycogen synthesis from La.

Increased H^+ and K^+ influx and HCO_3^- efflux were observed in response to lactacidotic perfusion. Sodium and Cl^- exchange patterns showed a net influx over 60 min of LP perfusion. Data from the ionic flux of the various strong ions and non-volatile H^+ suggested that La is transported into inactive skeletal muscle by various mechanisms, including HLa diffusion, La/H^+ cotransport, and possibly La/Cl^- exchange. The data also suggested that a number of regulatory mechanisms are activated in rat skeletal muscle to maintain intracellular $[H^+]$ and membrane potential during lactacidotic perfusion.

From this investigation it was concluded that, in inactive muscle of the isolated rat hindlimb perfused for 60 min with a lactacidotic perfusate, patterns of La uptake and metabolic elimination are different from those previously observed for active muscle. The metabolic fates of La appear to be related to the ionic disturbances associated with La and H^+ influx into inactive muscle. The net ionic movements across the inactive hindlimb appear to be related to the preferred metabolic pathways of La elimination, but whether or not a direct cause and effect relationship exists cannot be stated conclusively.

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FOREWORD

This thesis is being submitted in partial fulfillment of the requirements for the Master of Science degree in "Adapted Human Biodynamics". The presentation style of the thesis will differ from the traditional format in that the main body will consist of a series of papers being submitted for journal publication. The introduction (Chapter 1) will serve as both an introduction and a review of the literature relevant to lactic acid removal by inactive skeletal muscle as it contributes to recovery from muscle fatigue. Following the introductory chapter, a series of three independent manuscripts linked to the central issue will be presented. These papers will examine the effect of lactacidosis on inactive skeletal muscle with respect to carbohydrate metabolism and glyconeogenic control (Chapter 2), fat metabolism and triacylglycerol regulation (Chapter 3), and ionic regulation of lactate (Chapter 4).

A concluding chapter (Chapter 5) will integrate the significant findings from these various aspects of muscle cell function, and will discuss the interrelationship and importance of biochemical and physico-chemical (ionic) regulation of lactate during recovery from maximal exercise.

Through this approach, the processes of scientific publication and completion of a Master's thesis will be more congruent.

CHAPTER 1 INTRODUCTION

1.1 OVERVIEW

Muscle fatigue is conventionally defined as the failure to maintain a required force output (Edwards, 1981). Decreased tension development during maximal exercise has often been attributed to the accumulation of lactate and/or H^+ (Donaldson, 1983; Hermansen, 1981), which may potentially interfere with many aspects of muscle function: contractile, neural, and metabolic. The main focus of this thesis will be the lactacidosis generated during maximal exercise and its metabolic and ionic regulation by inactive skeletal muscle. More specifically, this thesis will examine the biochemical and physico-chemical changes occurring in non-exercised, inactive, skeletal muscle in an attempt to quantify the mechanisms by which it helps to remove lactate from the circulating plasma. The term "maximal exercise" will refer to high intensity exercise that leads to exhaustion in 30-60 seconds. The term inactive skeletal muscle should also be differentiated from resting skeletal muscle. Inactive skeletal muscle will refer to non-working muscle tissue which has not previously been exercised but which exists in vitro or in vivo in extracellular conditions of an exercised state. Resting muscle, on the other hand, will refer to muscle tissue which exists in an environment where no exercise-related changes have occured.

In order to discuss lactate removal and metabolic elimination by inactive skeletal muscle, it is considered necessary to outline normal muscle function with respect to muscle metabolism, and to review current hypotheses regarding the role of lactate and H^+ accumulation in muscle

fatigue. Subsequent consideration of biochemical pathways for lactate elimination, physico-chemical (ionic) removal of lactate, and their differences in skeletal muscle of various fibre types will provide insight into potential factors involved in recovery from fatigue. Certain areas of weakness in the literature, regarding inactive skeletal muscle metabolism and ion regulation will be outlined, thus providing the rationale for the problem investigated.

1.2 MUSCLE ENERGY SYSTEMS

1.2.1 Muscle ATP Requirement

Force development in skeletal muscle requires the transfer of chemically stored energy into mechanical energy. The immediate source of chemical energy, adenosine triphosphate (ATP), is rapidly hydrolysed during muscle contraction. While ATP is required to pump calcium (Ca^{++}) into the sarcoplasmic reticulum and to pump sodium (Na^{+}) and potassium (K^{+}) ions to maintain an appropriate environment for neural activation, the majority of ATP is required to activate actin-myosin cross-bridge interaction. Repetitive cycling of actin-myosin cross-bridges, to produce muscle shortening and force production, will rapidly breakdown all available sources of ATP.

ATP is hydrolysed in the reaction:

$$ATP + H_2O \text{-----} > ADP + Pi + H^{+}.$$

Due to its low concentration in skeletal muscle (approximately 5.9 $mM \cdot kg^{-1}$ wet weight (WW)) (Tesch et al, 1986), this high energy compound would rapidly be depleted and its end products ADP, P_i, and H^{+} would accumulate. To prevent this from occurring, ATP must constantly be resynthesized.

1.2.2 Muscle ATP Supply

In skeletal muscle cells, three metabolic, or energy transducing, systems function to provide a continuous supply of ATP. Systems of ATP regeneration are based on the principle of cyclical energy transfer, and not energy "production" per se. Energy transfer occurs by the coupling of two biochemical reactions - one, an energy-releasing and the other, an energy-capturing reaction. This allows various forms of energy substrates to fuel muscle contraction via ATP.

1.2.2 (i) High Energy Phosphagens

The initial and most immediate system for ATP resynthesis is the hydrolysis of a high energy phosphagen, phosphocreatine (PCr), which is coupled to ATP formation in the following two reactions:

$$\text{1)}\ PCr + H_2O \xrightarrow{CK} Creatine + P_i$$

$$\text{2)}\ ADP + P_i + H^+ \longrightarrow ATP + H_2O$$

(Newsholme and Leech, 1983). This reaction is catalyzed by the enzyme creatine kinase (CK), which has an altered equilibrium state favouring PCr breakdown under acidotic conditions (Sahlin et al, 1975). PCr recycling of ADP to ATP occurs in the absence of oxygen and, therefore, is considered an anaerobic energy-transducing pathway.

When intracellular ADP concentrations increase and ATP decrease, ATP can be resynthesized in the following reaction, which is catalyzed by the enzyme myokinase (MK):

$$2\,ADP \xrightleftharpoons{M.K.} ATP + AMP.$$

Adenosine monophosphate (AMP) is then broken down to inosine monophosphate (IMP) and ammonia (NH_3), catalyzed by the enzyme AMP-deaminase (Newsholme and Leech, 1983). This pathway of ATP regeneration, known as the nucleotide cycle, is activated when a high ATP turnover rate is coupled with low PCr levels, as occurs during maximal exercise (Green, 1986, Katz et al, 1986b).

1.2.2 (ii) Glycolysis

Glycolysis, the breakdown of glucose, and glycogenolysis, the breakdown of glycogen to pyruvate are rapidly initiated during skeletal muscle activity. Glycolytic ATP resynthesis involves a successive breakdown of the energy source, glucose, through a sequence of intermediates. The transfer of energy, from substrate degradation to ATP resynthesis, occurs at several stages in the glycolytic pathway (see Figure 1.1) with the net result being:

$$\text{Glucose} + 2\text{ADP} + 2P_i \text{ -----> } 2\text{ Pyruvate} + 2\text{ATP} + 4H^+$$

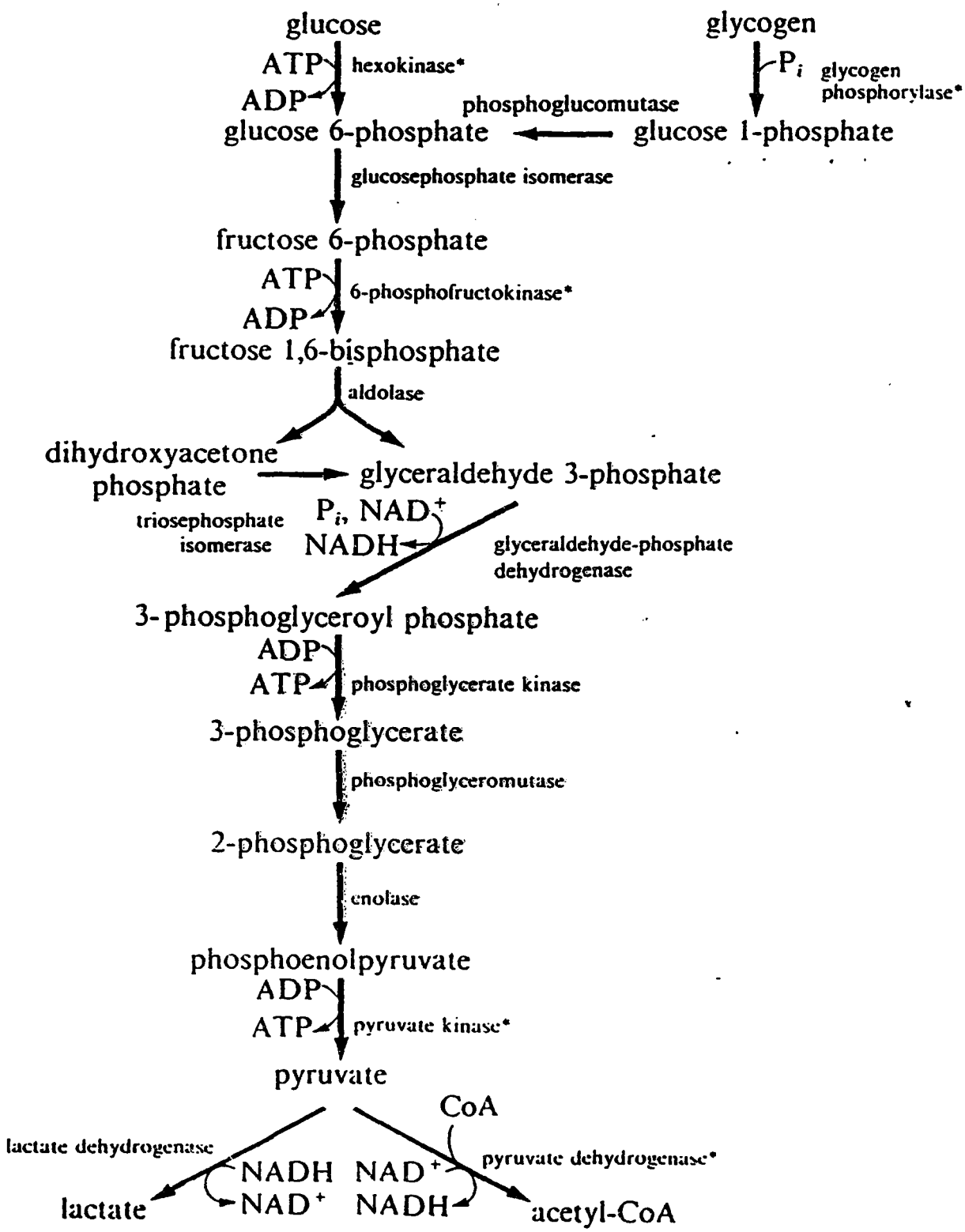
(Guyton, 1981). Glycogenolysis requires an additional step in this pathway. Initially, glycogen is phosphorylated to glucose-1-phosphate (G-1-P), the reaction catalyzed by glycogen phosphorylase, with G-1-P then following the same pathway as glucose.

Pyruvate, the end-product of glycolysis, has two metabolic fates. It will either be oxidized to $CO_2 + H_2O$ in a third system for metabolic ATP resynthesis, or it will be reduced to form lactate (La). This latter reaction involves the oxidation of the nucleotide NADH, as given by the following reaction:

$$\text{Pyruvate} + (\text{NADH} + H^+) \xrightarrow{\text{LDH}} (\text{Lactate} + H^+) + \text{NAD}$$

(Newsholme and Leech, 1983). Lactate formation has traditionally been

Figure 1.1 Schematic representation of the glycolytic pathway in skeletal muscle.
From: Newsholme and Leech (1983), p.180.
* Indicates enzymes that are not reversible under physiological conditions.


glucose
ATP
ADP
hexokinase*
glycogen
P_i
glycogen phosphorylase*
phosphoglucomutase
glucose 6-phosphate
glucose 1-phosphate
glucosephosphate isomerase
fructose 6-phosphate
ATP
ADP
6-phosphofructokinase*
fructose 1,6-bisphosphate
aldolase
dihydroxyacetone phosphate
glyceraldehyde 3-phosphate
triosephosphate isomerase
P_i, NAD^+
NADH
glyceraldehyde-phosphate dehydrogenase
3-phosphoglyceroyl phosphate
ADP
ATP
phosphoglycerate kinase
3-phosphoglycerate
phosphoglyceromutase
2-phosphoglycerate
enolase
phosphoenolpyruvate
ADP
ATP
pyruvate kinase*
pyruvate
CoA
lactate dehydrogenase
NADH
NAD^+
NAD^+
NADH
pyruvate dehydrogenase*
lactate
acetyl-CoA

thought to occur in an anaerobic environment, but current evidence suggests that muscle hypoxia and net La production are not a simple cause and effect relationship.

Muscle hypoxia was long believed to be the sole cause for lactate production during prolonged, intense muscle contraction (Hill, 1924; Margaria et al, 1933). It has recently been suggested, however, that increased lactate formation results from one of two mechanisms. Net lactate production may result from either: 1) an increased pyruvate concentration, due to an accelerated rate of glycolysis, without a proportional increase in pyruvate oxidation, or 2) an altered redox state of the muscle whereby the mitochondrial membrane proton shuttle is too slow to reoxidize the reduced cytosolic NADH (Wasserman et al, 1985).

Ward et al (1982, 1986) have indicated that pyruvate dehydrogenase (PDH), the enzyme complex catalyzing the decarboxylation of pyruvate to acetyl-coenzyme A (acetyl coA), is a rate limiting enzyme. Therefore the transfer of PDH to its active form may be the rate-limiting step in the oxidation of pyruvate. The activation of the PDH enzyme complex, regulated by its interconversion to an active non-phosphorylated form, may be the factor responsible for net lactate production during heavy exercise. Thus, it has been hypothesized that the imbalance between tha rate of glycolysis and the rate of pyruvate oxidation, controlled by active PDH, is responsible for net lactate production (Ward et al, 1982). Glycolytic lactate production has been found to occur in muscle where mitochondrial PO_2 did not drop below 2 Torr (Connett et al, 1984), significantly above the estimated critical mitochondrial O_2 tension of 0.1-0.5 Torr (Chance and Quistorff, 1978). Supporting these arguments, Connett et al (1986) have concluded that lactate production does not appear to be dependent upon tissue oxygen levels (PO_2).

1.2.2 (iii) Oxidative Phosphorylation

During prolonged muscular work, the contributions of PCr degradation and glycolysis decrease due to increases in blood flow and oxygen delivery to the tissues (Hultman and Sjoholm, 1986), and due to Ca^{++} activation of PDH and therefore pyruvate oxidation (Ward et al, 1982). Under these conditions, the glycolytic intermediate, pyruvate, is decarboxylated to acetyl-coA, rather than reduced to La. Acetyl-coA enters the Tricarboxylic Acid (TCA) cycle, resulting in: (1) direct ATP regeneration and (2) indirect ATP regeneration via subsequent channeling of reduced coenzymes NADH and $FADH_2$ through the electron transport chain (ETC).

Coenzymes NADH and $FADH_2$ do not react directly with oxygen, but donate electrons to other electron carriers (Newsholme and Leech, 1983). The ETC consists of a series of electron carriers which transfer electrons to its final acceptor, oxygen (O_2). In the process of O_2 reduction to water, energy is captured via a proton pump and utilized to synthesize 3 moles of ATP per mole of NADH or 2 moles of ATP per mole of $FADH_2$ (Hall, 1983). This final process of ATP formation is known as oxidative phosphorylation.

Aerobic oxidation of glucose by the TCA cycle and the ETC results in the following net energy exchange:

(1) Glucose + $6O_2$ -----> $6CO_2$ + $6H_2O$

(2) 36 ADP + 36 P_i + 36 H^+ -----> 36 ATP

(Hall, 1983). The series of biochemical reactions occurring in the step-wise degradation of pyruvate allow efficient transfer of energy into the usable form of ATP. These reactions, their intermediates and enzymes, are

summarized in Figure 1.2.

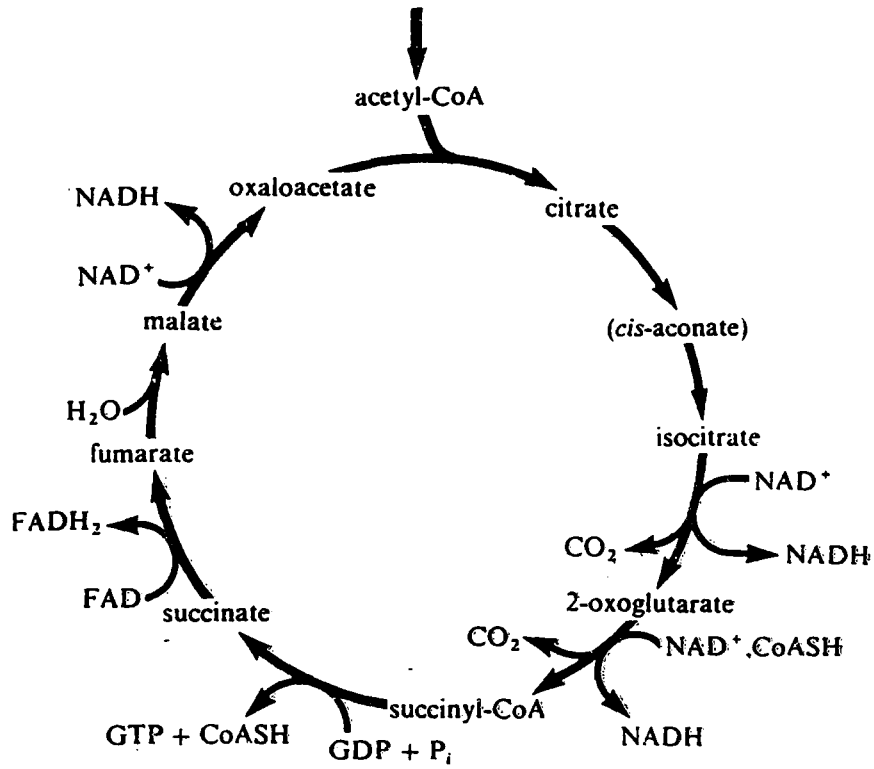
The integration of fuel sources occurs through the entry of substrates as intermediates within the glycolytic and oxidative metabolic pathways. Besides glucose /glycogen, another major energy source, triacylglycerol (TG), can enter these metabolic pathways. TG hydrolysis results in the release of non-esterified fatty acids (NEFA) and glycerol in a 3:1 stoichiometric relationship per mole of TG (Havel and Carlson, 1963). NEFA are catabolized in a step-wise process known as beta-oxidation, to generate acetyl-coA groups and reduced coenzymes NADH and $FADH_2$ (Hall, 1983). Acetyl-coA then enters the TCA cycle, and NADH and $FADH_2$ enter the ETC directly, for complete oxidation and ATP resynthesis. Glycerol, on the other hand, is phosphorylated to glycerol-3-phosphate and, subsequently, oxidized to dihydroxyacetonephosphate (DHAP), an intermediate in the glycolytic pathway.

A third potential energy source, protein, contributes minimally to total energy production during intense muscular activity (Haralambie and Berg, 1976, Viru, 1987), but can enter the energy transducing pathways through amino acid catabolism. Some amino acids can be catabolized to pyruvate or intermediates of the TCA cycle (i.e. acetyl-coA) (Newsholme and Leech, 1983). The extent of protein utilization as a fuel source in skeletal muscle is somewhat controversial.

1.2.3 Metabolic Limitations During Exercise

The relative contributions of the various metabolic fuels are in a constant state of flux. The factors controlling this flux appear to be dependent on energy demand, specifically ATP utilization, as well as energy supply from the various sources of ATP regeneration. During exercise, both

Figure 1.2 Schematic representation of the tricarboxylic acid cycle in skeletal muscle.
From: Newsholme and Leech (1983), p.101.


acetyl-CoA
citrate
(cis-aconate)
isocitrate
NAD+
NADH
CO2
2-oxoglutarate
CO2
NAD+, CoASH
NADH
succinyl-CoA
GDP + Pi
GTP + CoASH
succinate
FAD
FADH2
fumarate
H2O
malate
NAD+
NADH
oxaloacetate

of these factors are altered, and their degree of change dependent upon the intensity of muscular work required. Whether or not the ability of these systems to regenerate ATP is rate limiting during muscle contractions of maximal intensity, has not been clearly established.

1.2.3 (i) ATP and PCr Depletion

During sustained maximal force production (approximately 30 seconds), changes in muscle metabolites have been correlated with decreased tension and the onset of fatigue. Heavy exercise has been characterized by a rapid initial decrease in ATP and PCr stores (Hultman et al, 1967, Bergström et al, 1971, Saltin and Karlsson, 1971, Tesch et al, 1986). Investigations into metabolic changes during intense, prolonged resistance exercise have reported decreases in ATP concentration (ie. Tesch et al, 1986). The apparent decrease in concentration may be due to a fluid shift into the muscle. Hermansen and Vaage (1977) noted an approximate 11-14% increase in intracellular water following intermittent maximal exercise. Slight changes in total adenine nucleotides (ATP + ADP + AMP) may occur due to IMP formation (Edwards et al, 1972, Vaage et al, 1978, Green, 1986, Katz et al, 1986), however, it is currently thought that relatively little net change in ATP content occurs.

Decreased PCr content in muscle was thought to be causally related to fatigue based on findings that isometric force development was directly proportional to PCr concentration (Spande and Schottelius, 1970). More recent investigation using ^{31}P NMR (Dawson et al, 1978, Dawson and Wilkie, 1980, Dawson et al, 1980), isolated muscle preparations (Sahlin et al, 1981), and human biopsy samples (Karlsson et al, 1975), found no relation between muscle PCr levels and isometric tension. Phosphocreatine appears to

be an important energy source in maximal power output, as well as an important shuttle for inorganic phosphate to ADP. However, there appears to be no biochemical basis for hypothesizing PCr depletion as a direct cause of fatigue (Hermansen, 1981). This has lead to the conclusion that limitations in this energy system are probably not the major cause for reduced power output during short term maximal exercise.

1.2.3 (ii) **Muscle Glycogen Depletion**

Muscle glycolysis and glycogenolysis serve an important role in rapid ATP resynthesis during maximal exercise. Recent investigations into skeletal muscle fuel utilization have indicated that glycolysis can occur as rapidly as 1.26 sec into exercise, and can contribute as much as 50% to ATP resynthesis by 6.0 sec (Hultman and Sjöholm, 1986). Muscle glycogen stores have been found to decrease from approximately 90 to 40 mMole glucosyl units·kg^{-1}WW during maximal exercise (3 times 1-min exercise to exhaustion with 4-min rest periods between) (Hermansen and Vaage, 1977). Since this 50 mMole utilization represents about half of the glycogen stores available, it appears that during this form of exercise muscle fatigue occurs prior to glycogen depletion. Mechanical failure during short term high intensity work, therefore, is not likely attributed to lack of muscle glycogen.

1.2.3 (iii) Metabolite Accumulation

Glycogen utilization, under conditions of maximal exercise, will cause rapid La formation and subsequent H^+ release. Muscle La levels have been found to increase from 1-2 mM·kg^{-1} WW at rest to 25-30 mM·kg^{-1} WW following high intensity exercise (Hermansen and Vaage, 1977, Karlsson, 1971). Many authors have suggested that La accumulation is the direct or

indirect cause of muscle fatigue (Asmussen et al, 1948, Karlsson 1971, Karlsson et al 1975, Mainwood and Renaud, 1985). It has also been demonstrated that a close relationship exists between H^+ and La concentration at the end of exercise (Sahlin, 1978), suggesting that cell acidification may be the cause of muscle failure.

Studies measuring muscle homogenate pH have indicated a change from 7.0 at rest to 6.4 at exhaustion with maximal exercise (Hermansen and Osnes, 1972, Sahlin, 1978, Sahlin et al, 1981). This corresponds to an increase in H^+ concentration ($[H^+]$) of approximately 20-25 umole$\cdot g^{-1}$ (Mainwood and Renaud, 1985). Some of the released H^+ is buffered by muscle bicarbonate (HCO_3^-) (Sahlin, 1978, Beaver et al, 1986), however, most of this proton load remains in the muscle or blood. It has been hypothesized that an increase in intracellular $[H^+]$ may be the common regulating factor for the maximal rate of ATP utilization and the maximal rate of ATP resynthesis (Hermansen, 1981, Sahlin et al, 1981, Donaldson, 1983). Controversy continues to exist as to whether the ultimate cause of fatigue is, in fact, a metabolic limitation.

1.3 MUSCLE FATIGUE

1.3.1 Overview

Power output by skeletal muscle is dependent upon the optimal and dynamic interaction of neural recruitment, muscle contractile process, and metabolic sources of ATP regeneration (Green, 1986). Failure at the level of any of these components of muscle function represents a weak link, which may be responsible directly for muscle fatigue. Interference with the

normal biochemical and physico-chemical processes, which can occur at several sites along the nerve-muscle pathway, appear to be the direct cause of such failure.

1.3.2 Sites of Fatigue

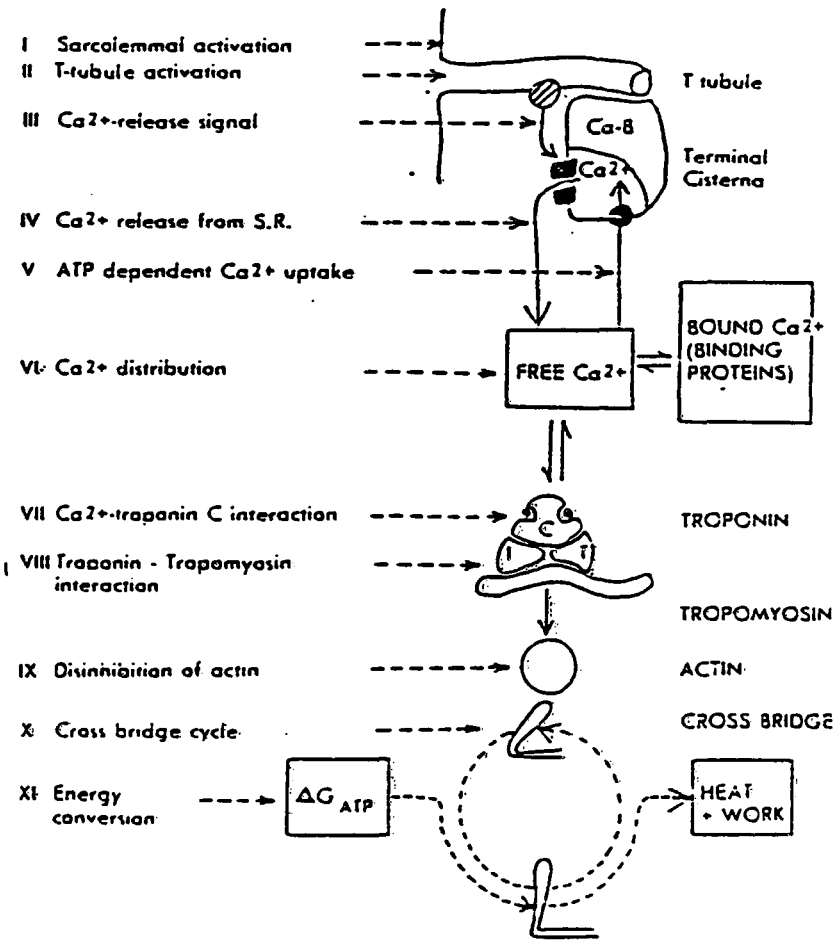
Seven possible sites of fatigue have been outlined by Bigland-Ritchie (1986a) and are listed in Table 1.1. The potential sites of fatigue can be grouped into two major categories - central, involving the brain to muscle pathway (sites 1-3) and peripheral, within the muscle cell itself (sites 4-7). Changes in the neural drive component have not been rigorously investigated during dynamic exercise, therefore the role of central fatigue in decreased dynamic force production is not known. Current evidence indicates, however, that the neural drive is not the weak link during maximal isometric exercise of less than 60 seconds duration (Merton et al, 1981, Bigland-Ritchie et al, 1986b). Thus, only peripheral sites of fatigue will be considered in this section.

Fatigue at the periphery can result from many factors, including changes in electrical properties of muscle membrane, changes in function of the contractile proteins, or from changes in the Ca^{++} kinetics which link electrical to mechanical events. The latter process is known as excitation-contraction (E-C) coupling. The steps linking electrical excitation to mechanical events are illustrated in Figure 1.3, as depicted by Mainwood and Renaud (1985). The relationship between La and H^+ accumulation, and peripheral loci of fatigue, is complex and involves a number of biochemical and biophysical changes which occur at the same time. Examination of the changes occurring at the subcellular sites gives some indication of the direct and indirect role which may be attributed to La and H^+ during muscle

Table 1.1. Possible Sites of Muscle Fatigue

1. Excitatory drive to higher motor centers (ie. motivation, effort).
2. Balance of excitatory and inhibitory pathways converging on lower motoneuron pool.
3. Changes in spinal motor neuron excitability.
4. Integration of electrical transmission from nerve to muscle and over muscle sarcolema and t-tubule system.
5. Excitation-contraction coupling.
6. Local muscle energy supplies.
7. Accumulation of metabolites that may interfere with both metabolic and electrical events.

Figure 1.3 Schematic representation of the possible sites of muscle fatigue within the excitation-contraction coupling mechanisms.
From: Mainwood and Renaud (1985).


I Sarcolemmal activation
II T-tubule activation
III Ca^{2+}-release signal
IV Ca^{2+} release from S.R.
V ATP dependent Ca^{2+} uptake
VI Ca^{2+} distribution
VII Ca^{2+}-troponin C interaction
VIII Troponin - Tropomyosin interaction
IX Disinhibition of actin
X Cross bridge cycle
XI Energy conversion
T tubule
Ca-S
Ca^{2+}
Terminal Cisterna
FREE Ca^{2+}
BOUND Ca^{2+} (BINDING PROTEINS)
TROPONIN
TROPOMYOSIN
ACTIN
CROSS BRIDGE
ΔG_{ATP}
HEAT + WORK

fatigue and recovery.

1.3.3 Mechanisms of Fatigue

1.3.3 (i) Indirect Effects of H^+

It has been suggested that the biochemical processes involved in E-C uncoupling and contractile failure may be a direct result of decreased ATP supply relative to utilization (Edwards et al, 1974, Karlsson et al, 1975, Fitts and Holloszy, 1976, Dawson et al, 1978, Dawson and Wilkie, 1980). A decreased ATP supply during intense exercise could result from the inhibition of glycolysis by the inactivation of key enzymes hexokinase (HK) (Newsholme and Leech, 1983), phosphofructokinase (PFK) (Danforth, 1965, Edwards et al, 1972), or phosphorylase (Chasiotis et al, 1982). This may result from either an increase in $[H^+]$ (Hill, 1955, Edwards et al, 1972) or product inhibition (increase in ADP, P_i, or G-1-P concentration) (Edwards et al, 1972, Donaldson, 1983). Decreased ATP supply will ultimately limit Ca^{++} uptake by the sarcoplasmic reticulum (SR) and actin-myosin cross-bridge formation.

Release and uptake of Ca^{++} by the SR, and Ca^{++} binding to troponin, specifically to the troponin C (TnC) subunit, are important processes in E-C coupling of electrical and mechanical events. Interference with Ca^{++} kinetics during maximal exercise could occur through H^+ interaction at a number of steps (IV-VIII in Figure 1.3). Increased intracellular $[H^+]$ ($[H^+]_i$) can affect the amount of Ca^{++} released by the SR by increasing the amount of Ca^{++} bound to the intra-SR binding proteins (Nakamura and Schwarz, 1972, Fabiato and Fabiato, 1978). Alterations in $[H^+]_i$ may also interfere with muscle relaxation by preventing a re-uptake of

Ca^{++} by the SR (Fabiato and Fabiato, 1978). The latter is most likely due to a failure of an ATPase pump, which moves Ca^{++} against a concentration gradient into the SR.

Intracellular H^{+}, measured as intracellular pH (pH_i), has also been found to affect Ca^{++} function at the contractile level. Studies using the skinned fiber technique have indicated that as pH_i decreases, an increased free Ca^{++} concentration is required by the myofilaments to produce 50% maximal tension (Fabiato and Fabiato, 1978, Robertson and Kerrick, 1979). Mechanisms of altered Ca^{++} sensitivity are still not clear, but a direct interference of H^{+} with Ca^{++} at the TnC binding site has been suggested (Donaldson and Hermansen, 1978).

Nosek et al (1987) have recently suggested that increased $[H^{+}]$ indirectly contributes to muscle fatigue by increasing the fraction of inorganic phosphate (P_i) in the diprotonated form ($H_2PO_4^{-1}$). Phosphate exists in either the monoprotonated (HPO_4^{-2}) or diprotonated form, with the ratio dependent upon proton concentration. It has been suggested that an increase in the diprotonated form of P_i, observed at a decreased pHi (Nosek et al, 1987), depresses force output by direct action on the cross-bridges (Dawson et al, 1986).

The suspected mechanisms by which La and/or H^{+} exert inhibitory effects during muscle fatigue, have been directly linked to failure of ATP-dependent processes, and to disruption of Ca^{++} regulation. In a complex interaction, metabolic (ATP supply and utilization) and ionic (Ca^{++} release and uptake) factors are controlled through metabolic (lactic acid production) and ionic (La and H^{+} accumulation) feedback. This feedback system may function as a protective mechanism to prevent irreversible muscle rigour (Bigland-Ritchie, 1986a). It has been suggested that the single most

important factor in this control cascade is intracellular H^+ concentration.

1.3.3 (ii) Direct Effects of H^+

Decreased force development during fatigue has also been attributed to several mechanisms where H^+ acts directly on the contractile machinery. These direct H^+-mediated processes may account for up to 30% of the force decrease during maximal exercise (Sahlin et al, 1978, Mainwood and Alward, 1982, Mainwood and Renaud, 1985). Hypotheses to explain this direct proton interaction include: 1) H^+ binding and interference with myofibrillar ATPase activity (Fitts and Holloszy, 1976, Fabiato and Fabiato, 1978); and 2) H^+ interaction with Mg^{++} on the contractile machinery, most likely on the myosin filament (Donaldson and Hermansen, 1978).

1.3.4 Summary of Fatigue Mechanisms

These direct and indirect H^+-mediated mechanisms of muscle fatigue are still not fully understood. Although a number of studies associate acidosis with fatigue (see Mainwood and Renaud, 1985, for recent review), this association does not necessarily prove that increased $[H^+]$ is a causative factor of fatigue. In fact, it has recently been suggested that rate of fatigue development is pH-independent, and that it is the recovery process which is dependent upon muscle pH (Mainwood and Renaud, 1985, Renaud and Mainwood, 1985).

1.4 RECOVERY FROM MUSCLE FATIGUE

1.4.1 Model of Muscle Fatigue and Recovery

It has recently been hypothesized that H^+ interferes with the

recovery of muscle from a fatigued back to a normal state (Renaud and Mainwood, 1985). The transition in muscle from a normal to a fatigued state appears to be dependent upon frequency and duration of stimulation, and not on $[H^+]$. Only during the reverse process of recovery, is H^+ implicated. Furthermore, it has been suggested that extracellular pH (pH_o), rather than intracellular pH (pH_i), determines rate of recovery from muscle fatigue.

Renaud and Mainwood (1985) suggest that failure in the Ca^{++} release mechanism by the SR may be the ultimate step in the development of muscle fatigue and impairment. The reversal of this fatigue process involves a Ca^{++} release/uptake - H^+ interaction. Recovery from a fatigued state (a pH-dependent process), however, is thought to be directly related to changes in the extracellular environment of the muscle. Thus, in order to understand the mechanisms underlying muscle recovery from fatigue, the inter-relationship between intra- and extracellular $[H^+]$, should also be clarified.

1.4.2 Fatigue, Recovery and Extracellular $[H^+]$

1.4.2 (i) H^+ Accumulation

Intracellular acidosis is a result of proton accumulation. During maximal exercise, protons are generated via metabolic production of both lactate and CO_2 in the glycolytic pathway. The former is a strong acid and readily dissociates to its ionic form ($HLa \text{-----} > La + H^+$), while the latter results in formation of a weak acid (H_2CO_3) which will release H^+ and HCO_3^-. During maximal activity of short duration, proton production from CO_2 is minimal compared to the relative increase in protons from lactate production.

Proton removal, to counter its production, is dependent upon two processes: 1) transmembrane flux of acid (or base) and, 2) removal of acid (or supply of base) from extracellular fluids by capillary blood flow. It has been found that extracellular [H^+] determines intracellular [H^+] (Mainwood and Worsely-Brown, 1975, Renaud and Mainwood, 1985) by altering rate of proton efflux. By maintaining a low extracellular [H^+] and therefore an increased transmembrane pH gradient, H^+ efflux appears to be enhanced. Proton efflux will, to some extent, determine the magnitude of intracellular acidosis and, ultimately this may determine skeletal muscle functional impairment.

It should be noted that although extracellular [H^+] has been found to influence intracellular [H^+], the effect of extracellular [H^+] on intracellular [H^+] does not appear be as important in the normal physiological pH range of 7.1-7.4. Adler et al (1965a, 1965b) found that extracellular acidification did not significantly effect intracellular pH until extracellular pH was below 7.0, an acidosis induced by a PCO_2 of 70 mmHg or a [HCO_3^-] of < 7 mMol·l^{-1}. Therefore, it has been observed that large changes in extracellular [H^+] are required to produce even small changes in intracellular [H^+].

1.4.2 (ii) Extracellular [H^+] and Fatigue Mechanisms

Extracellular pH has been found to influence the recovery transition from a fatigued back to a normal muscle state by its effect on several E-C coupling and contractile elements. Muscle pH_o appears to influence t-tubule function and transmission of Ca^{++} release signals. Sites on skeletal muscle t-tubules that are inhibited at low pH_o levels have been identified (Dorrsheidt-Kafer, 1983), and these may prevent excitation of the

SR. Changes in pH_o from an acidotic extreme of 5.5 to an alkalotic extreme of 10.5 have been found to inhibit contractile function (Robertson and Kerrick, 1979) possibly due to changes in membrane macromolecules associated with activation of Ca^{++} release.

The process of muscle fatigue, and its regulation by metabolic and ionic factors, is a complex interaction of changes in several variables ($[H^+]_i$, La, ATP, Ca^{++}, etc.). The determining factor in fatigue-onset of experimental models appears to be related to rate and frequency of stimulation. Recovery from muscle fatigue may involve the same variables, but the determining factor is thought to be $[H^+]_o$. The pH_o dependency of recovery has been supported by evidence from numerous studies (see Mainwood and Renaud, 1985). This bulk of evidence suggests that proton efflux and proton removal mechanisms, which help to maintain a high extracellular pH, will determine the status of the immediate extracellular fluid and therefore play a key role in determining intracellular proton balance.

1.4.3 Lactic Acid and Recovery from Fatigue

1.4.3 (i) Relationship of Lactate to H^+

Many investigators argue that the decrease in muscle performance at high intensities is primarily linked to lactic acid production and accumulation (Hermansen, 1981, Donaldson, 1983, Hultman and Sjöholm, 1986). This association is more than coincidental. Since H^+ accumulation is linked to La, through metabolic production of lactic acid, the presence of La indirectly indicates presence of H^+. The relationship between La concentration ([La]) and $[H^+]$ becomes clearer when considering their ionic interdependence.

Because lactic acid is completely dissociated at a physiological pH (pKa = 3.7), and because the anion La is a strong ion and therefore an independent variable in Stewart's model of acid-base equilibria (Stewart, 1981, 1983), [La] will directly determine $[H^+]$. According to this view of physical chemistry, $[H^+]$ is a dependent variable and changes in $[H^+]$ can only occur from changes in the independent variables. These independent variables include: 1) strong ion difference (difference between all positive cations and all negative anions); 2) total weak acid concentration (termed $[A_{tot}]$; and 3) partial pressure of CO_2 (PCO_2). The strong ion difference, of which La is an important constituent, is calculated as:

$$[SID] = [Na^+] + [K^+] + [Mg^{++}] + [Ca^{++}] - [La] - [Cl^-]$$

(Stewart, 1981, 1983). Based on this relationship, it appears that $[H^+]$ can only be altered through changes in concentration of a strong ion, changes in A_{tot} and/or changes in PCO_2. Recent investigation has determined that the single most important variable in determining acid-base equilibria during and following high-intensity exercise, is [La] (Kowalchuk, 1985, Lindinger et al, 1987).

1.4.3 (ii) Lactate, H^+ and Recovery from Fatigue

In accordance with the model of muscle fatigue and recovery introduced by Renaud and Mainwood (1985), it is apparent that control of the extracellular $[H^+]$ is important in the pH_o-dependent transition of muscle from a fatigued to a normal state. Also, based on the reported dependence of $[H^+]$ on [La], it should hold that recovery from fatigue is dependent on the extracellular [La] ($[La]_o$). Building from this premise, the production, accumulation, and removal of La may become significant variables during recovery. If skeletal muscle is to recover from a fatigued state,

then it is hypothesized that La must be removed from the direct surroundings of the muscle cell, and eliminated by various other cells of the body.

The ionized form of La is a strong ion and also a metabolite. Discussion of La (and, therefore, H^+) regulation in the extracellular fluid should consider the biochemical and physico-chemical properties of La. As a substrate, La can be metabolically eliminated by several pathways, and at several metabolically active sites. As an ion, La must be transported across membrane barriers and will interact in physiological solutions as outlined by the equations of Stewart (1981, 1983). If recovery from muscle fatigue is dependent on how/when pH_o changes, then it may also be dependent on how/when $[La]_o$ changes. The ability of the organism to ionically and metabolically remove La from the extracellular space of the active muscle may determine its ability to recover from fatigue.

1.5 LACTATE REMOVAL FOLLOWING MAXIMAL EXERCISE: IONIC CONSIDERATIONS

1.5.1 Introduction

Traditional theories regarding lactic acid accumulation and removal following exercise have focused on the "acid-base" disturbances caused by this molecule. By the accepted definition, lactate is an "acid" because it is a proton donor. For the sake of clarity, the ion lactate will continue to be referred to as 'La', recognizing the fact that it carries a negative charge (i.e. La^-), and the protonated molecule will be referred to as 'HLa'.

At a physiological intracellular pH of 7.0, the concentration of La is

2000-fold greater than the concentration of HLa (Hultman and Sahlin, 1980). This warrants the consideration of La as a strong ion, rather than an acid per se. It has recently been suggested that "acid-base" regulation is ionic regulation (Lindinger, 1987), and that H^+ regulation can only occur in conjunction with exchanges in strong ions, and CO_2 regulation, between muscle and extracellular components (Heigenhauser et al, 1986).

Mechanisms by which La permeation across a membrane can occur include: 1) passive diffusion; 2) passive mediated transport; and 3) active mediated transport. Since either the La or HLa species can cross the muscle and capillary membrane barrier, theoretically, six possible methods of translocation could exist (Hultman and Sahlin, 1980). Differentiation between the two forms of La translocation (ie. protonated versus unprotonated) have been assessed by comparison of La versus H^+ flux rates (Benade and Heisler, 1978, Seo, 1984), while differentiation between mechanisms of translocation has involved the use of channel blockers amelioride (blocks Na^+/H^+ exchange), and 4-acetomide-isothicyanostilbene-2, 2'-disulphonic acid (SITS) (blocks anion exchange) (Mainwood and Alward, 1982, Kuret et al, 1986, Juel, 1987). Controversy continues to exist as to the precise mechanisms for translocation of La and H^+ across the muscle membrane.

1.5.2 Lactate Flux by Lactic Acid Translocation

Early theories of HLa translocation across muscle cell membranes were based on observations by Fenn (1936) that muscle was impermeable to anions. It was postulated that HLa movement occurred by simple diffusion of the protonated molecule, or that cell injury occurred, allowing enhanced membrane permeability. More recent work continues to support the idea of

HLa transport, as it has repeatedly been shown that La and H^+ release from muscle occurs in a 1:1 stoichiometric relationship (Hirche et al, 1975, Roos, 1975, Mainwood and Worsley-Brown, 1975, Sahlin, 1978, Seo, 1984).

Hirche et al (1975) investigated La and H^+ efflux in perfused, supramaximally stimulated, dog gastrocnemius and found that La and H^+ efflux followed the same time course. By inducing metabolic acidosis and alkalosis, it was also found that permeation increased with an increased HCO_3^- concentration and low pH. Support for this proposed pH-gradient dependency was given by Roos (1975), who investigated steady state La distribution over 2-5 hours and suggested that HLa translocation acted as a carrier system to transport H^+ down an electrochemical gradient. Recent work by Connett et al (1986) has supported this conclusion.

Studies with exercising humans (Sahlin, 1978), as well as stimulated frogs (Seo, 1984) and rats (Mainwood and Worsley-Brown, 1975, Mainwood and Alward, 1982) have recently confirmed the equimolar and pH-dependent release of La and H^+. Seo (1984), using ^1H NMR, has quantified this efflux mechanism. It was observed that H^+ efflux increased linearly with La efflux up to 70 umole·min^{-1} ·$litre^{-1}$ H_2O. This study (Seo, 1984) suggested, however, that both the unprotonated and protonated forms of La contribute to La efflux following maximal exercise, a conclusion supported by the work of Sahlin et al (1978) and Connett et al (1986).

1.5.3 Lactate Flux by Lactate and Hydrogen Ion Translocation

Heisler et al (1973) were the first to suggest that differences in rate of La and H+ efflux exist between La and H^+. This observation of a non-stoichiometric release of La and H^+ was later confirmed in both animal (Benade and Heisler, 1978, Barbee et al, 1983, Seo, 1984, Chirtel et al,

1985) and human (Sahlin et al, 1976, Sahlin, 1978, Jorfeldt et al, 1978) skeletal muscle. Benade and Heisler, (1978) reported H^+:La release ratios of 14:1 and 50:1 in stimulated rat diaphragm and sartorius muscle, respectively. Heigenhauser et al (1986) reported a ratio of 4.5:1 in isolated rat hindlimb muscle, while Barbee et al (1983) found the non-CO_2 acid output rates exceeded La output rates by a factor of 4.7 during isometric twitch contractions of dog gastrocnemius-plantaris muscle. Discrepancies between these studies may be due to differences in fibre type of the muscle(s) investigated. Nonetheless, these authors have concluded that separate exchange mechanisms for La and H^+ appear to exist in skeletal muscle.

Hultman and Sahlin (1980) have postulated that La, as an ion, is the permeating species due to its relatively higher concentration in muscle and interstitial spaces. These authors caution, however, that due to problems in membrane permeability of charged particles through a lipid bilayer, the controversy over La versus HLa transport remains unresolved.

1.5.4 Mechanisms of Lactate and/or Lactic Acid Transport

If translocation of La or HLa occurs by a passive mechanism, there must be a release of energy during the process. Analysis of the energetics of permeation has helped to distinguish between passive and active transport mechanisms (see Hultman and Sahlin, 1980 for calculations). Free diffusion of HLa will occur as a protonated molecule from a compartment with a higher concentration to a compartment with a lower concentration. The rate of HLa flux is therefore dependent upon the relative concentrations of La and H^+ ions on either side of the membrane. Under resting conditions, free energy change for HLa movement across muscle is zero when the intracellular to

extracellular La ratio ($[La]_i/[La]_o$) is 0.54 (Hultman and Sahlin, 1980). The ratio [La]i/[La]o of 0.54 appears to be the steady state distribution ratio for La between the intracellular and extracellular fluids of muscle. Thus, passive efflux of HLa would occur at a ratio of $[La]_i/[La]_o$ greater than 0.54 and passive influx of HLa would occur at ratios of $[La]_i/[La]_o$ lower than 0.54.

The energy released from movement of charged particles (La and H^+) between biological compartments with different membrane potentials is dependent upon: 1) concentration gradients, and 2) differences in electrical potential. The negatively charged intracellular compartment can exert an electrical force to drive negatively charged anions (i.e. La) out of the cell. The reverse direction, for tissues removing La from circulating fluids in vivo, however, cannot occur passively (Hultman and Sahlin, 1980).

Connett et al (1986) determined that passive transport is the principal mechanism controlling the outward flux of HLa in dog red gracilis muscle, at stimulation rates of 1-8 Hz. Calculations of efflux were based on surface area available for exchange (capillary and muscle), and concentration gradients. The rate limiting step in passive HLa efflux was concluded to be the capillary density of the working muscle. Capillary density was critically lowered only at a stimulation rate of 6-8 Hz, which is where La efflux reached a plateau. Previous work had suggested that the muscle membrane barrier and/or diffusion of HLa through the interstitial space were rate limiting in La efflux (Hirche et al, 1975).

Passive-mediated and active-mediated transport of La are both carrier mechanisms. They are thought to involve membrane bound proteins, with specific binding sites for La and/or H^+, or pores through the membrane where passage is facilitated (Hultman and Sahlin, 1980). These carrier

mechanisms may consist of anion exchange mechanisms (La/Cl^- or La/HCO_3^-) or cotransport mechanisms (La/H^+) (Juel, 1987). Other transport mechanisms thought to be involved in pH_i regulation include Na^+/H^+ and Cl^-/HCO_3^- exchangers (Mainwood and Alward, 1982). Thus, there may be a variety of mechanisms which regulate intra- and extracellular ion concentrations, each of which may be involved in La removal.

Carrier mechanisms do not depend on free energy exchange across a membrane (Hultman and Sahlin, 1980), but passive-mediated transport only occurs when net energy is released while active-mediated transport requires net energy input. Both types of mediated transport are rate-regulated, similar to enzymatic reactions, and act as catalysts to increase permeation rates compared to passive diffusion.

These carrier mechanisms characteristically show some type of saturation at elevated substrate levels. This has been demonstrated in studies where La efflux levelled off at 4-5 $mMol \cdot min^{-1}$ (Jorfeldt et al, 1978) and 8 $mMol \cdot min^{-1}$ (Connett et al, 1986). Lactate influx, into La metabolizing tissues, has also been reported to be a saturable process (Karlsson et al, 1972). These data indirectly implicate carrier mechanisms in the translocation of La across muscle cell membranes.

A more direct approach to investigating the function of carrier mechanisms for La transport has been to use channel blocking chemicals. Kuret et al (1986) found no saturation of La influx over the physiological range of 1-50 mMol when no inhibiting agents were used. These authors did find, however, that within the same range, La influx was decreased 30% with the anion-transport inhibitor, SITS. An irreversible inhibition (90%) was found when mersayl acid (2mM) was used, leading to the conclusion that a channel protein of known chemical composition was responsible for La and H^+

cotransport (Kuret et al, 1986).

These findings have been challenged by Juel (1987) who found that no large change in La efflux occurred in response to inhibitors used to block La/$Cl^-$and La/HCO_3^- anion exchange. Work by Juel (1985) suggested that a Na/H^+ exchange mechanism is activated at a faster rate than any La efflux mechanism following exercise. This may explain the non-stroichiometric efflux rates found for La and H^+, as reported in various studies (Benade and Heisler, 1978, Sahlin, 1978, Jorfeldt et al, 1978, Seo, 1984, Heigenhauser et al, 1986). The integration of the various ionic transport mechanisms involved in La and H^+ flux across muscle membrane is still not fully understood.

1.5.5 Factors Modifying Lactate Transport

Lactate transport has been found to be dependent on a number of external variables. Of greatest significance are the following factors:

1) transmembrane pH-gradient (and therefore pHo) (Hirche et al, 1975, Roos, 1975, Mainwood and Worsley-Brown, 1975, Mainwood and Alward, 1982, Connett et al, 1986)
2) La concentration gradient (Jorfeldt et al, 1978, Sahlin, 1978, Seo, 1984)
3) buffer concentration (Mainwood and Worsley-Brown, 1975, Beaver et al, 1986, Juel, 1987)
4) membrane potential (Mainwood and Worsley-Brown, 1975)
5) work rate (Freund et al, 1986)
6) time into recovery (Sahlin, 1978, Beaver et al, 1986)

and

7) muscle fibre type (Essén et al, 1975).

Based on the interaction of these variables, different mechanisms of removal may predominate under different conditions. This would account for some discrepancies in the results reported in the literature. Differences may also be due to methodological problems, such as H^+ efflux calculation, which has been criticized in several studies.

1.5.6 Methodological Considerations

The classical approach to "acid-base" regulation has been to examine changes in La content and changes in CO_2-combining capacity (Barr and Himwich, 1923), or base deficit (Osnes and Hermansen, 1972, Benade and Heisler, 1978). Measurements of the latter have utilized the Henderson-Hasselbalch equation to equate changes in HCO_3^- to changes in H^+. Hydrogen ion buffering by the HCO_3^-/CO_2 buffering system occurs according to the following reaction:

$$H^+ + HCO_3^- \xleftarrow{CA} H2CO_3 \xleftarrow{CA} CO_2 + H_20$$

as catalyzed by the enzyme carbonic anhydrase (CA) (Hultman and Sahlin, 1980). Values obtained for changes in pH and H^+ are then related to La measurements to evaluate relative rates of release of the various forms of La (Osnes and Hermansen, 1972, Benade and Heisler, 1978).

Shortcomings of the classical approach have been recognized by Lindinger (1987) based on the physico-chemical model of $[H^+]$ determination proposed by Stewart (1981, 1983). According to this approach, all weak acids (proteins, phosphates, phosphorylated metabolites, and amino acids), and all strong acids (ions) (La, Na^+, K^+, Mg^{++}, Ca^{++}, Cl^-), can associate and dissociate H^+, thereby acting as potential mechanisms to remove H^+ from solution various biological compartments. Strong acids are always

dissociated in physiological solution, thus their contribution to H^+ regulation appears to be dependent on their net ionic charge ([SID]). Weak acids (A) buffer H^+ in the general reaction:

$$HA \text{ <-----> } H^+ + A^-$$

(Stewart, 1981, 1983), with the rate of H^+ elimination dependent upon the dissociation constant for the reaction :

$$[H^+]*[A^-] = K_A*[HA]$$

where K_A is the dissociation constant. Bicarbonate is just one of the buffers that remove free protons from a solution. Other physico-chemical buffers involved in H^+ removal in skeletal muscle during intense exercise include: creatine, P_i, and glutamine (Sahlin, 1978) and the histidine groups. Evidence that other buffers are actively involved during exercise-induced acidosis has been obtained in studies where recovery from fatigue was not [HCO_3^-]-dependent (Mainwood and Alward, 1982, Juel, 1987). Thus, considerations of base deficit, base excess, and CO_2-combining capacity alone is not sufficient to accurately calculate H^+ efflux.

1.5.7 Summary of Ionic Mechanisms of Lactate Removal

Carrier mechanisms involved in La efflux include cotransport of La and H^+, or anion exchange, which may be coupled with Na^+/H^+ cation exchange mechanisms. Cotransport of La and H^+ would explain the 1:1 relationship in efflux of these ions, and the apparent diffusion of HLa. Following intense exercise, activation of the Na^+/H^+ exchange mechanism has been suggested to occur (Juel, 1985). This increase in H^+ efflux, relative to La removal, would account for the non-stoichiometric efflux of H^+ and La. Thus, various mechanisms may interact to remove La and H^+ from the intracellular compartment following heavy exercise, with the predominating system

apparently dependent upon external factors (pH_o, $[H+]_o$, etc.).

Based on current evidence, it appears that La translocation in both the ionic and non-ionic species occurs. According to Seo (1984), initial La removal involves passive and/or carrier-mediated transport of La across the muscle membrane. Beyond a blood La concentration of 25 mMol, where exercise limitations begin to occur, carrier mechanisms may become saturated, and optimal La removal is hindered. Saturation of mechanisms of La transport into tissues that remove La have been examined in vitro (Juel, 1987), but the mechanisms of La influx have not been rigorously investigated.

1.6 LACTATE REMOVAL FOLLOWING MAXIMAL EXERCISE: METABOLIC CONSIDERATIONS

1.6.1 Overview

The ultimate fate of La in circulation, or of La accumulating in the muscle cell, is dependent upon the pathway by which it is metabolized. Controversy exists over the metabolic pathway most important in La removal following maximal exercise. Disagreement also exists as to the most significant sites of La elimination. Control of both site and pathway are dependent on a number of factors, including substrate supply, hormonal condition, and key enzyme activity levels, which in turn are governed by the metabolic state. The interaction between changes in these regulating factors, and changes in pathway and site of La elimination following exercise will be reviewed.

1.6.2 Lactate Elimination : Early Theories

Theories regarding La utilization following exercise have existed since the early work of Meyerhof et al (1922, 1925) and Hill (1925). The Hill-Meyerhof theory of oxygen-debt (0_2-debt) was developed to explain elevated post-exercise 0_2 consumption, lactate disappearance, and glycogen resynthesis. It was thought that for every mole of La utilized, one-fifth was oxidized, providing energy for the remaining four-fifths to be converted to glycogen (Hill, 1925). The 5:1 ratio for La removed to La oxidized was later revised by Margaria et al (1933) and Bendall and Taylor (1970), who reported values of 10:1 and 6.2:1, respectively. This ratio represents a high proportion (80-90%) of La utilization for glycogen resynthesis.

A challenge to this early theory came from Cori and Cori (1927), Eggleton and Evans (1930), and Himwich et al (1930), who demonstrated that glycogen synthesis from La did not occur directly within the muscle. Measurements of La in the circulating blood following exercise lead to the conclusion that La was transported to the liver for conversion to glucose and/or glycogen. Hepatic glucose was then released and transported back to muscle tissue for synthesis of glycogen, which would ultimately be rephosphorylated for La production. The completed cycle is known as the Cori cycle.

Glyconeogenesis from La is thought to occur via a reversal of the glycolytic pathway, with the exception of alterations at three regulating steps. These regulating steps provide control of metabolic flux along the degradation or synthesis pathways. During glycogen synthesis, the three rate limiting steps are:

1) pyruvate ----> phosphoenolpyruvate (PEP) catalyzed by the enzymes PEP-carboxykinase (PEP-CK) and pyruvate carboxylase (PC)

2) fructose-1,6-diphosphate (F-1,6-diP) ----> fructose-6-phosphate (F-6-P) catalyzed by F-1,6-diphosphatase (FBP)
3) glucose-1-phosphate (G-1-P) ----> UDP glucose ----> glycogen catalyzed by glycogen synthetase (Hall, 1983).

Arguments against glyconeogenesis occuring directly from La in muscle were based on earlier studies which reported that skeletal muscle cells had inadequate concentrations of the key enzymes (PEP-CK, PC, and FBP) for glyconoeogenesis (Krebs and Woodford, 1964, Opie and Newsholme, 1967).

1.6.3 Lactate Elimination: Current Theories and Controversies

Recent evidence has indicated that the key glyconeogenic enzymes do exist in skeletal muscle tissue, but that their concentrations vary widely across muscles of varying fiber type distribution (McLane and Holloszy, 1979, Shiota et al, 1984). Experimental data attempting to confirm intramuscular glycogen resynthesis from La have been obtained from several human studies (Hermansen and Vaage, 1977, Astrand et al, 1986), as well as in animal studies (McLane and Holloszy, 1979, Shiota et al, 1984) in which muscle glycogen was previously depleted. These findings argue against the Cori cycle being the only pathway for restoration of muscle glycogen.

In a recent review, however, Brooks (1986) re-emphasized that La removal patterns contribute to total carbon flux in the body by integrating anabolic functions in some tissues (i.e. liver gluconeogenesis) with catabolic functions in others (muscle glycogenolysis). This concept would support the original Cori cycle hypothesis. Thus, controversy continues to exist as to the precise pathway for La to glycogen conversion.

Brooks and co-workers (1973, 1980, 1986) have challenged the concepts underlying the entire 0_2 debt-glycogen reconversion theory.

Following an infusion of ^{14}C La, these authors concluded that the primary fate of La following exhaustive exercise was CO_2 and HCO_3^-. Following one hour of recovery, 65% of the radioisotope appeared as endpoints of oxidation, and very little was found as glycogen. These findings agreed with previous work with dogs (Depocas et al, 1969) and rabbits (Drury and Wick, 1956, Issekutz et al, 1976).

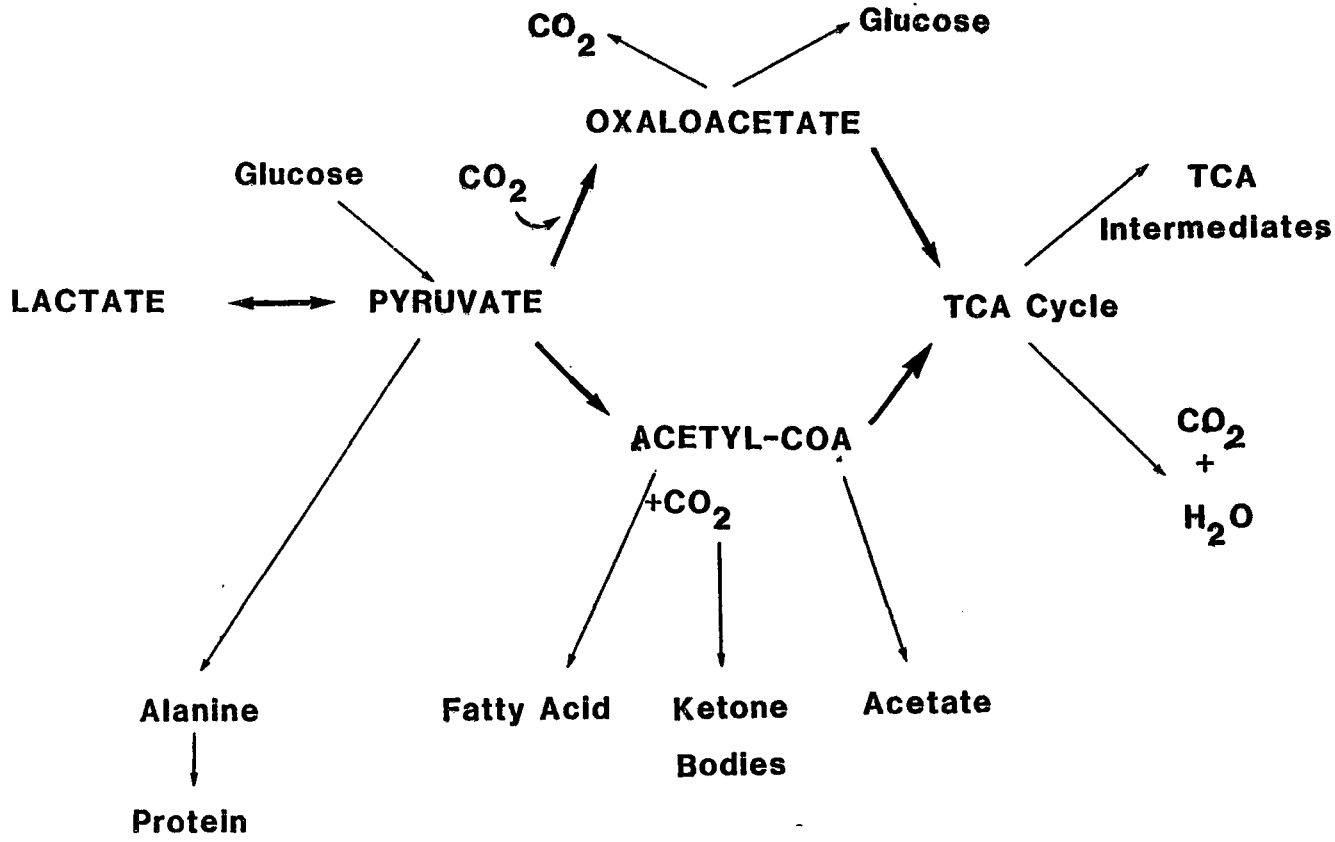
Even greater controversy exists when considering the numerous other postulated pathways for La elimination. The importance of these pathways have been suggested from studies measuring substrate and/or gas exchange in tissues consuming La post-exercise. The various metabolic pathways that may be involved in La elimination are illustrated in Figure 1.4. The alanine cycle, proposed by Malette et al (1969) and Felig et al (1970), accounted for La elimination by its oxidation to pyruvate and subsequent transamination of pyruvate to alanine. Fatty acid synthesis, during recovery from supramaximal exercise, has also been suggested as an end product of La (Kowalchuk, 1985). The latter finding was based on gas exchange data and not direct quantification of muscle TG content or NEFA release. It is not known, however whether alterations in NEFA biosynthesis would be occurring intramuscularly or within adjacent adipose cells.

1.6.4 Regulation of Metabolic Pathways for Lactate Elimination

The relative importance of each metabolic pathway is dependent upon the metabolic state (previously exercised versus rested; fasted versus fed), and the site of removal. The two major pathways for La removal appear to be: (1) oxidation to CO_2 and H_2O with some release of HCO_3^-; and (2) synthesis of glycogen. Various proportions of La eliminated by each pathway have been reported in the literature. A summary of the major

Figure 1.4 Illustration of the major biochemical pathways for lactate metabolism.
Modified from: Katz et al, (1986).

MAJOR PATHWAYS FOR LACTATE METABOLISM



studies investigating La elimination, under various conditions and in various populations, can be seen in Table 1.2.

As revealed by Table 1.2, large discrepancies exist in studies examining the major metabolic pathways in La removal. Some of the discrepancy can be explained by the use of different populations, different models, and different techniques of analysis. These differences will not be evaluated. Some of the noted differences in La elimination pathway can be explained by two major factors: metabolic state, and muscle fibre type. The second factor, muscle fibre type, will be reviewed in Section 1.7. Differences due to changes in substrate supply, hormonal conditions, and enzyme activity levels, as altered by the metabolic state, will now be discussed.

1.6.4 (i) Substrate Supply

The relative proportion of La being utilized for glycogen resynthesis is dependent upon blood glucose and blood La availability. Under fasted conditions, hypoglycemia promotes La extraction and increased conversion of glucose to La (Kriesburg et al, 1970). High La and glucose levels (i.e. following maximal intensity exercise) enhance glyconeogenesis from La, with La being the preferred substrate (McLane and Holloszy, 1979, Åstrand et al, 1986). In an investigation by Bendall and Taylor (1970), optimal conditions for glycogen synthesis were found to occur when La concentrations were 38-40 mMol, a value not within the physiological range. The interdependence of substrate supply on metabolic rate is critical, since oxidation of La will not increase simply due to increased blood La concentration. Both pyruvate dehydrogenase (PDH) and citrate synthetase are flux-generating steps, and elevated La (and, therefore, pyruvate)

Table 1.2. Relative Roles of Oxidation and Glyconeogenesis in Lactate Elimination

CONDITIONS	POPULATION	%LACTATE APPEARING AS			REFERENCE
		GLYCOGEN	CO_2 + HCO_3^-	OTHER	
Theoretical	---	80	20		Hill (1924)
Ex.	Man	90	10		Margaria (1933)
Rest	Rabbit	4-5	80-90		Drury and Wick (1956)
Ex.	Dog	10	74		Depocas et al (1969)
	Frog/Rabbit	83.8	16.2		Bendall & Taylor (1970)
Ex.	Man		38 @ 10min 52 @ 40min	20*	Jorfeldt (1970)
Fasted + Ex.	Rat		20-25 @ 20min 65 @ 1hr		Brooks et al (1973)
Fasted	"		71 @ 1hr		
Rest	Dog	18-19	50		Issekutz et al (1976)
Ex.	"	25	55		
Ex.	Man	75	15		Hermansen & Vaage (1977)
Rest (24 hr post-ex.)	Rat	44		35* 20**	McLane & Holloszy (1979)
Ex. + 12hr fast	Rat	19	63 @ 4hr		Brooks & Gaesser (1980)
Rest	"	< 2	91		
Ex.	Man	43	40		Åstrand et al (1986)

* Other PCA extracted metabolites
** Pyruvate

concentration will not, in itself, increase oxidation rate (Newsholme and Leech, 1983).

1.6.4 (ii) Hormonal Control

Alterations in hormonal state of the body have been found to govern the metabolic fate of La. With moderate exercise, insulin levels decrease and catecholamines increase, to directly stimulate hepatic gluconeogenesis and glycogenolysis in an attempt to maintain blood glucose levels. Following this, a second priority would be to re-establish normal muscle glycogen levels by stimulating glycogen synthesis (Katz et al, 1986a).

Following intermittent, high intensity work, hyperglycemia and hyperinsulinemia have been reported (Pruett, 1970). Insulin supports glyconeogenesis. Thus elevated insulin levels may stimulate glyconeogenesis immediately following exercise. With the high La levels, found post exercise, this carbon source can serve as a precursor for muscle glycogen replenishment. Rapid glycogen repletion, in absence of food intake, has been documented in several studies (Hermansen and Vaage, 1977, MacDougall et al, 1977).

1.6.4 (iii) Enzyme Activity Levels

During high intensity exercise of 30-60 second duration, changes in the extracellular environment will affect key enzymes activities and, thereby control the pathways of La metabolism. Calcium release from the SR is thought to be the activating factor in conversion of the inactive 'a' form of the phosphorylase enzyme to the active 'b' form (Chasiotis et al, 1983). Muscle contraction therefore stimulates glycogenolysis. When

contractile level reaches a steady state, phosphorylase activation reverses and glyconeogenic inhibition is released. Basal levels of glycogen synthetase in the active I form (34%) will predominate and stimulate glycogen synthesis (Conlee et al, 1979, Constable et al, 1984). Brooks and Gaesser (1980) have provided evidence that supports the increased role of glycogen synthesis in La removal following exercise.

Following La oxidation to pyruvate, pyruvate is: (1) carboxylated to oxaloacetate, requiring CO_2, ATP and the enzyme PC, or 2) decarboxylated to acetyl-coA, catalyzed by the 'malic enzyme' (L-malate NADP oxidoreductase) and requiring CO_2, but no ATP (Bendall and Taylor, 1970). ATP availability, as well as enzyme activation, will control the entry point for La metabolism. Changes in energy state and pH are two variables altering enzyme activity as a result of exercise.

Subsequent to oxaloacetate or acetyl-coA formation, other regulatory enzymes include citrate synthase (controls flux in TCA cycle), PEP-CK, and FBP (control glyconeogenesis). The pathways and rate limiting steps in oxidative phosphorylation are fairly well understood (Katz et al, 1986a), but the exact pathway and rate limiting steps in La to glycogen conversion are still unclear (Constable et al, 1984, Shiota et al, 1984).

1.6.4 (iv) **Metabolic State**

Relative proportions of La oxidized and La converted to glycogen depend on substrate supply, hormonal conditions, and on key enzyme activity, all of which are modified by the acidosis and changes in energy state related to maximal exercise. Several studies have directly compared the preferred metabolic pathways under rest and exercise conditions. Under resting conditions, oxidation was found to account for 55% and 90% of the La

removed in dogs (Issekutz et al, 1976) and rats (Brooks and Gaesser, 1980), respectively. Following exercise, oxidation contributed only 50% and 63% to the La elimination process. Compared to resting conditions, oxidation accounted for less of the total La removed following exercise. Thus, following heavy exercise, glycogen synthesis from La appears to be relatively more important as a pathway for La removal than it is at rest. The findings of Brooks and Gaesser (1980) have been criticized due to incorrect interpretation of ^{14}C release data (Katz et al, 1986a), causing predicted percentage of La oxidized to be overestimated by as much as 50%.

1.6.5 Sites of Lactate Elimination

Most tissues are capable of producing La, however, the liver, kidney, heart and skeletal muscle appear to be the most important sites for its removal (Newman et al, 1937, Ahlborg et al, 1975, Hultman and Sahlin, 1980). The relative importance of these tissues also differs under rest and exercise conditions. Due to exercise-related acidosis and shifts in blood flow, skeletal muscle becomes the most important metabolic disposal site for La following exercise.

It has been established that, at rest, the liver, heart + kidney, and skeletal muscle consume up to 10, 20, and 35% of infused La, respectively, with 35% remaining as La in fluids of the body (Ahlborg et al, 1976). The liver will utilize La for glucose synthesis, while the heart prefers La as a substrate for oxidation. In skeletal muscle, La can be oxidized and/or converted to glycogen, since enzymes for both pathways exist in effective concentrations in some fiber types (McLane and Holloszy, 1979, Shiota et al, 1984). Activation of these enzymes is dependent on metabolic status.

During exercise, large amounts of La are taken up and metabolized by the active skeletal muscle despite net La release (Jorfeldt, 1970, Stanley et al, 1986). Approximately 25% of the total La produced recirculates and is taken up by the active muscle (Kowalchuk, 1985). Partial explanation of the simultaneous production and removal of La is the metabolic profiles of the different muscle fibre types. Brooks (1986) refers to a "lactate shuttle" system between fibres. Diffusion and transport of La from La-producing type IIb fibres, to La-consuming type I and type IIa fibres occurs with, and without, recirculation of La through the body. This La shuttle system is compatible with LDH isoenzyme patterns in these fibre types (Peter et al, 1971).

Other sites of La elimination during exercise are the heart, liver, kidney and non-working skeletal muscle, the importance of which are determined by regional blood flow. Cardiac perfusion is high during exercise, so cardiac cells will continue to oxidize La. With high intensity exercise, however, blood flow to the splanchnic area is compromised (Gollnick and Hermansen, 1973) and La uptake of the liver and kidney are diminished as a result.

1.6.6 Lactate Elimination in Inactive Skeletal Muscle

Skeletal muscle tissue, in the non-obese individual, is estimated to account for 45% of total body weight (Poortmans et al, 1978) and, therefore, represents the largest tissue in the body. If one assumes that up to 50% of the muscle mass is utilized during exercise, then inactive skeletal muscle also represents a large fraction of total body mass. As a metabolically active tissue, inactive skeletal muscle can play a significant role in buffering the lactacidosis transported by the blood from the

exercising muscle. The contributions of the non-working muscle mass is dependent on blood flow to the tissue, and the blood-muscle La gradient.

Barr and Himwich (1923) were first to recognize the importance of inactive muscle in buffering the acid load from working tissues. More recent investigations have indicated that inactive muscle can account for up to 35% of the total La removed during or following exercise (Ahlborg et al, 1976, Kowalchuk, 1985). Increased La uptake across the non-working limb is due to increases in arterial - venous (a-v) La difference (Bergström and Hultman, 1966, Freyschuss and Strandell, 1967, Poortmans et al, 1978), coupled with increases in blood flow to an inactive muscle bed (Bevegard and Sheppard, 1966, Ahlborg et al, 1976).

Lactate taken up by inactive skeletal muscle will partially be metabolized (Poortmans et al, 1978), although the fate of La in this tissue has not been quantitatively determined. Due to alterations in blood flow, differences in metabolic state exist between resting skeletal muscle as opposed to inactive, non-working muscle during exercise. For this reason, studies of the body at rest (Ahlborg et al, 1976, Issekutz, 1976) are not compatible with those observing non-working skeletal muscle during or following exercise (Ahlborg et al, 1975, Freyshuss and Strandell, 1967, Poortmans et al, 1978).

Based on a comparison of non-exercised leg and arm muscle metabolism during single legged exercise (Ahlborg et al, 1975), it has been suggested that important metabolic adaptations occur in non-working tissues. In addition to increased blood flow, a shift in substrate utilization was found, with a predominant use of NEFA at rest switching to an increased use of carbohydrates, without changes in metabolic demand. This might be expected based on findings that lipolytic enzymes are inhibited under

acidotic conditions (Jones et al, 1980). The metabolic changes in non-working skeletal muscle during lactate accumulation are still not fully understood.

1.6.7 Summary of Metabolic Elimination of Lactate By Skeletal Muscle

Metabolic removal of La may occur in various biological tissues. Skeletal muscle has been found to actively dispose of La by a number of elimination pathways. The major metabolic pathways appear to be oxidation of La to CO_2 + H_2O and resynthesis of La to glycogen. Other pathways such as alanine production (Felig et al, 1970) and fat synthesis (Ahlborg et al, 1976, Kowalchuk 1985) may also be involved in La removal. Current evidence indicates that the above pathways may be activated in skeletal muscle under varying metabolic conditions. Their existence, in some cases, has been demonstrated under extreme conditions in vitro not applicable to in vivo interpretation (ie. Shiota et al, 1984). Demonstration of several metabolic pathways for La elimination awaits confirmation under conditions representing the normal physiological range.

Discrepancies also exist as to La elimination in various muscle groups. Inactive muscle may or may not be involved in metabolic La removal, and the extent of its removal may be dependent on a third factor: fibre type. The relative roles of inactive skeletal muscles of various fibre types (i.e. soleus, plantaris), may vary based on differences in blood flow and enzymatic and ionic profiles of the respective types. A final consideration of these differences, and how they influence patterns of La removal, may clarify the conflicting literature as to metabolic and ionic removal systems for La.

1.7 LACTATE REMOVAL FOLLOWING MAXIMAL EXERCISE: CONSIDERATION OF DIFFERENCES DUE TO FIBRE TYPE

1.7.1 Metabolic Differences

The enzymatic profiles of type I and type II skeletal muscle fibers dictate the preferred metabolic pathway for La elimination in each muscle fiber. Type I fibers contain elevated concentrations of oxidative enzymes succinate dehydrogenase (Essén et al, 1975, Henriksson and Reitman, 1976), citrate synthase (Essén-Gustavsson and Henriksson, 1983), and the heart-type LDH isozyme (H-LDH) (Sjödin, 1976, Tesch, 1980). Type II fibers, however, are characterized by increased concentrations of the glycolytic enzymes phosphorylase (Piehl and Karlsson, 1977), PFK (Essén et al, 1975, Henriksson and Reitman, 1976), and total LDH (Sjodin, 1976, Essén-Gustavsson and Henriksson, 1983). Type II fibers also contain a relatively higher concentration of muscle type LDH (M-LDH).

Based on LDH isozyme distribution, differences would be expected with respect to La elimation. The high H-LDH isozyme concentration in type I fibers indicates that La will remain in equilibrium with pyruvate,suggesting a preference for oxidative metabolism. Type II fibers, with a high M-LDH isozyme content, favour pyruvate reduction to La, and therefore net La formation.

Both oxidation and glycogen synthesis, the two major pathways involved in the metabolic removal of La, may occur in skeletal muscle. Type I fibers are well suited for oxidative removal of La. Type II fibers, however, have low concentrations of oxidative enzymes. Recent findings of sufficient concentrations of glyconeogenic enzymes PEP-CK, PC, and FBP in type II

muscle fibers (McLane and Holloszy, 1979, Shiota et al, 1984), would predict La removal via glyconeogenesis in these fibers. Preliminary data from Nordheim and Vollestad (1987) have suggested that La may be oxidized in type I fibers, and simulateously resynthesized to glycogen in type II fibers during low intensity recovery from high intensity exercise. Whether or not this occurs in resting muscle following high intensity work has not been investigated.

The extent to which each metabolic pathway is involved in La elimination may be dependent upon the fiber type composition of the muscle. Interaction between fiber types may also occur. It has been suggested that La "flow" or a La shuttle exists between type II and type I fibers, which may be mediated by membrane-bound M-LDH (Sjodin, 1976).

1.7.2 Morphological Differences

Differences in capillarization between muscle fiber types will influence their relative rate of perfusion and therefore their capacity to remove La. It has been demonstrated that type I fibers have an elevated capillary density (Anderson, 1975) representing an increased perfusion per unit fiber area. Increased blood flow to type I fibers has been documented in the perfused rat hindlimb (Mackie and Terjung, 1983, Gorski et al, 1986).

Perfusion of type II muscle fibers had been thought to be several orders of magnitude less than that of type I fibers based on measurements of capillary density (Brodahl, 1977, Andersen, 1975). Capillarization of type IIb sub-group was found to be the lowest among the skeletal muscle fiber types with type IIa being intermediate and type I being the highest. Recent quantification of blood flow by microsphere technique, however, has demonstrated that perfusion is highest to type IIa fibers, intermediate to

type I fibers, and lowest to type IIb fibers (Mackie and Terjung, 1983). Based on blood flow distribution, La uptake and elimination in type I and type IIa muscle fibers may be of greatest significance to recovery from exercise.

1.7.3 Ionic Differences

The ionic properties of skeletal muscle have recently been characterized at rest (Lindinger and Heigenhauser, 1987) and following intense exercise (Lindinger et al, 1987) in the rat hindlimb. Muscles examined included the soleus (SOL), plantaris (PLT), red and white gastrocnemius (RG and WG). The fiber type composition of these muscles differ, with the SOL, RG, and WG being predominantly type I, type IIa, and type IIb respectively, and with the PLT representing a combination of all fiber types of the hindlimb group.

Fiber type differences in ionic properties are found in resting muscle strong ion difference ([SID]). At rest, [SID] is lowest in type I SOL muscle (137 $meq \cdot l^{-1}$) and highest in type II fibers of the PLT, RG and WG (161 $meq \cdot l^{-1}$). In general it has been shown that liberation of free H^+ is more pronounced at low [SID] values (Lindinger et al, 1987). For example, for the same 50 $meq \cdot l^{-1}$ drop in [SID], a smaller increase in $[H^+]$ will result if [SID] is initially high, as it is in type II fibers. These differences in ionic characteristics of the type II- predominated muscles (PLT, RG, WG) has been suggested to function as a "protective effect", to prevent large inhibitory changes due to increased [La] with exercise.

1.7.4 Summary of Fiber Type Differences

A close relationship has been shown to exist between the metabolic and

ionic properties of different muscle fiber types and their metabolism and function (Tesch, 1980, Green, 1986, Lindinger et al, 1987). It has been suggested that metabolic and ionic changes occuring with exercise will have differing effects on La removal patterns in different muscle groups, depending on their fiber type distribution. Type II fibers may synthesize glycogen from La more readily than type I fibers. These type II fibers may also incur less translocation hinderance for La due to a high resting [SID] and protection from changes in carrier mechanism function. Type I fibers will oxidize La more readily, remove more La from circulation, and may experience greater inhibition of La transport mechanisms due to changes in La and H^+ following maximal exercise. Although the metabolic and ionic changes in previously active and depleted muscle of various fiber types have been investigated, the respective adaptations in muscle metabolism and ionic La regulation in inactive skeletal muscle, under conditions of lactacidosis, remains unknown.

1.8 PURPOSE OF INVESTIGATION

The dependency of muscle on extracellular [H^+] for recovery of E-C coupling and contractile processes has been documented (Renaud and Mainwood, 1985). The inter-relationship and dependency of [H^+] on [La] has also been established (Stewart, 1981, 1983). Thus, the importance of maintaining a low [La] and [H^+] in the extracellular space of the active muscle bed for recovery from fatigue has been recognized. Transport of La to, and metabolic elimination by La metabolizing tissues is an important process in lowering extracellular La. Metabolic removal of La represents a permanent

disposal mechanism since both La and H^+ are utilized. Whether by oxidation or direct synthesis of muscle glycogen, the net result is a reduced acidosis due to removal of a strong ion, and a reduction in [SID].

Removal of La by inactive skeletal muscle may play a crucial role in the recovery process by decreasing extracellular [La] and thus extracellular [H^+]. The role of inactive muscle in recovery from fatigue has been given little attention. Therefore, it was the purpose of this study to investigate the mechanisms of metabolic and ionic elimination of La by inactive skeletal muscle. The direct effects of the induced extracellular acidosis, on non-exercised skeletal muscle metabolic activity, and on muscle ion regulation was examined in an isolated rat hindlimb preparation for this purpose.

CHAPTER 2 EFFECTS OF LACTACIDOSIS ON CARBOHYDRATE METABOLISM IN INACTIVE SKELETAL MUSCLE OF THE PERFUSED RAT HINDLIMB

2.1 ABSTRACT

An isolated rat hindlimb perfusion model was used to test the hypothesis that lactate (La) conversion to glycogen will occur in inactive skeletal muscle perfused with high arterial La. Male Sprague-Dawley rats were perfused for 60 min with either a normal perfusate (NP) (N = 8) or a high La perfusate simulating metabolic and ionic conditions following high intensity exercise (LP) (N = 8). The latter was acheived by altering perfusate pH (7.15) and La (11.0 mM), hemoglobin (16.7 $g \cdot dl^{-1}$), and K^+ (7.88 mM) concentrations. Soleus, plantaris, and white gastrocnemius muscles were sampled pre and post perfusion, and tissues analyzed for ATP, CP, La, glycogen, and glycolytic intermediate concentrations. Ratios of glycolytic intermediates at controlling points in the oxidative and glyconeogenic pathway were calculated. Metabolite exchange was calculated from blood flow and arterio - venous content difference.

Pre and post tissue analysis revealed no significant ($p < .05$) change in muscle ATP, CP, glycogen, pyruvate (Pyr) or F-6-P concentrations. Significant increases were found in muscle La (NP vs LP, and pre vs post), with differences between all muscle groups being significant. Muscle La/Pyr ratios were not elevated. In the LP group, muscle Pyr/F-1,6-diP ratios were elevated pre vs post perfusion in the PLT and WG muscles. Changes in muscle F-1,6-diP/F-6-P ratios were found in the LP vs NP group post perfusion. This observation was not consistent in all muscle groups, suggesting a

difference due to fiber type. Muscle G-1-P, G-6-P, and glucose levels decreased post perfusion in both groups, with the exception of glucose in the LP group. Total calculated La uptake by the 3 muscles in th LP group was 61.0 umole, with 14% accumulating as tissue La post perfusion. Of the remaining 86 %, 12-33% could be accounted for by oxidative metabolism. The remaining 67-88% may have been involved in metabolic cycling along the glycolytic/glyconeogenic pathway. Greatest inhibition along the glycogen synthesis pathway in muscle occurred at the F-1,6-biphosphatase and PEP-carboxykinase and pyruvate carboxylase steps. This study rejects the hypothesis of glycogen synthesis from La in undepleted, inactive skeletal muscle, but suggests that changes in other glycolytic intermediates will result from the lactacidosis caused by high intensity exercise.

2.2 INTRODUCTION

Following high intensity exercise of short duration (30-60 sec), reduction of the exercise-induced lactacid load appears to be important in recovery from muscle fatigue. Both site and metabolic pathway for lactate (La) elimination have been widely investigated (see Brooks, 1986 for recent review). Important sites for La elimination following maximal exercise are cardiac as well as skeletal muscle, with both active (Hermansen and Vaage, 1977, Brooks and Gaesser, 1980, Stanley et al, 1985) and inactive (Barr and Himwich, 1923, Freyschuss and Strandel, 1967, Ahlborg et al, 1975, Ahlborg et al, 1976, Poortmans et al, 1978, Kowalchuk, 1985) skeletal muscle contributing to La removal.

Controversy exists regarding the relative importance of various metabolic pathways for La elimination. During recovery from exhaustive exercise, the major pathways for La elimination include glycogen resynthesis (Bendall and Taylor, 1970, Hermansen and Vaage, 1977, McLane and Holloszy, 1979, Shiota et al, 1984, Åstrand et al, 1986), oxidation to CO_2 + H_2O (Depocas et al, 1969, Issekutz et al, 1976, Brooks et al, 1973, Brooks and Gaesser, 1980, Mazzeo et al, 1986), and transamination to alanine (Malette et al, 1969, Felig et al, 1970). Based on differences in enzymatic profiles of slow oxidative (SO), fast oxidative glycolytic (FOG), and fast glycolytic (FG) muscle fibers, the pathway for metabolic removal of La may differ in various muscles depending on their fiber type distribution. It has been suggested that different end products of La may simultaneously be produced in different fibers of the same muscle (Norheim and Vollestad, 1987).

Previous work has shown that rate-limiting enzymes in the glyconeogenic pathway (pyruvate carboxylase (PC), PEP-carboxykinase (PEP-CK), and

fructose-1,6-biphosphatase (FBP)) exist in effective concentrations in fast but not in slow muscle (McLane and Holloszy, 1979, Shiota et al, 1984). Thus FOG and FG fibers may preferentially remove La through glycogen synthesis. Slow oxidative fibers, with a high proportion of the H-LDH isozyme (Peter et al, 1971), and elevated concentrations of oxidative enzymes (Essen et al, 1975) have a higher oxidative capacity than FOG or FG fibers (Baldwin et al, 1978) and thus may preferentially oxidize La to pyruvate.

Skeletal muscle constitutes a relatively large proportion of total body mass (45 %) (Poortmans et al, 1978), and therefore is quantitatively more important than other tissues in La disposal following exercise (Ahlborg et al, 1976). Not all muscle groups, and fibers within these muscle groups, are activated during high intensity exercise such as cycling. Inactive skeletal muscle therefore represents a significant amount of physiologically inactive but metabolically active muscle mass. This tissue has been found to contribute significantly to the recovery of whole body homeostasis following maximal exercise (Ahlborg et al,1976, Sahlin et al, 1976, Kowalchuk 1985), but the extent of its metabolic activity during recovery remains controversial.

It has been suggested that inactive skeletal muscle acts primarily as a sink for La removal (Poortmans et al, 1978). Alterations in substrate metabolism and blood flow to resting skeletal muscle, however, have been observed following La infusion (Ahlborg et al, 1975, Ahlborg et al, 1976). It has also been shown that there is an increased affinity for La as a substrate for glycogen synthesis in the non-exercised, non-depleted rat muscle at La levels above 7 mM (Shiota et al, 1984). Thus, the role of inactive muscle in the metabolic removal of La, under the physiological conditions of post-maximal exercise, remains uncertain.

The purpose of this study was two-fold: 1) to quantify the amount of La that is metabolically removed in inactive skeletal muscle by the oxidative and glyconeogenic pathways during lactacidosis, and 2) to examine the differences in removal patterns in inactive muscles of varying fiber type composition.

Conditions of recovery, from high intensity exercise of short duration, in inactive skeletal muscle were simulated by perfusing resting, isolated rat hindlimb muscle with a lactacidotic perfusate. Tightly controlled metabolic and ionic manipulations in the arterial perfusion medium allowed metabolic flux across the muscle to be measured and quantified. Changes in muscle metabolism in response to the lactacid load could therefore be assessed in resting skeletal muscle.

2.3 METHODS

2.3.1 Animals

Sixteen male Sprague-Dawley rats weighing 334.3 + 6.0 grams ($\bar{X}$ + SE) were used. The animals were housed in a controlled environment with 12 hr of day and night and allowed to feed on Purina laboratory chow ad libitum up until the time of the experiment. The rats were not fasted prior to experimentation in order to maintain normal muscle glycogen levels. The animals were randomly assigned to a control group, which received a normal perfusate (NP), or to an experimantal group receiving a lactacidotic perfusate (LP).

2.3.2 Animal Preparation

The rats were anaesthetized with an intraperitoneal injection of sodium pentobarbitol (6 mg·100 mg^{-1} body weight). Muscle samples of the soleus

(SOL), plantaris (PLT), and white gastrocnemius (WG) were removed pre perfusion from the right leg. The left hindlimb was surgically prepared for perfusion of the gastrocnemius-plantaris-soleus (GPS) muscle group as described by Spriet et al (1986) (see Figure 2.1). Briefly, the left knee and ankle were stabilized with support brackets, and the GPS tendon anchored to hold the muscle at resting length. The femoral vessels were exposed above the knee and the artery and vein cannulated with 22 and 18 gauge teflon catheters (Deseret Medical Inc.) respectively. The arterial catheter was inserted as far toward the popliteal space as possible to restrict perfusion to the lower portion of the limb. The limb vasculature was flushed with 1 ml of sterile, heparinized (100 $U \cdot ml^{-1}$) 0.9% saline and the animal killed with an intracardiac injection of sodium pentobarbital (20 mg). The rat was placed in a 37°C perfusion chamber where perfusion began immediately. Total time required for surgery was 20-25 minutes.

2.3.3 Perfusion Medium

The arterial perfusate consisted of a Krebs-Henseleit buffer with fresh bovine erythrocytes as characterized by Lindinger et al (1986). For the NP group the perfusate was comparable to normal rat blood (Lindinger et al, 1986) while the LP group perfusate was manipulated to contain increased concentrations of La, K^+, and hemoglobin (Hb) and a decreased pH, PCO_2 and bicarbonate (HCO_3^-) concentration. The latter perfusate was developed to simulate the metabolic and ionic characteristics of arterial blood as previously measured in rats following exhaustive swimming exercise (Lindinger et al, 1987). The blood perfusate characteristics for both groups are given in Table 2.1.

The perfusion apparatus consisted of a one-pass perfusion system

Figure 2.1 Schematic representation of rat hindlimb perfusion set-up. GPS = Gastrocnemius-Plantaris-Soleus. From Lindinger (1987), p.63.

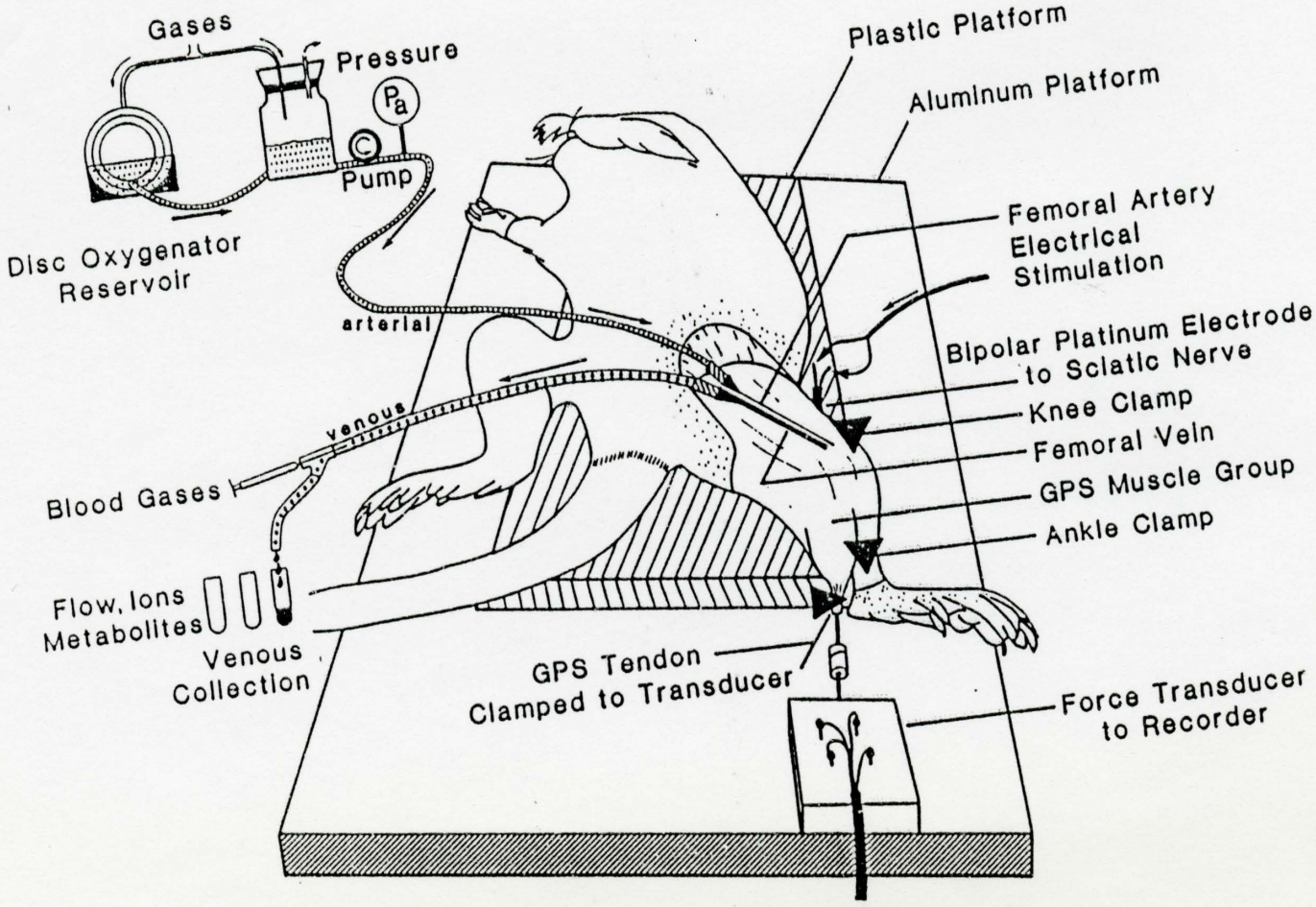

Gases
Pressure
Pa
C
Pump
Disc Oxygenator
Reservoir
arterial
venous
Blood Gases
Flow, Ions
Metabolites
Venous
Collection
Plastic Platform
Aluminum Platform
Femoral Artery
Electrical
Stimulation
Bipolar Platinum Electrode
to Sciatic Nerve
Knee Clamp
Femoral Vein
GPS Muscle Group
Ankle Clamp
GPS Tendon
Clamped to Transducer
Force Transducer
to Recorder

Table 2.1 Metabolic and Physico-Chemical Characteristics of the Bovine Erythrocyte Perfusion Medium

	NORMAL	LACTACIDOTIC	
Whole Blood			
pH	7.433 ± .007	7.149 ± .012	
PO2 (mm Hg)	145.8 ± 2.8	116.6 ± 2.3	**
PCO2 (mm Hg)	40.9 ± 0.5	30.6 ± 0.8	**
HCO3- (mMol/L)	21.5 ± 0.2	9.2 ± 0.3	**
Hb (g/dl)	14.8 ± 0.04	16.7 ± 0.10	**
Lactate (mMol/L)	1.29 ± 0.03	11.00 ± 0.11	**
Glucose (mMol/L)	2.37 ± 0.11	2.41 ± 0.10	
Plasma			
Na+ (mMol/L)	155.4 ± 3.1	150.8 ± 3.6	
K+ (mMol/L)	6.31 ± 0.23	7.88 ± 0.41	**
Ca++ (mMol/L)	0.99 ± 0.06	1.13 ± 0.09	
Cl- (mMol/L)	107.2 ± 1.2	102.0 ± 1.0	**
La- (mMol/L)	1.05 ± 0.09	9.98 ± 0.16	**
Protein (g/dl)	3.96 ± 0.33	4.14 ± 0.35	

* $p < .05$ ** $p < .01$

(Spriet, 1984). A physiological force transducer (Statham-Gould no. 48703) coupled to a chart recorder (Hewlett Packard no. 8805B) was used to continuously monitor arterial perfusion pressure. The perfusate flow rates were those at which O_2 uptake was independent of flow rate and averaged 1 $ml \cdot min^{-1}$ for all animals.

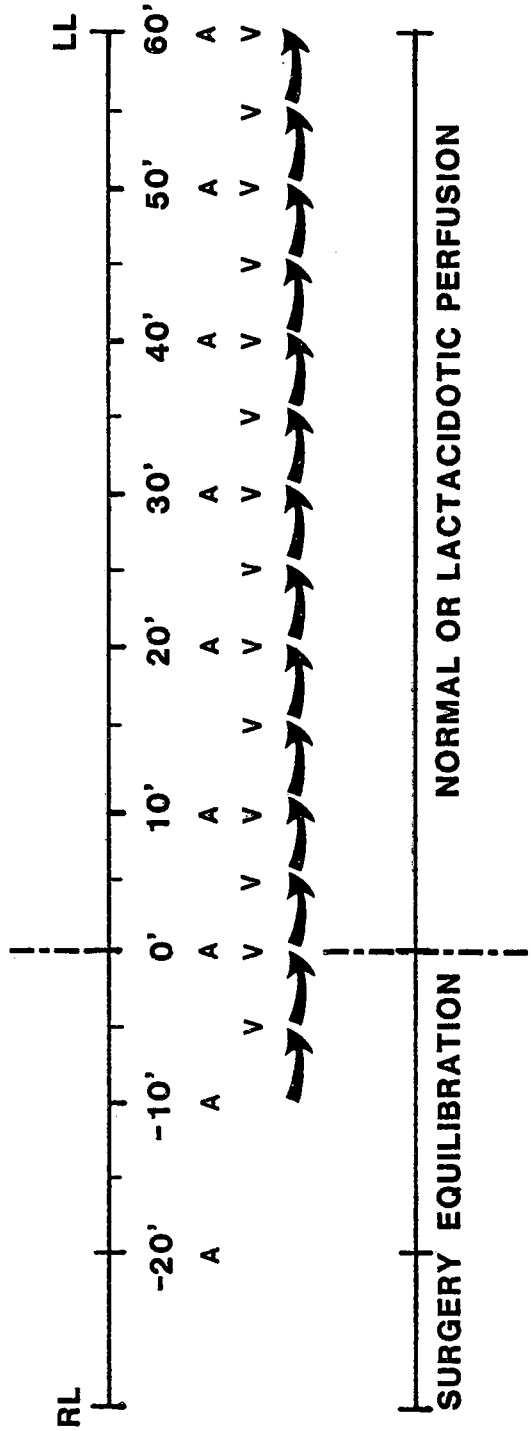
2.3.4 Sampling of Perfused Skeletal Muscle

Muscle samples were taken post-perfusion from the GPS muscle group. The entire SOL and PLT muscle, and superficial sample of WG were sequentially removed and immediately frozen in large metal tongs precooled in liquid nitrogen. The SOL consists mainly of SO fibers, and a small portion of FOG fibers (Armstrong and Phelps, 1984). The PLT contains approximately 9% SO, 41% FG, and 50% FOG fibers, and best represents the fiber population of the entire hindlimb musculature (Armstrong and Phelps, 1984). The WG contains a large proportion of FG fibers, some FOG fibers, but no SO fibers. During sampling of muscle tissue, flow rates remained constant until removal was complete (90-120sec).

2.3.5 Perfusion and Sampling Protocol

Perfusion began within 3 minutes of interuption of normal limb blood flow. A 20 min equilibration period preceeded the 60 min of experimental perfusion in both NP and LP groups. Arterial and venous samples were collected as indicated in Figure 2.2. Samples were immediately analyzed for blood acid-base status to ensure constant arterial pH, PO_2, and PCO_2 levels. Arterial and venous perfusate samples were acidified using PCA, and frozen for later analyses. Flow rate was set at approximately 1 $ml \cdot min^{-1}$ and remained at that rate throughout the total perfusion period for both NP and

Figure 2.2 Schematic representation of experimental protocol indicating 20 min equilibration and 60 min experimental perfusion periods.
A = Arterial blood sample
V = Venous blood sample
↘ = Venous blood flow collection
RL = Right limb muscle biopsy
LL = Left limb muscle biopsy.


LL
RL
-20'
-10'
0'
10'
20'
30'
40'
50'
60'
SURGERY EQUILIBRATION
NORMAL OR LACTACIDOTIC PERFUSION

LP groups.

The LP group required a special adaptation to the normal set-up. Two blood perfusates were prepared: one normal, the other with the exercise-associated modifications. The two perfusates were gased in separate oxygenators and held in separate resevoirs. One line leading from each resevoir converged blood into the arterial line perfusing the hindlimb. Initially, the line from the lactacidotic perfusate was clamped at the junction to the arterial line. The normal perfusate perfused the hindlimb for the first 20 min period, in both control and experimental groups. In the LP group experiments, at 19 min the normal perfusate line was clamped off and the line from the lactacidotic perfusate opened. The one minute advance changeover was made to compensate for the previously measured one minute transit time from the junction point to the catheter entrance into the hindlimb. This ensured that the lactacidotic perfusion began by time zero without having to increase the flow rate.

2.3.6 Analytical Methods

Electrodes were used to measure PO_2, PCO_2 and pH (Radiometer BM 3). The hemoglobin (Hb) O_2 saturation and concentration were measured by a hemoximeter (Radiometer OSM 2). Total O_2 content of arterial and venous blood were calculated from measured values of [Hb], percent O_2 saturation, and PO_2. Hindlimb O_2 uptake was calculated according to the Fick principle. Oxygen uptake measurements are reported as $umole \cdot min^{-1} \cdot hindquarter(HQ)^{-1}$. Arterial perfusate ion concentrations were measured using ion selective electrodes (Radiometer KNA1 Sodium/Potasium Analyzer and Radiometer ICA1 Ionized Calcium Analyzer).

Frozen muscle samples (50-500 mg wet weight) were wrapped in aluminum

foil and stored in liquid nitrogen until freeze dried and analyzed. Each sample was pulverized in liquid nitrogen, the visible connective tissue removed, and weighed to determine wet weight. The samples were subsequently freeze dried to a powder, reweighed, wet:dry weight ratio calculated, and visible connective tissue removed.

Muscle samples were analyzed for ATP, CP, La, glycogen and various glycolytic intermediates (pyruvate (Pyr), F-1,6-diP, F-6-P, G-6-P, G-1-P, and glucose). Metabolites were extracted in ice cold PCA, neutralized with potassium carbonate, centrifuged, and the supernatant removed. Muscle extracts were analyzed for metabolites using standard enzymatic techniques (Bergmeyer, 1965). Muscle metabolites are expressed in umole·g^{-1} dry weight (DW).

Acidified arterial and venous blood samples were analyzed for La and glucose concentration using enzymatic techniques of Bergmeyer (1965). Metabolite exchange across the hindlimb was calculated as a product of the arterio-venous (a - v) difference (umole·ml^{-1}) and flow rate (ml·min^{-1}) and are reported as umole·min^{-1}·HQ^{-1} of substrate exchange.

2.3.7 Calculation of Glyconeogenic Rate Limiting Steps

Glyconeogenesis is primarily a reversal of the glycolytic pathway with the exception of differences at 3 steps. At these steps, reverse reactions are catalyzed by different enzymes. These steps are found between Pyr and phosphoenol pyruvate (PEP), F-1,6-diP and F-6-P, and between G-6-P and glycogen. Analysis of inhibition at these steps involved calculating ratios between measured glycolytic intermediates at each step. Since reactions from PEP to F-1,6-diP are reversible, and intermediates between them in equilibrium, inhibition at the Pyr ---> PEP step was indicated by changes in

the Pyr/F-1,6-diP ratio. La/Pyr ratios were also calculated for indications of possible inhibition at the LDH step.

2.3.8 Correction for Fluid Shift

During resting muscle perfusion in both NP and LP groups, a fluid shift from blood to muscle was observed, as indicated by changes in venous Hb concentration. The calculated shift of fluid into the muscle was approximately 5% in the LP group and 1.5% in the NP group. The underlying cause for the fluid shift appeared to be the elevated flow rate which created an increased perfusion pressure. The increased perfusion pressure without an increased reabsorption pressure may have resulted in net movement of water from extracellular to intracellular compartments.

Correction for the movement of water across the muscle membrane was made for all perfusate metabolites (La, glucose, glycerol) and ions (Na^+, K^+, Cl^-, HCO_3^- and non-volatile H^+) analyzed. Normalization of venous concentration to a constant volume was made in order to assess exchange of metabolites and ions across the muscle according to their content and not concentration per se. Venous concentrations were corrected by a factor equivalent to the arterial:venous Hb concentration ratio (see Appendix I). The corrected venous values were then used to calculate (a-v) difference and net metabolic and ionic flux.

2.3.9 Statistical Analyses

Reported values are given as mean ± standard error of the mean (SE). Separate two factor ANOVAs with one repeated measure were used to test for differences in O_2 uptake, La and glucose exchange over the perfusion time

period. Separate three factor ANOVAs with two repeated measures (for time and muscle group) were used to test for differences in muscle metabolites between perfusate groupings (LP and NP). A Tukey post-hoc test was used to compare means when a significant F ratio was found. Statistical significance was accepted at $p < .05$.

2.4 RESULTS

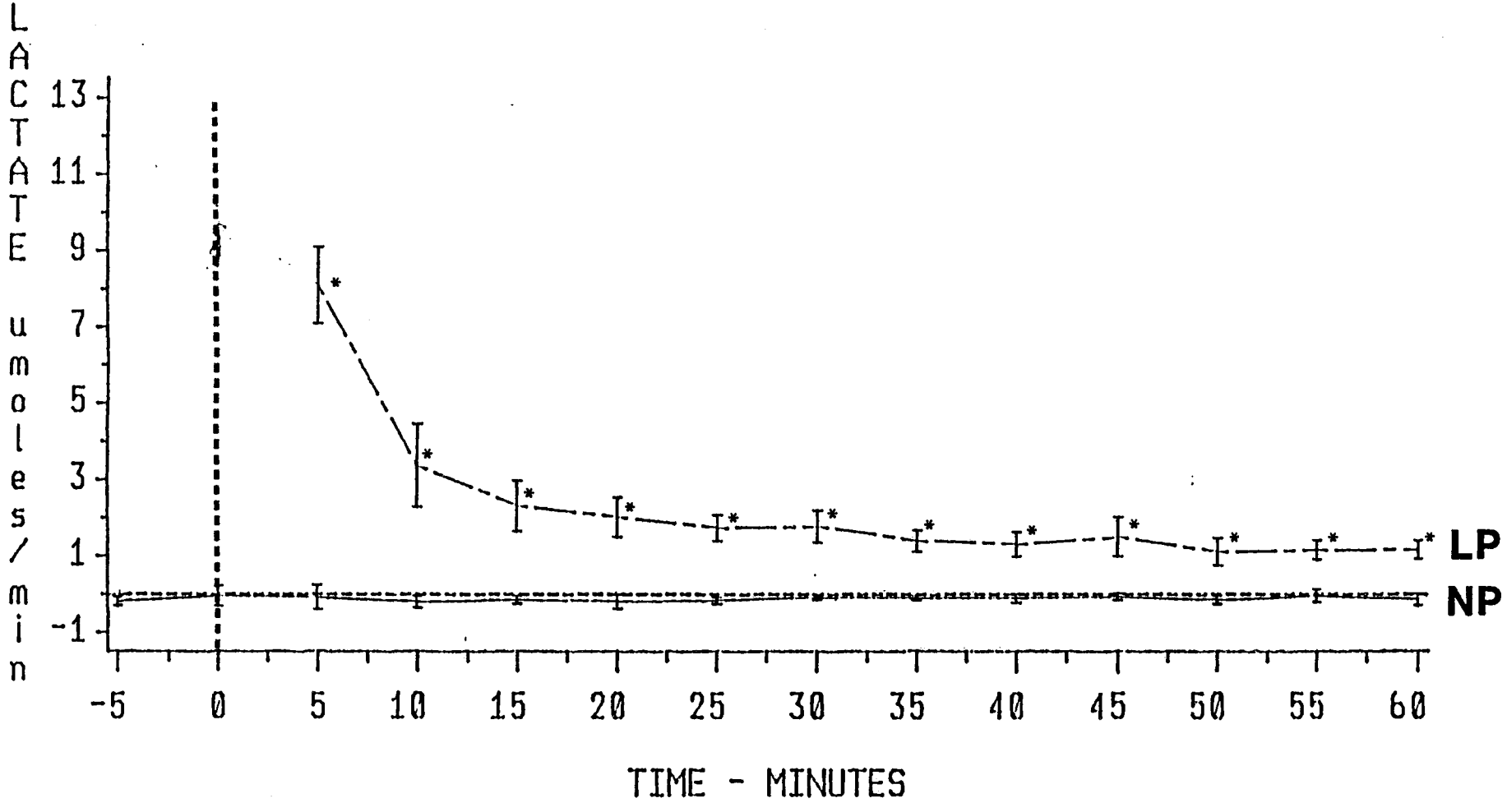
The average mass of perfused muscle tissue in the rat hindlimb was previously determined to be 1.54 $\pm$ 0.1 $g \cdot 100g^{-1}$ body weight (Spriet et al, 1985). Thus, the perfused muscle mass in the present study averaged 5.13g, and the flow rate 19.5 $ml \cdot 100g^{-1} \cdot min^{-1}$ for both groups throughout the perfusion period.

2.4.1 Metabolite and Gas Exchange

Skeletal muscle of the rat hindlimb normally exhibits a net production of La, as indicated by the average negative La exchange value in the NP group (-0.12 $\pm$ 0.02 $umole \cdot min^{-1} \cdot HQ^{-1}$). A net release of La was also observed during the equilibration period in the LP group (-0.18 $\pm$ .03 $umole \cdot min^{-1} \cdot HQ^{-1}$). Following the changeover to the lactacidotic perfustate there was an immediate uptake of La by the inactive muscle (Figure 2.3). It is recognized that calculations at time zero would tend to overestimate (a-v) difference for La because venous concentration would still reflect the normal perfusate. The uptake of La, however, was still pronounced after 5 min of perfusion - long after complete clearance of the normal perfusate would have occurred. The inactive muscle receiving the LP continued to remove La throughout 60 min of perfusion, reaching steady state values of

Figure 2.3 Lactate exchange across inactive rat hindlimb in lactate (LP) and normal (NP) perfusate groups.

* Indicates significant difference between groups.


LACTATE umoles/min
13
11
9
7
5
3
1
-1
-5
0
5
10
15
20
25
30
35
40
45
50
55
60
LP
NP
TIME - MINUTES

1.0-2.0 umole·min^{-1}·HQ^{-1} by 20 min. La exchange was significantly different between LP (net uptake) and NP (net release) groups at all time points measured.

Glucose uptake by the GPS muscle group was found to average 0.1 to 0.3 umole·min^{-1}·HQ^{-1} in both the NP and LP groups. The low uptake values are thought to be due to the absence of insulin in the perfusate. No significant change in glucose exchange was found over time, or between perfusate groups.

Oxygen uptake by the rat skeletal muscle was found to average 1.85 + 0.03 umole·min^{-1}·HQ^{-1} in the control, NP group (see Figure 2.4). These values are similar to previous values obtained utilizing the same rat hindlimb perfusion set-up (Lindinger, 1987). Hindlimb O_2 uptake by the LP group increased at time zero to 3.2 + 0.3 umole·min^{-1}·HQ^{-1} but was no longer significantly above control levels at 5 min. The initial, unsustained increase in calculated O_2 uptake may have occurred due to the rapid changeover in arterial perfusates, and thus may reflect an artifact from the experimental model rather than a real effect of the induced lactacidosis on gas exchange.

2.4.2 Muscle Metabolites

Muscle La levels increased significantly following the 60 min perfusion period in the LP group only (Figure 2.5). Tissue La concentrations for the perfused limb in the LP group were 39.6 + 1.1, 33.1 + 2.2, and 28.8 + 1.7 umole·g^{-1} DW in the SOL, PLT, and WG respectively. The differences were significant between all muscles with the SOL being 16.5% greater than PLT and 27.2% greater than WG, and PLT being 12.9% greater than WG. Tissue La levels of the NP group post-perfusion were significantly lower than in the respective muscle of the LP group, averaging 7.8 + 1.5, 5.8 + 1.8, and 6.6 +

Figure 2.4 O_2 uptake across inactive rat hindlimb muscles in lactate (LP) and normal (NP) perfusate groups. No significant differences between groups were found between 5-60 min of experimental perfusion.

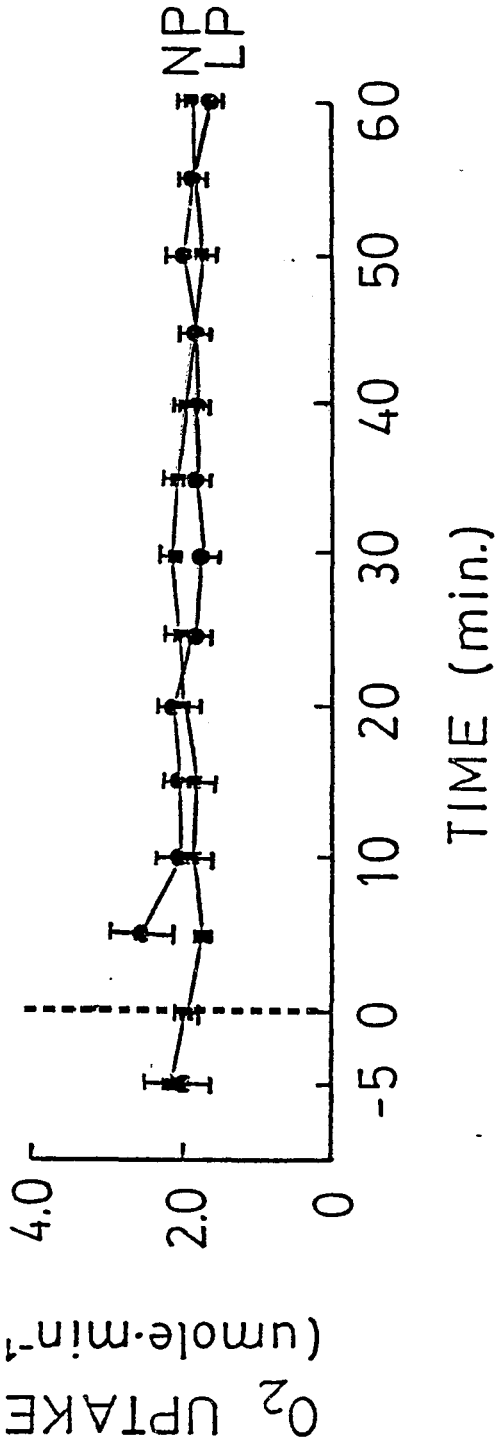
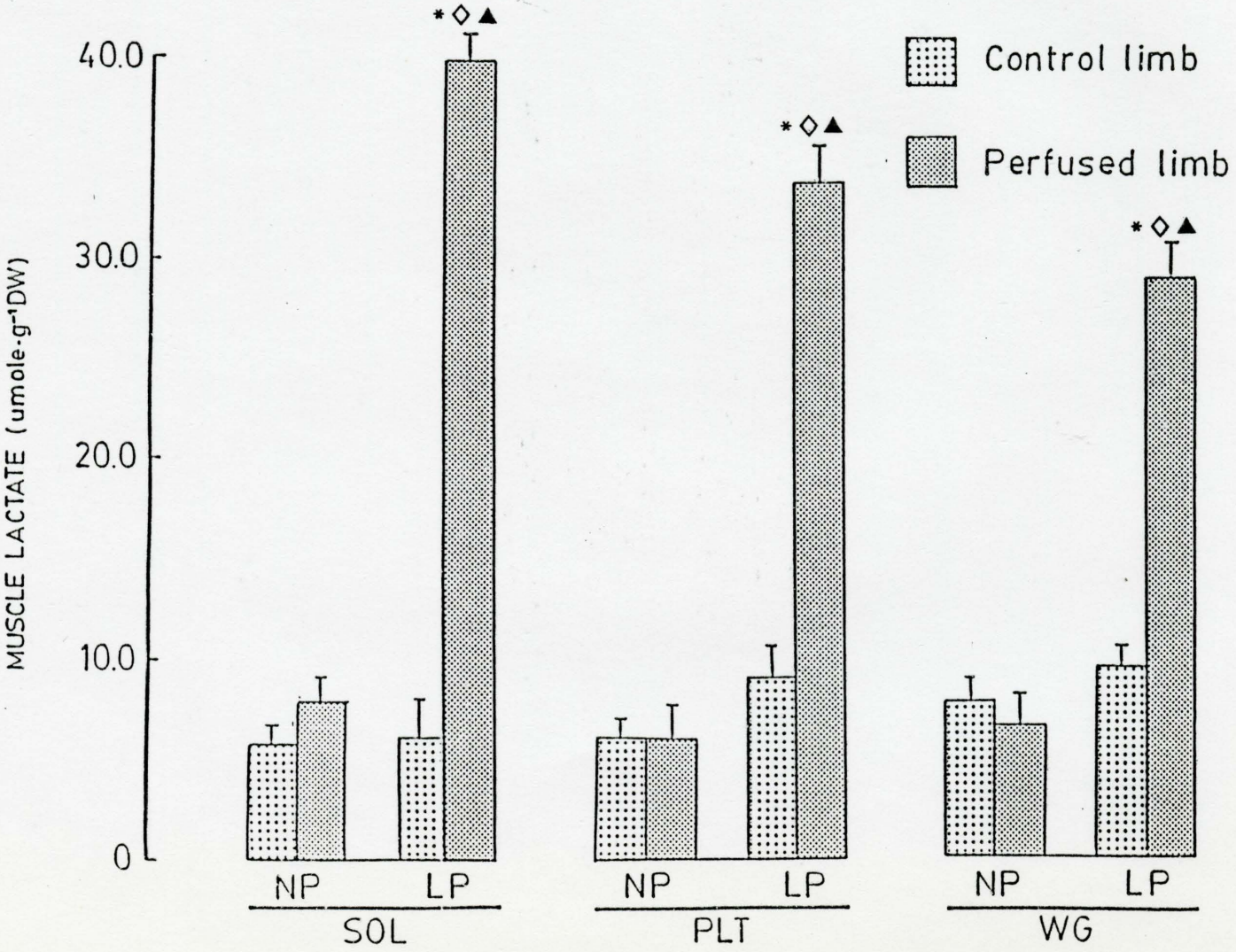

NP
LP
4.0
2.0
0
-5
0
10
20
30
40
50
60
TIME (min.)
O_2 UPTAKE
(umole·min^{-1})

Figure 2.5 Muscle lactate concentration in rat hindlimb muscles pre and post perfusion in lactate (LP) and normal (NP) perfusate groups. SOL = soleus, PLT = plantaris, WG = white gastrocnemius.

* Indicates significant difference in control vs perfused limb.
◇ Indicates significant difference in perfused limb of LP vs NP.
▲ Indicates significant difference in hindlimb between SOL, PLT and WG.


MUSCLE LACTATE (umole·g⁻¹DW)
40.0
30.0
20.0
10.0
0
* ◇ ▲
Control limb
Perfused limb
NP
LP
SOL
PLT
WG

1.3 umole·g^{-1} DW in SOL, PLT, and WG.

Muscle glycogen content did not change pre vs post perfusion in either the LP or NP group (Figure 2.6) suggesting that no net synthesis of glycogen from La occurred. Significant differences were found between the SOL, PLT and WG muscles in both treatment groups at both time periods measured. Average glycogen concentration in the SOL, PLT, and WG were 105.9 + 3.9, 156.1 + 5.3 , and 168.3 + 5.0 umole·g^{-1} DW respectively, with glycogen levels in the SOL being significantly lower than in PLT (32.2%), and WG (37.1%).

No significant changes in the high energy phosphates, ATP and CP, were found between LP and NP groups post perfusion, suggesting no change in the energy state of the muscle due to the lactacidosis. Differences were noted between muscles, with both ATP and CP being highest in the WG, followed by the PLT and SOL.

Changes in the various glycolytic intermediates are shown in Table 2.2. No significant difference following 60 min of perfusion with either LP or NP was observed in Pyr or F-6-P levels in the three muscles examined. High variability in these intermediates, due to their low concentration in resting muscle, may explain the non-significant findings.

Significant differences were found in muscle F-1,6-diP in the PLT and WG. Differences in pre vs post perfusion concentration were only significant in the WG, however this decrease occurred in response to both the normal and lactacidotic perfusion. Differences between PLT and WG were found pre perfusion, with WG having an average F-1,6-diP concentration 51.2% higher than PLT. This pre-perfusion difference may have been related to the fiber-type characteristics of this muscle, but it did not persist following 60 min of perfusion with either perfusate. The decrease in F-1,6-diP concentration

Figure 2.6 Muscle glycogen concentration in rat hindlimb muscles pre and post perfusion in lactate (LP) and normal (NP) perfusate groups. SOL = soleus, PLT = plantaris, WG = white gastrocnemius.
No significant differences in glycogen content were found following either type of perfusion.

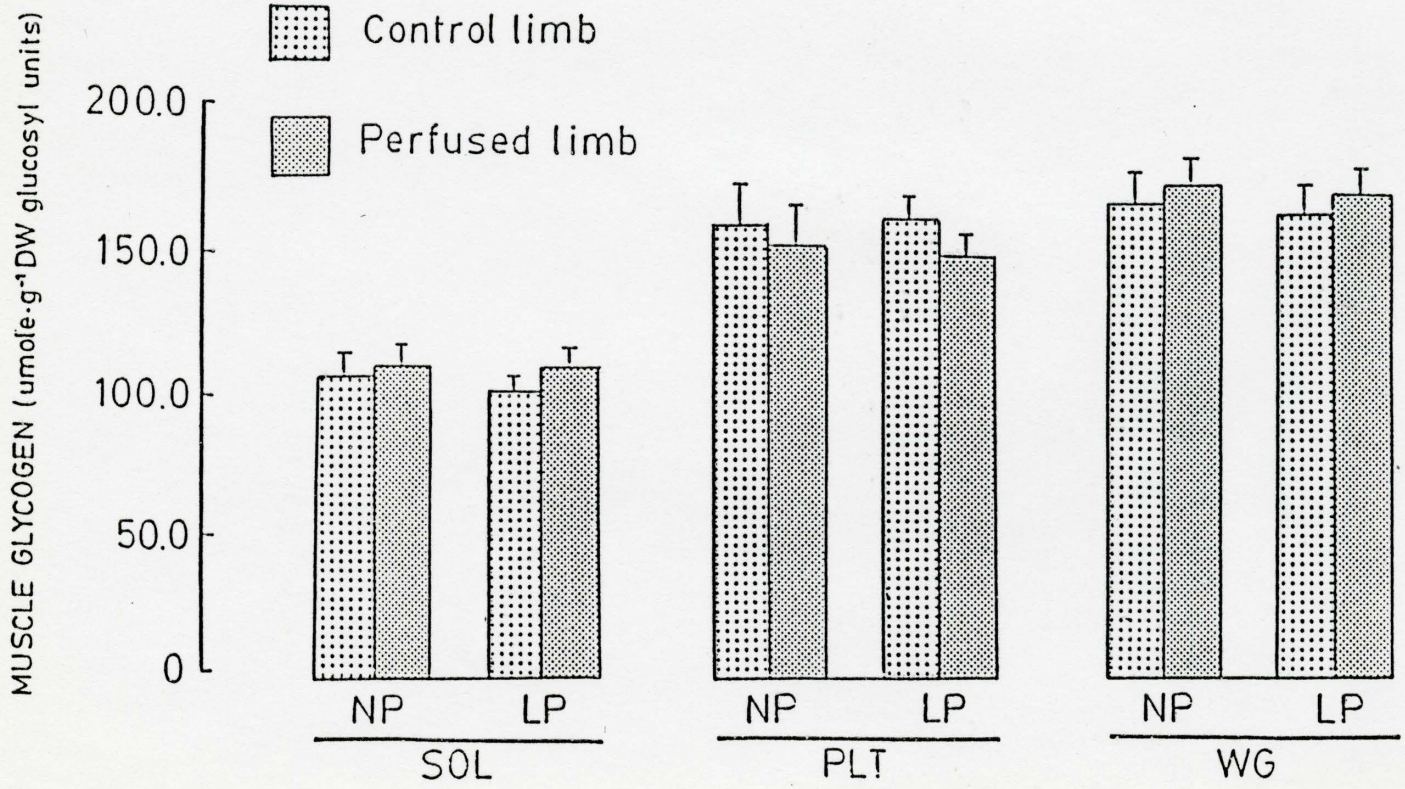

MUSCLE GLYCOGEN (umole·g⁻¹DW glucosyl units)
Control limb
Perfused limb
200.0
150.0
100.0
50.0
0
NP
LP
SOL
NP
LP
PLT
NP
LP
WG

Table 2.2 Glycolytic Intermediate and High Energy Phosphate Concentrations

Metabolite	SOLEUS		PLANTARIS		W. GASTROCNEMIUS	
	PRE	POST	PRE	POST	PRE	POST
Pyruvate						
NP	2.43 ± .74	1.87 ± .56	2.68 ± .63	2.40 ±1.49	2.85 ± .63	2.05 ± 1.02
LP	1.68 ± .99	2.07 ± .90	1.31 ± .80	1.75 ± .67	1.06 ± .60	2.83 ±1.42
F-1,6-diP						
NP	--	--	0.38 ± .17	0.13 ± .06	0.90 ± .34	0.20 ± .10
LP	--	--	0.76 ± .22	0.44 ± .14	1.44 ± .26	0.34 ± .10
F-6-P						
NP	--	--	0.48 ± .09	0.24 ± .10	0.57 ± .09	0.23 ± .09
LP	--	--	0.16 ± .06	0.12 ± .04	0.23 ± .05	0.14 ± .04
G-6-P						
NP	--	--	0.87 ± .15	1.23 ±	0.23 ± .08	0.67 ± .23
LP	--	--	0.42 ± .21	0.19 ± .07	0.57 ± .26	0.19 ± .08

Table 2.2 (Continued)

Metabolite	SOLEUS		PLANTARIS		W. GASTROCNEMIUS	
	PRE	POST	PRE	POST	PRE	POST
G-1-P						
NP	--	--	0.32 ± .06	0.19 ± .04	0.45 ± .08	0.23 ± .04
LP	--	--	0.11 ± .02	0.20 ± .03	0.28 ± .05	0.17 ± .08
Glucose						
NP	1.30 ± .20	0.85 ± .15	1.04 ± .11	0.47 ± .10	0.99 ± .17	0.40 ± .05
LP	1.41 ± .31	1.21 ± .47	0.91 ± .33	0.63 ± .04	0.76 ± .14	0.70 ± .07
ATP						
NP	11.8 ± 1.8	9.5 ±0.9	18.9 ± 2.7	16.7 ± 3.2	25.6 ± 0.8	22.6 ± 3.2
LP	14.5 ± 2.5	8.8 ±1.1	20.5 ± 1.0	22.3 ± 0.5	26.3 ± 1.6	20.6 ± 4.3
CP						
NP	29.8 ± 4.8	24.7 ± 2.8	65.0 ± 6.8	43.3 ±14.3	66.3 ± 4.8	50.0 ±11.0
LP	29.2 ± 5.1	28.2 ±10.5	43.2 ± 3.3	42.7 ± 8.2	49.8 ± 3.1	42.4 ± 9.2

over time appears to be a result of the perfusion itself, and not the type of perfusate per se.

Muscle G-1-P and G-6-P concentrations were lower post perfusion in both LP and NP groups when collapsed across muscle groups. Differences over time were not significant for PLT and WG separately. Soleus concentrations of G-1-P and G-6-P were too low for the sensititvity of the assay, therefore these values were eliminated. Intramuscular glucose concentrations were significantly lower in SOL, PLT and WG muscles following 60 min of perfusion with the normal perfusion medium. Glucose concentrations were maintained in all muscles of the GPS group despite 60 min of perfusion with a lactacidotic medium, suggesting an adaptation in metabolic fuel source in the LP group. Significant differences were also found between muscles, with SOL glucose concentration being 35% and 39% higher than PLT and WG respectively.

2.4.3 Metabolite Ratios

Changes in glycolytic intermediate ratios were found between pre and post perfusion values and between perfusate groups (see Table 2.3). Due to the small cell size for F-6-P and F-1,6-diP concentrations in the SOL, no Pyr/F-1,6-diP or F-1,6-diP/F-6-P ratios were obtained for this muscle.

Following 60 min of perfusion, the muscle La/Pyr ratio showed a trend towards being elevated in the LP group (18.9 $\pm$ 4.5 post perfusion versus 5.6 $\pm$ 0.8 pre perfusion) but this increase was not statistically significant. This lack of significance was likely due to the high variability in muscle Pyr levels, resulting in magnified variability of the La/Pyr ratio.

Changes in muscle Pyr/F-1,6-diP ratio were found pre (4.2) vs post (18.1) perfusion in the lactacid perfused group but not in the NP group. No significant differences between PLT and WG were found. Therefore, for

Table 2.3 Glycolytic Intermediate Ratios

Ratio	SOLEUS		PLANTARIS		W. GASTROCNEMIUS	
	PRE	POST	PRE	POST	PRE	POST
La / Pyr						
NP	5.2 ±2.3	5.3 ±1.3	5.0 ±1.9	7.1 ±2.3	5.6 ±2.5	6.0 ±1.1
LP	8.3 ±3.4	12.8 ± 3.2	8.7 ±3.0	17.1 ± 7.6	10.7 ± 3.1	24.1 ± 8.8
Pyr / F-1,6-diP						
NP	--	--	6.8 ±1.6	10.7 ± 3.8	3.3 ±0.5	9.2 ±2.3
LP	--	--	3.9 ±1.5	26.6 ±12.2	1.4 ±0.5	12.8 ± 6.3
F-1,6-diP / F-6-P						
NP	--	--	2.1 ±1.4	0.3 ±0.1	1.9 ±0.4	0.7 ±0.3
LP	--	--	3.4 ±0.8	3.8 ±1.4	6.3 ±2.0	8.9 ±7.4

purposes of greater statistical significance, these values were collapsed across muscle groups.

Changes in the F-1,6-diP/F-6-P ratio were not consistent in the two muscles analyzed. The F-1,6-diP/F-6-P ratio was elevated post perfusion in the LP vs NP group in the PLT but not the WG muscle. No significant differences in this ratio were found pre vs post perfusion in the NP or LP group.

2.5 DISCUSSION

The present study has indicated that despite net La influx into a non-working muscle, no net change in oxygen uptake or muscle glycogen resulted. Altered levels of some glycolytic intermediates, and intermediate ratios, between LP and NP were found, indicating possible involvement of the oxidative and/or glycolytic/glyconeogenic pathways. A shift in substrate utilization and metabolic cycling within the glyconeogenic pathway are possible explanations for the responses observed.

2.5.1 Methodological Considerations

The effects of lactacidosis on carbohydrate metabolism in inactive skeletal muscle has been investigated in the isolated perfused rat hindlimb. The underlying purpose was to investigate the La elimination patterns in non-working skeletal muscle under conditions which simulated high intensity exercise. The isolated, perfused muscle preparation allowed tight control over the concentration of substrates and hormones in the circulating blood. Lack of insulin and glucagon, the two major hormones controlling glycogen degradation and synthesis, and lack of catecholamines, important in

stimulating glycolysis, prevented hormonal regulation of metabolic activity. Manipulations in La and H^+ concentration, as well as other exercise-related changes were therefore the dependent variables in the investigation. Changes in carbohydrate metabolism in the inactive muscle could then be attributed to the altered substrate concentration (La) and/or acidosis in the arterial blood.

The closed metabolic system in the one-pass perfusion model represents an artificial physiological state, however it does allow controlled analysis of isolated mechanisms of metabolic regulation. Regulation of blood flow, through the use of a roller pump, allowed observation of changes in metabolic activity independent of changes in blood flow. The flow rates used in the present study averaged 1.0 $ml \cdot min^{-1}$, a value corresponding to 19.4 $ml \cdot 100g^{-1} \cdot min^{-1}$. This rate of perfusion is slightly higher than previously reported values for resting skeletal muscle (Terjung et al, 1985), but nonetheless represents a physiological flow rate.

2.5.2 Percent of Lactate Metabolized

A primary objective of the study was to determine whether or not inactive skeletal muscle is metabollically active in response to perfusion with a lactacidotic medium. Percent of La metabolized was determined by calculating the ratio of total cumulative La disappearing between 5 and 60 min of perfusion (TDLa) to total cumulative La removed by the hindlimb muscles over the 60 min perfusion period (TRLa). TDLa was defined as the difference between the TRLa by the GPS muscle group and total tissue La remaining at the end of 60 min of lactacidotic perfusion in the GPS group (TTLa). Thus, the following equations were utilized:

$$TDLa = TRLa - TTLa$$

%La metabolized = TDLa / TRLa.

TTLa at the end of 60 min of perfusion was calculated from the muscle La concentration, measured post perfusion in umole·g^{-1} wet weight (WW), and the average muscle mass for each respective muscle group, as determined by previous investigations (Lindinger and Spriet, unpublished data). La removed by the inactive muscle was calculated as a product of La removal rate (per 5 min interval between 5 min and 60 min), and duration of the interval (5 min). Individual La uptake values in umole per 5 min period were then summated to determine total La removed (TRLa).

It had previously been determined that the GPS group represents 44% of the perfused hindlimb muscle mass (Lindinger and Spriet, unpublished data). Thus, TRLa was corrected for the proportion actually taken up by the SOL, PLT and WG muscles. Total La removed by the inactive perfused hindlimb was 181.6 umole, with approximately 61.0 umole removed by the GPS group.

Total tissue La remaining at the end of 60 min of perfusion in the LP group was calculated to be 8.7 umole, with 0.8, 1.5, and 6.4 umole in the SOL, PLT, and WG respectively. Although the SOL was found to have the highest La concentration following perfusion, its contribution to the total amount of La removed was minimal due to its relatively small muscle mass.

TRLa and TTLa in response to 60 min of perfusion were 61.0 and 8.7 umole respectively. Therefore, only 14% of the La taken up by the inactive skeletal muscle was still found in the GPS portion of the hindlimb post perfusion in the LP group. The remaining 86% is assumed to have been metabolically eliminated. Observation of a net La uptake over the entire 60 min of lacatacidotic perfusion supports the finding of a high degree of metabolic clearance. This value, however, does not agree with the data of Poortmans et al (1978) which indicated that only 19% of the La taken up by

inactive muscle was metabolized directly within the tissue.

Inactive muscle of the isolated perfused rat hindlimb appears to metabolize the majority of the La removed by it. This finding is in agreement with earlier work which suggested that non-working skeletal muscle is metabolically active during contractile activity of other muscle groups (Freyschuss and Strandel, 1967) and following La infusion at rest (Ahlborg et al, 1975, Ahlborg et al, 1976). Quantification as to the extent of metabolic elimination of La was not made in these earlier studies, making a comparison of results difficult.

Ahlborg and co-workers (1975, 1976) also reported changes in blood flow and O_2 uptake in response to La infusion. The increased flow rate in these studies would increase La uptake without increasing La extraction or La metabolism. Since constant blood flow and O_2 uptake values in the present study indicate an unchanged metabolic rate, the increased uptake of La was most likely due to increased La extraction and/or metabolism in the inactive muscle. This is unlike the findings of Ahlborg et al (1975, 1976) where metabolic rate increased during La infusion. An increased rate of La oxidation, without a significant change in O_2 uptake during lactacidotic perfusion, suggests that a shift in substrate utilization, from other carbohydrate or triacylglycerol (TG) sources to La, has likely occurred in the perfused rat hindlimb.

Intramuscular glucose concentration decreased in the SOL, PLT and WG in response to 60 min of perfusion with a normal perfusate but did not change with 60 min of lactacidotic perfusion. Free muscle glucose was spared as an energy source in the LP group, indicating an altered substrate utilization pattern in the LP vs NP group. The absolute amount of glucose preserved was small (0.216 umole, based on WW calculations) and therefore probably

contributed little to the overall shifts in metabolism.

2.5.3 Percent of Lactate Oxidized

To determine the proportion of metabolically removed La that could be accounted for by oxidation, total La uptake (TDLa), total O_2 uptake, and total glucose uptake by the GPS muscle group over the 60 min of perfusion were determined. Oxygen and glucose uptake were calculated as described above for TDLa.

Total exchange of oxygen across the GPS portion of the hindlimb over the 60 min perfusion protocol represented a net uptake of 51.5 umole of O_2 in the LP group. Assuming that 3 mole of O_2 are required per mole La oxidized (McGilvrey, 1983), the maximal amount of La removed by oxidation was 17.2 umole. Thus, if no other substrate was metabolized by the GPS muscle group of the lactate perfused hindlimb, then a maximum of 33% of the TDLa could be accounted for by oxidative metabolism.

Glucose uptake by the GPS muscles, however, totalled 5.5 umole. No net change in muscle glycogen content or intramuscular glucose concentration (Figure 2.4 and Table 2.2) occurred, suggesting that any glucose extracted by the inactive muscle was metabolically removed via oxidation and/or cycling into other glycolytic intermediates. Assuming that 6 umole of O_2 are required per umole of glucose oxidized (McGilvery, 1983), and that all glucose which disappeared was oxidized, a maximum of 33 umole of O_2 would be utilized for glucose oxidation. Of the total 51.5 umole O_2 taken up by the GPS muscles, only 18.5 umole would then be available for La oxidation. Thus, the minimum amount of La oxidized by the GPS group would be 6.2 umole, representing 12% of the La metabolically removed.

Without the use of a labelled tracer it was not possible to determine

the extent to which either substrate could account for the metabolic activity of the LP hindlimb. Depending on the relative proportion of La and glucose oxidized, a minimum of 12 and a maximum of 33% of the La metabolically removed can be attributed to oxidative metabolism. Free fatty acids (FFA) were eliminated as a potential substrate since neglible concentrations were found in the perfusate and because muscle RQ values of > 1.0 indicated metabolism of pure carbohydrates. The remaining 67 - 88% of the La metabolized is unaccounted for.

2.5.4 Lactate Removal By Oxidation

The present study suggests that only a minimal amount (range = 12-33%) of the La removed by inactive muscle of a lactacidotic perfused rat hindlimb was oxidized to CO_2 + H_2O. This value agrees with previously obtained values of 16.2 % in frog and rabbit (Bendall and Taylor, 1970), 20-25% in rat (Brooks et al, 1973), and 15% in human (Hermansen and Vaage, 1977) skeletal muscle. The latter studies, however, investigated La metabolism following exercise in the previously active muscle.

Studies of La metabolism in resting muscle tissue with infused, labelled La, indicate much higher values for the oxidative elimination of La. In dogs (Issekutz et al, 1976), rats (Brooks and Gaesser, 1980), and humans (Ahlborg et al, 1975), the percent of La oxidized in physiologically inactive muscle tissue was found to be 55, 91, and 46 % respectively.

From the values obtained in the present study, it would appear that the inactive hindlimb muscles responded to the lactacidotic perfusion in a manner similar to that of previously exercised tissue. The relatively small proportion of La oxidized is closer to the reported values for La metabolism in exercised than in resting muscle. The exercise-induced state of muscle

metabolism in the LP hindlimb may have resulted from inhibition of oxidative metabolism by the arterial acidosis (arterial perfusate pH = 7.15). Findings of unchanged La/Pyr ratios at the end of 60 min of perfusion, however, indicate that inhibition of La oxidation did not occur at the La ---> Pyr step.

2.5.5 Latate Removal by Other Metabolic Pathways

Various other metabolic pathways for La elimination exist in skeletal muscle. Other major pathways reported for La removal include glycogen synthesis (Bendall and Taylor, 1970, Hermansen and Vaage, 1977, McLane and Holloszy, 1979, Shoita et al, 1984, Åstrand et al, 1986), lipogenesis (Ahlborg et al, 1975, Kowalchuk, 1985) (although this most likely occurs in adjacent adipose cells), and transamination to alanine (Felig et al, 1970, Malette et al, 1969, Brooks and Gaesser, 1980). McLane and Holloszy (1979), utilizing a ^{14}C labelled La to perfuse rat skeletal muscle, determined that of the La disappearing, 35% was found in the form of PCA extracted metabolites and 25 % as Pyr.

The endpoints of La metabolism cannot accurately be determined since a radioactive label was not utilized. Based on the present study, which found 67 -88% of the removed La unaccounted for by tissue La or oxidation, it appears that a major portion of the La metabolized by inactive skeletal muscle during lactacidosis is taken up in various carbon-cycling steps of glycolysis/glyconeogenesis or possibly FFA/TG cycling. Alanine, a potential metabolic fate of La, was not quantified in the present analysis, but this endpoint may also account for some of the La that has disappeared.

2.5.6 Muscle Glycogen Regulation

Measurements of muscle glycogen concentration indicated that no net change in carbohydrate storage occurred in the LP hindlimb muscles. This suggests that net glycogen synthesis does not occur simply in response to an excess of available substrate. Previous studies demonstrating La to glycogen conversion in skeletal muscle have depleted initial muscle glycogen stores with intense exercise and/or fasting (McLane and Holloszy, 1979, Hermansen and Vaage, 1977, Astrand et al, 1986). Others (Fell et al, 1980) have shown preferential glycogen resynthesis in previously depleted muscle. These studies suggest that glycogen depletion may be a pre-requisite for direct glycogen repletion from La.

The biochemical basis for these findings appear to be related to the activation of the glycogen synthase enzyme. The active form, glycogen synthase 'a', is activated by increased concentrations of G-6-P, Ca + +, cyclic AMP, and epinephrine (McGilvrey, 1983, Newsholme and Leech, 1983), and inhibited by high glycogen concentrations. It is thought that glycogen inhibits the removal of an inorganic phosphate group from the inactive glycogen synthase 'b' enzyme (McGilvery, 1983). Without the hydrolysis reaction to activate the anabolic form of glycogen synthase, glyconeogenesis will not occur.

Muscle G-1-P and G-6-P concentrations were found to decrease over the 60 min of perfusion in both LP and NP groups. This decrease indicates a net downward flux higher up in the glycolytic pathway. The lowered [G-6-P] in the LP group is one of the factors responsible for lack of glycogen synthase 'a' activation, and therefore lack of glycogen synthesis.

The present study supports the argument that muscle glycogen level is regulated by its own glycogen concentration (Fell et al, 1982). It appears

that glycogen synthesis from La in non-working, non-depleted skeletal muscle does not occur under lactacidotic conditions simulating those following high intensity exercise.

2.5.7 Lactate Cycling Within the Glycolytic/Glyconeogenic Pathway

Although no net change in muscle glycogen content was found in any of the muscles investigated, differences in concentration of various glycolytic intermediates were observed in response to 60 min of perfusion with the lactacidotic or normal perfusate. No changes pre to post perfusion were found in muscle Pyr or F-6-P levels in either the LP or NP group. A trend toward increased Pyr concentrations in the LP muscles was noted, but these findings were not statistically significant.

It has been established that PEP-CK, PC, and FBP are the major rate-limiting enzymes along the metabolic pathway for La conversion to glycogen (Hers and Hue, 1983, Newsholme and Leech,1983). The present study indicates that inhibition at the level of Pyr ---> F-1,6-diP occurred in the GPS muscle group. Enzymes PEP-CK and PC regulate the reactions at this point in the glyconeogenic pathway. In liver, PEP-CK is distributed between cytosol and mitochondria (Hers and Hue, 1983), with the proportions variable between species. The relative distributions in muscle have not been reported. Depending on the subcellular location of this enzyme, the upward flux of the La ---> Pyr ---> PEP reactions may be more or less inhibited when La moves into muscle from the extracellular space, as occurs in inactive muscle. The greater the mitochondrial fraction of PEP-CK, the greater the potential inhibition of glycogen synthesis from La in skeletal muscle at this step.

Inhibition may have resulted from altered ionic concentrations in the

cytosol. The ionic composition of the interstitial space may have been altered in response to elevated perfusate K^+ levels, and resultant transmembrane ion fluxes. Both PC (Hers and Hue, 1983) and FBP (Nakashima and Tuboi, 1976) require optimal concentrations of monovalent cations Na^+ and K^+. Any changes in the intramuscular [Na^+] or [K^+] would alter key enzyme activity levels. Induced changes in intracellular [H^+] may also affect the activity level of the key enzymes.

Differences in F-1,6-diP/F-6-P ratios were found between the PLT and WG muscles in the LP group. Elevations in this ratio were only significant when compared between groups post perfusion, and not when compared over time. Inhibition at the FBP step would be expected in SO fibers but not in FG and FOG fibers (McLane and Holloszy, 1979). It is unclear why this inhibition occurred in the PLT, a predominantly FG/FOG muscle.

It has been suggested that metabolic control of the glycolytic/glyconeogenic pathway occurs at the F-6-P <---> F-1,6idiP and PEP <---> Pyr steps (Hers and Hue, 1983, McGilvrey, 1983). A futile cycle exists at both steps, since non-equilibrium reactions are coupled. Due to the requirements for ATP in the upstream reaction, metabolic cycling allows both reactions to occur at the same rate, without a net change in metabolite concentration, but a futile loss of energy as heat. At both the F-6-P <---> F-1,6-diP and PEP <---> Pyr levels, futile cycling of the La carbon may have occurred, explaining some of the discrepancies found between percent of La metabolically removed and percent of La oxidized.

Both oxidative and glycolytic/glyconeogenic pathways are involved in La elimination, with the relative fractions of each likely dependent upon muscle fiber type distribution (Nordheim and Vollestad, 1987). Muscle fiber type differences in La uptake and O_2 uptake could not be isolated in the

present experimental model due to the mixed venous drainage from all muscle groups in the femoral venous effluent.

The results of these data indicate that during exercise La may not only be removed by inactive skeletal muscle, but may also be metabolized by it. This would tend to suggest that inactive muscle might play an important role in delaying fatigue of the active muscle by permanently removing a potential fatigue factor from the circulating plasma. Previous observations, however, have indicated that a limited amount of La efflux (less than 10%) occurs from the active muscle following high intensity, intermittent exercise (Hermansen and Vaage, 1977). Thus, the significance of La removal by inactive muscle in an in vivo situation may only be minor.

2.6 CONCLUSION

In isolated inactive skeletal muscle of the rat hindlimb, perfused with a lactacidotic perfusate, patterns of La metabolism appeared to resemble those found in previously exercised skeletal muscle. The majority (86%) of the La extracted by the resting perfused hindlimb was metabolically eliminated, 12-33% of which could be accounted for by the oxygen uptake across the GPS muscle group. The remaining 67-88% may have been involved in metabolic cycling at the F-1,6-diP <---> F-6-P and Pyr <---> PEP steps along the glycolytic/glyconeogenic pathway. The present study does not support the hypothesis that La to glycogen conversion occurs in non-working, non-depleted skeletal muscle during lactacidosis. It does, however, support the argument for metabolic involvement of inactive muscle under conditions of recovery from high intensity exercise. Finally, some of the La unaccounted for may have entered FFA/TG cycling pathways via acetylcoA or oxaloacetate. Further investigation into TG regulation under lactacidotic conditions is

required to understand the integration of various metabolic pathways of La elimination in inactive skeletal muscle.

CHAPTER 3 EFFECTS OF LACTACIDOSIS ON TRIACYLGLYCEROL METABOLISM IN INACTIVE SKELETAL MUSCLE OF THE PERFUSED RAT HINDLIMB

3.1 ABSTRACT

The possible contribution of triacylglycerol/free fatty acid (TG/FFA) cycling to the disposal of lactate (La) in inactive skeletal muscle was investigated in the isolated perfused rat hindlimb. It was previously determined (Chapter 2) that 67-88% of the La metabolically removed by inactive perfused muscle remained unaccounted for. It was suggested that La removal involved substrate cycling along the glyconeogenic/glycolytic pathway, and possibly within TG-metabolizing pathways. Thus, TG metabolism was assessed within the same experimental protocol involving 60 min of rat hindlimb perfusion with either a normal perfusate (NP) (N = 8), or a lactacidotic perfusate (LP) (N = 8). The experimental perfusate (LP) was characterized by elevated concentrations of La, K^+, and hemoglobin, and a decreased pH. Soleus, plantaris, and white gastrocnemius muscles were sampled pre and post perfusion and analyzed for TG content. Carbon dioxide, HCO_3^-, and glycerol exchange across the muscle were calculated from blood flow and arterio-venous content differences.

Analysis revealed an elevated CO_2 output (2.5-4.3 umole·min^{-1}·HQ^{-1}) and sustained elevations in muscle RQ (1.5-1.8) suggesting that net substrate synthesis occurred. A release of HCO_3^- across the hindlimb suggested that some non-metabolic CO_2 may have been released. A gradual increase in glycerol release by the hindlimb muscles was observed in the LP group from 30-60 min indicating possible glycerol synthesis. No significant change in

muscle TG was found pre vs post perfusion in any of the muscles investigated. The stable TG levels, muscle RQ greater than unity, and positive glycerol release suggests that TG/FFA substrate cycling and/or glycerol synthesis was elevated during lactacidosis. This pathway appears to account for approximately 7% of the La removed. These findings indicate that, in the rat hindlimb model investigated, La taken up by inactive skeletal muscle enters TG-metabolizing pathways to stimulate glycerol release, however, the contributions of this pathway appear to be minimal.

3.2 INTRODUCTION

Heavy exercise associated with a high degree of glycolytic metabolism results in an acidosis due to the increased production of lactate (La) and H^+. It has previously been observed that elevated La concentrations inhibit TG turnover and/or fatty acid metabolism (Fredholm, 1971, Issekutz et al, 1975, Jones et al, 1980). Decreased FFA turnover rates have been found with heavy exercise (Issekutz et al, 1975, Jones et al, 1980). However, in dogs infused with La following heavy exercise, glycerol turnover rates were not found to be compromised despite decreased FFA turnover (Issekutz et al, 1975). This non-stoichiometric change in glycerol and FFA turnover suggests an adaptation in substrate cycling rates in response to increased La and/or H^+ levels with changes in glycerol and FFA metabolism occuring in opposite directions.

Glucose infusion has also been shown to decrease FFA oxidation and increase its rate of re-esterification to glycerol (Christopherson et al, 1983, Wolfe and Peters, 1983). This suggests that substrate availability, and not La or H^+ per se, will alter TG turnover rates. The interaction between lipid and carbohydrate metabolism has been referred to as a glucose/fatty acid cycle (Randle et al ,1962, Lillioja et al, 1985). Pathways for lipid and carbohydrate metabolism cross over at a number of common intermediates, including acetyl coenzyme A and glyceraldehyde-3-phosphate, providing several potential entry points for La and/or other glycolytic intermediates.

Carbohydrate sources can serve as lipogenic substrates for either fatty acid (Granneman and Campbell, 1984, Schmidt etal, 1984) or glycerol synthesis (Hems et al, 1975, Kalderon et al, 1984, Hahn, 1986). Glycolytic

intermediates can serve as substrates for glycerol synthesis (Neptune et al, 1963, Issekutz et al, 1975, Miller et al, 1983). As well, glyceride-glycerol can serve as a gluconeogenic substrate (Neptune et al, 1963, Lavau and Susini, 1975). It has been suggested that the main substrate for lipogenesis is not glucose, but 3 carbon compouds such as La (Hems et al, 1975, Bloxham et al, 1977).

The importance of inactive skeletal muscle in the elimination of La following maximal exercise has previously been recognized (Ahlborg et al, 1975, Ahlborg et al, 1976, Poortmans et al, 1978, Kowalchuk, 1985) and its role in the metabolic removal of La along the glyconeogenic/glycolytic pathway discussed (Chapter 2). Suggestions of La involvement in a triacylglycerol/free fatty acid (TG/FFA) and/or glucose/FFA cycle has been examined in previously exercised muscle, but not in the non-active muscle mass. This prompted investigation into the interaction between lactacidosis following heavy exercise and TG regulation in non-depleted, inactive skeletal muscle. Therefore, it was the purpose of this investigation to examine changes in TG metabolism in inactive muscle of the isolated perfused rat hindlimb under conditions simulating recovery from high intensity exercise .

The rat hindlimb perfusion model previously described (Chapter 2) was utilized to investigate the interaction between La removal patterns and TG regulation. Analysis of CO_2 output, HCO_3^- and glycerol release, and muscle respiratory quotient (RQ) and TG content were carried out in rats perfused at rest with an arterial lactacidosis. Increased glycerol release rates and stable muscle TG levels indicated that elevated substrate cycling and/or glyceroneogenesis may have occurred in the inactive skeletal muscle.

3.3 METHODS

The experimental animals, surgical technique, perfusion mediums (normal (NP) and lactacidotic (LP)), sampling protocols, and statistical analyses procedures were those previously described (see Section 2.2). Additional analyses of arterial and venous blood perfusates for CO_2, HCO_3^-, and glycerol, and muscle for TG content, were carried out in order to investigate the possible contributions of triacylglycerol metabolism to the metabolic clearance of La in inactive skeletal muscle.

3.3.1 Gas and Metabolite Exchange

Arterial and venous perfusates were sampled throughout the 20 min equilibration and 60 min experimental perfusion period. An electrode was used to measure PCO_2 and pH (Radiometer BMS 3). CO_2 content and HCO_3^- concentration were determined using an acid-base alignment nomogram developed for the artificial bovine erythrocyte perfusion medium (Lindinger et al, 1986) (see Appendix II). An acidified extract (PCA; 2/1, vol/vol) from arterial and venous perfusate samples was analyzed for glycerol concentration by enzymatic procedures (Garland and Randle, 1962). Hindquarter CO_2 output, glycerol and HCO_3^- exchange were calculated according to the Fick principle. Muscle RQ was derived from the quotient of CO_2 output/O_2 uptake, using O_2 uptake values previously reported (Chapter 2).

3.3.2 Muscle Triacylglycerol Analysis

Freeze dried muscle samples (see Section 2.3.6) were analyzed for TG content by methods described by Spriet et al (1986) with minor

modifications. Briefly, dried muscle samples were weighed and added to acid-washed glass tubes to which 5 ml of chloroform-methanol (2/1, vol/vol) extraction solution were added. Following overnight extraction (8-10 hrs) at 4°C, 3 ml of 4 mM $MgCl_2$ was added. The tubes were centrifuged at 1000g, 4°C, for 1 hr for separation of organic and inorganic phases. Two ml of the lower organic phase was removed without disrupting the muscle powder "cake" at the interface of the two layers. The organic extract was placed in a clean glass tube, evaporated, and the residue redissolved in 5 ml pure chloroform. Phospholipids were removed by adding 500mg of silicic acid to each tube. Following mixture and centrifugation, the supernatant was evaporated and saponified with ethanolic KOH (0.5 ml of 4% KOH in 95% ethanol per tube; 5 parts of aqueous 80% KOH in 95 parts ethanol) at 60°C for 1 hr. One ml of 0.15M MgSO4 was added and the mixture centrifuged for 15 min. The supernatant was decanted and analyzed within 12 hr. for glycerol by the same enzymatic procedure used for perfusate samples.

Reduplication of the glycerol assay following 24 hrs of storage at 4°C revealed a high degree of reliability ($r = 0.94$) suggesting that glycerol concentration was not altered during short periods of storage. A triolein standard processed through the extraction procedure gave a glycerol recovery of 89-100 %, similar to the 94-100% recovery values previously reported for this procedure (Spriet et al, 1986).

3.4 RESULTS

Gas and metabolite exchange data for time zero of the experimental perfusion period were disregarded in the LP group, since 0 min exchange values would have overestimated the (a-v) differences. Venous blood at time

zero would have reflected the normal perfusate from the 20 min equilibration period, while arterial blood, having rapidly been altered, would have reflected the new LP characteristics. The transit time for perfusion through the hindquarter vasculature was estimated from rinse solution volume (ml) and designated flow rate ($ml \cdot min^{-1}$). At the flow rates utilized (approximately 1.0 $ml \cdot min^{-1}$), the LP would have cleared the hindlimb vasculature in 2-3 min. A clearance of time of 5 min was alotted for. Thus, results are presented for the equilibration period (-5 min), and then from 5-60 min in the experimental period.

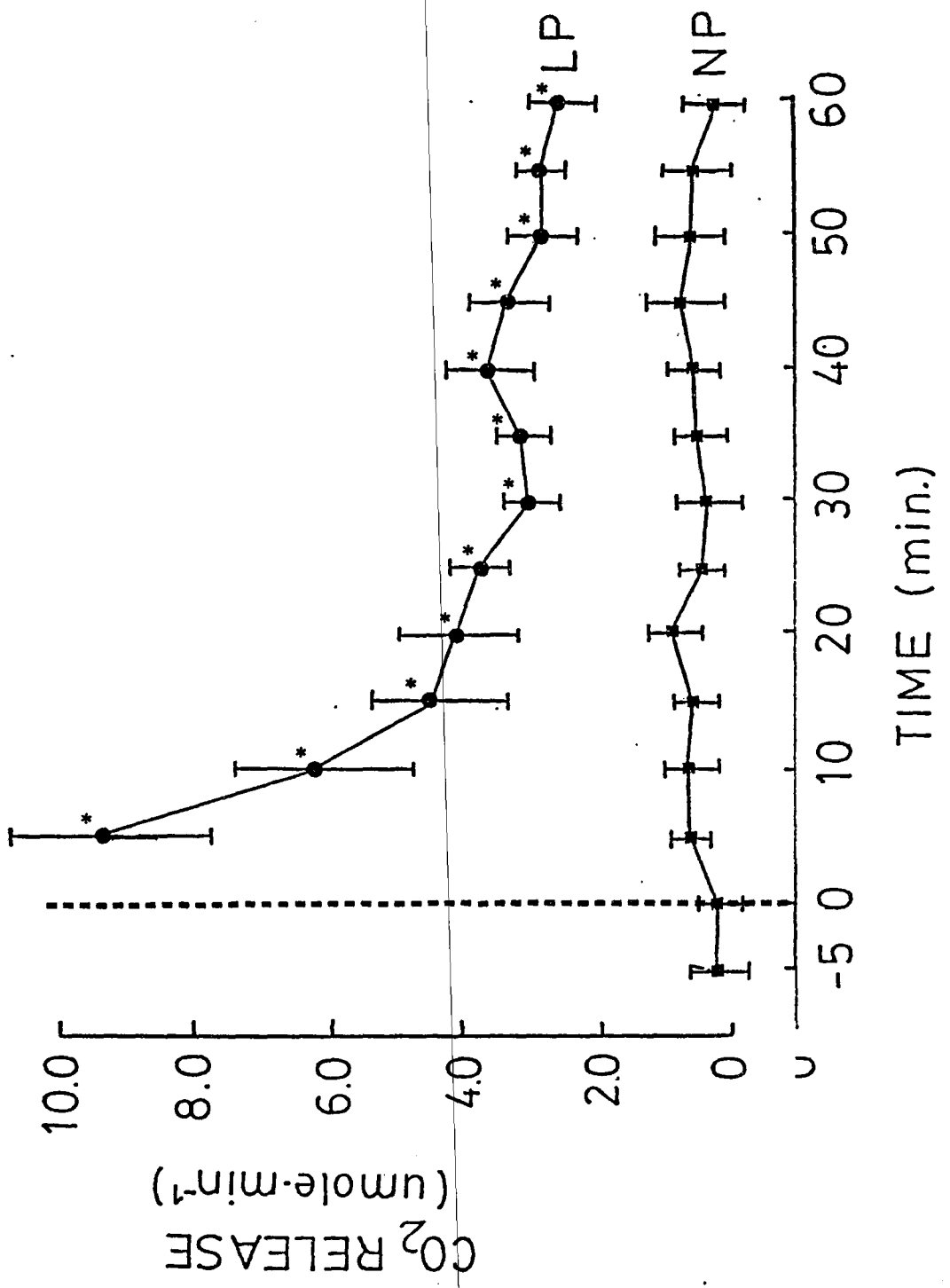
3.4.1 Gas Exchange

Carbon dioxide output by the hindlimb muscle group averaged $0.4 \pm .05$ $umole \cdot min^{-1} \cdot HQ^{-1}$ in the normal perfusion experiments (see Figure 3.1). A similar value was obtained during the 20 min equilibration period in the LP group (0.2 ± 0.4 $umole \cdot min^{-1} \cdot HQ^{-1}$). Carbon dioxide release increased to 9.2 ± 1.5 $umole \cdot min^{-1} \cdot HQ^{-1}$ by 5 min of LP perfusion and remained significantly above respective values in the NP group for each of the time intervals measured. Steady state CO_2 release values were observed from 20-60 min, averaging 3.11 ± 0.17 $umole \cdot min^{-1} \cdot HQ^{-1}$. During this time, CO_2 release values within the LP group did not change significantly.

Respiratory quotient (VCO_2/VO_2) values across the hindlimb muscles in the LP group differed significantly from values obtained in the NP group. Average RQ in the NP group was 0.55 ± 0.04, ranging from 0.45 during the initial 20 min equilibration period, to 0.79 during the 60 min of experimental perfusion. A high degree of variability was noted, and most likely reflected the variability in both VCO_2 and VO_2 values. In the LP group, muscle RQ values were significantly greater than respective NP group

Figure 3.1 CO_2 release across inactive rat hindlimb in lactate (LP) and normal (NP) perfusate groups.

* Indicates significant difference between groups.


LP
NP
10.0
8.0
6.0
4.0
2.0
0
-5
0
10
20
30
40
50
60
TIME (min.)
CO_2 RELEASE
(umole·min^{-1})

values from 0-20 min. Although RQ values from 20-60 min were no longer statistically different between NP and LP, there remained a qualitative difference. NP values remained within, or slightly below, the normal 0.7-1.0 range for net catabolism of substrate. LP values, however, were sustained above 1.0 (range 1.4-1.8), suggesting net anabolic activity in the hindlimb muscles.

3.4.2 Metabolite Exchange

In resting muscle of the perfused hindlimb, glycerol release occured at low rates (0.015 $\pm$ .003 umol$\cdot$min$^{-1}\cdot$HQ^{-1}) under normal perfusate conditions (see Figure 3.2). During perfusion with the lactacidotic perfusate, glycerol release remained within the normal range for resting muscle (0.011 - 0.065 umole$\cdot$min$^{-1}\cdot$HQ^{-1}) until 20 min. Between 20 and 60 min, glycerol release rates demonstrated a slow ramped increase, reaching values of 0.170 $\pm$ .020 umole$\cdot$min$^{-1}\cdot$HQ^{-1} by 60 min. Differences between LP and NP groups in glycerol output by the hindlimb muscles were significant from 30-60 min.

3.4.3 Bicarbonate Exchange

The non-metabolic release of CO_2 was measured as the apparent flux of HCO_3^- across the hindlimb muscles (see Figure 3.3). In the LP group, venous HCO_3^- concentrations dropped from 21.5 $\pm$ 0.6 mMol$\cdot$l^{-1} during the equilibratrion period, to steady values of 11.5-12.0 mMol$\cdot$l^{-1} from 30-60 min. Despite a decreased venous [HCO_3^-] in the LP effluent, HCO_3^- flux was found to be negative (ranging from -2.1 to -3.0 umole$\cdot$min$^{-1}\cdot$HQ^{-1} from 30-60 min), indicating net bicarbonate release. The calculated net HCO_3^- release was due, in part, to a large decrease in arterial [HCO_3^-] from 21.5

Figure 3.2 Glycerol release across inactive rat hindlimb in lactate (LP) and normal (NP) perfusate groups.

* Indicates significant difference between groups.

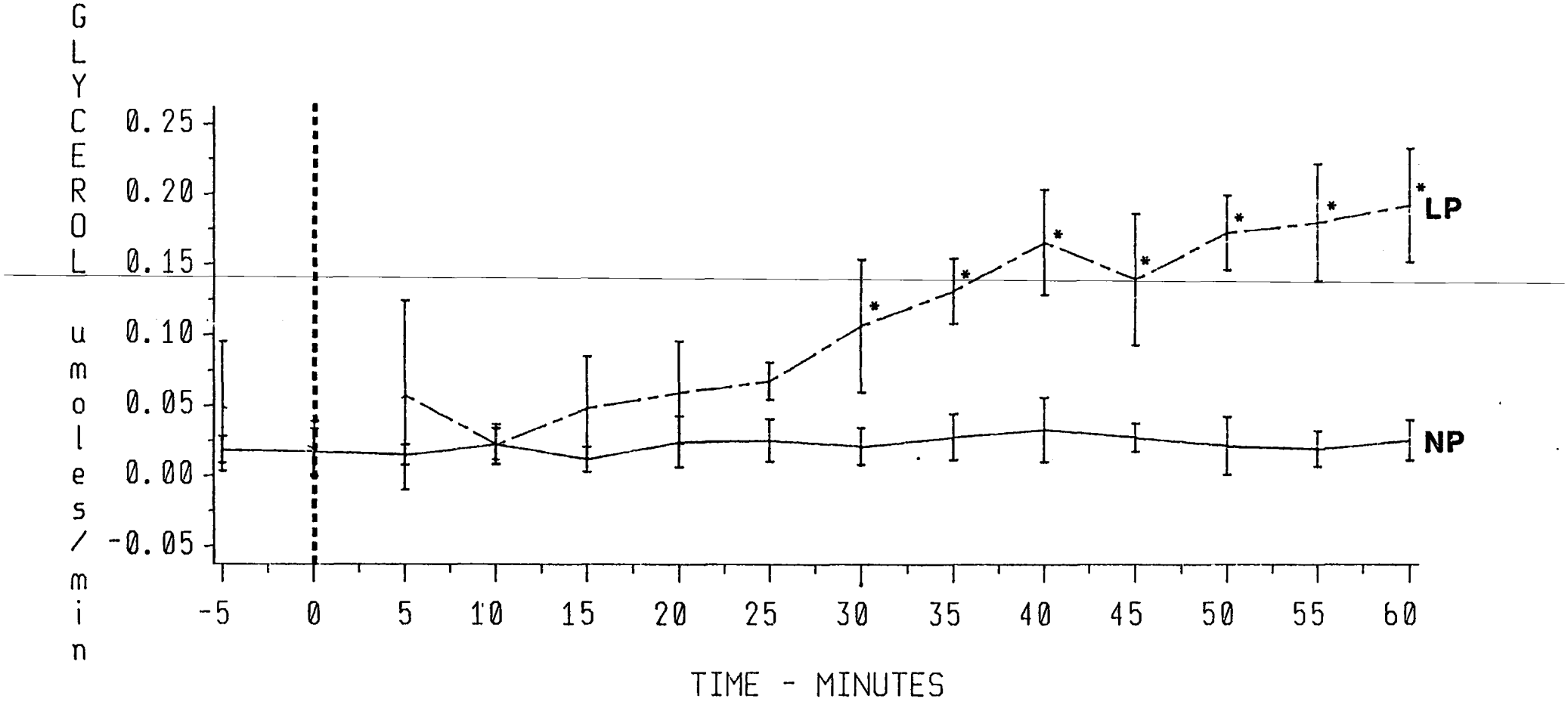
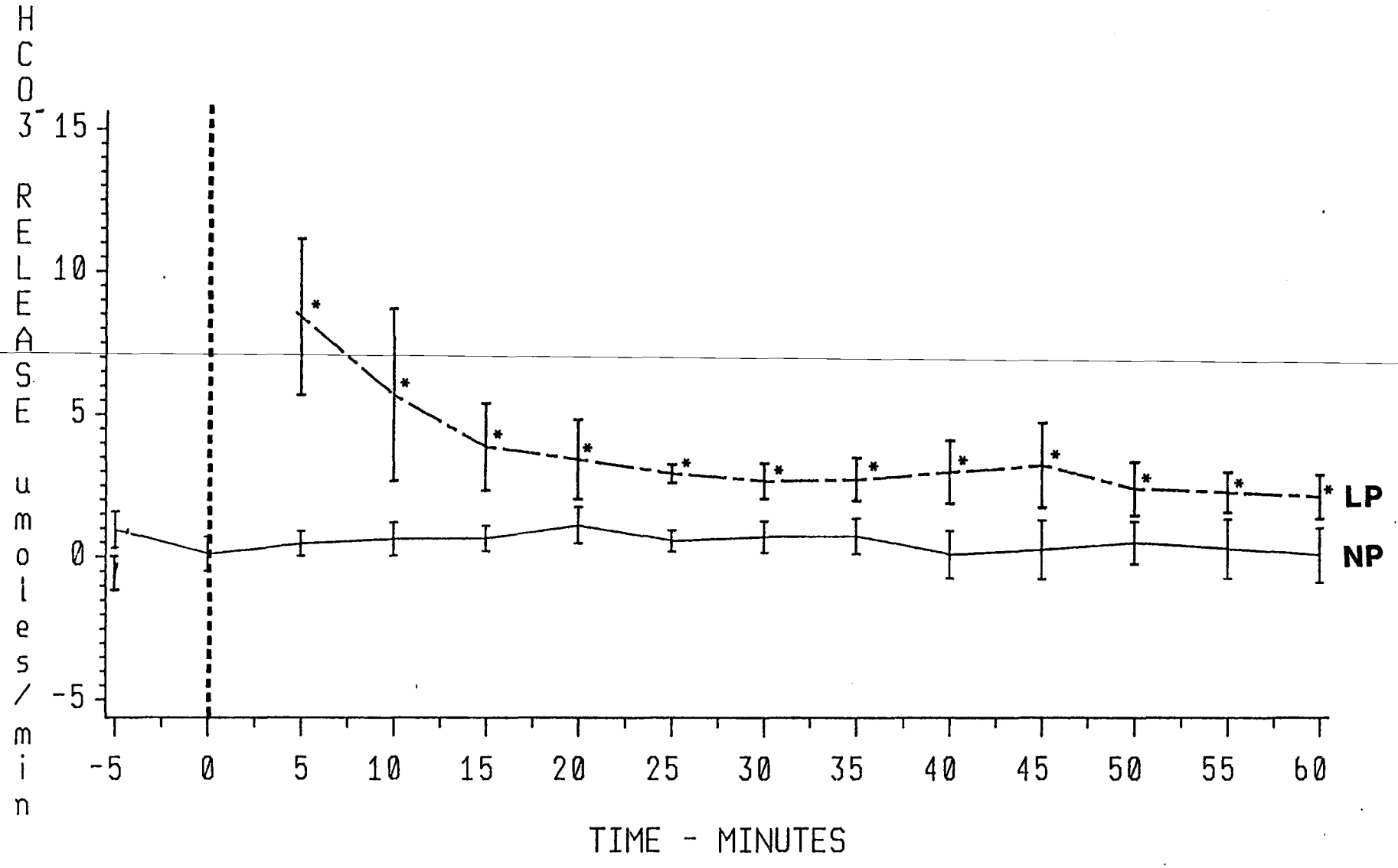

GLYCEROL umoles/min
0.25
0.20
0.15
0.10
0.05
0.00
-0.05
-5
0
5
10
15
20
25
30
35
40
45
50
55
60
TIME - MINUTES
LP
NP

Figure 3.3 Bicarbonate efflux from inactive rat hindlimb in lactate (LP) and normal (NP) perfuaste groups.

* Indicates significant difference between groups.


HCO3- RELEASE umoles/min
15
10
5
0
-5
-5
0
5
10
15
20
25
30
35
40
45
50
55
60
TIME - MINUTES
LP
NP

$\pm$ 0.2 mMol to 9.2 $\pm$ 0.3 mMol during the 20 min equilibration period and the 60 min LP perfusion period respectively.

3.4.4 Muscle TG Content

No significant differences in muscle TG content were found pre vs post perfusion in either the LP or NP groups (Figure 3.4). Differences were significant between muscle groups, with average TG content in the SOL being 73% higher than the PLT and WG. No difference in TG content between PLT and WG was found. A trend towards decreased TG concentrations, pre vs post perfusion, was noted in the SOL muscle (15.1 $\pm$ 4.0 to 11.5 $\pm$ 3.6 umole·g^{-1} DW in the LP group and 10.5 $\pm$ 2.0 to 9.3 $\pm$ 2.9 umole·g^{-1} DW in the NP group), but this trend was not significant.

3.5 DICUSSION

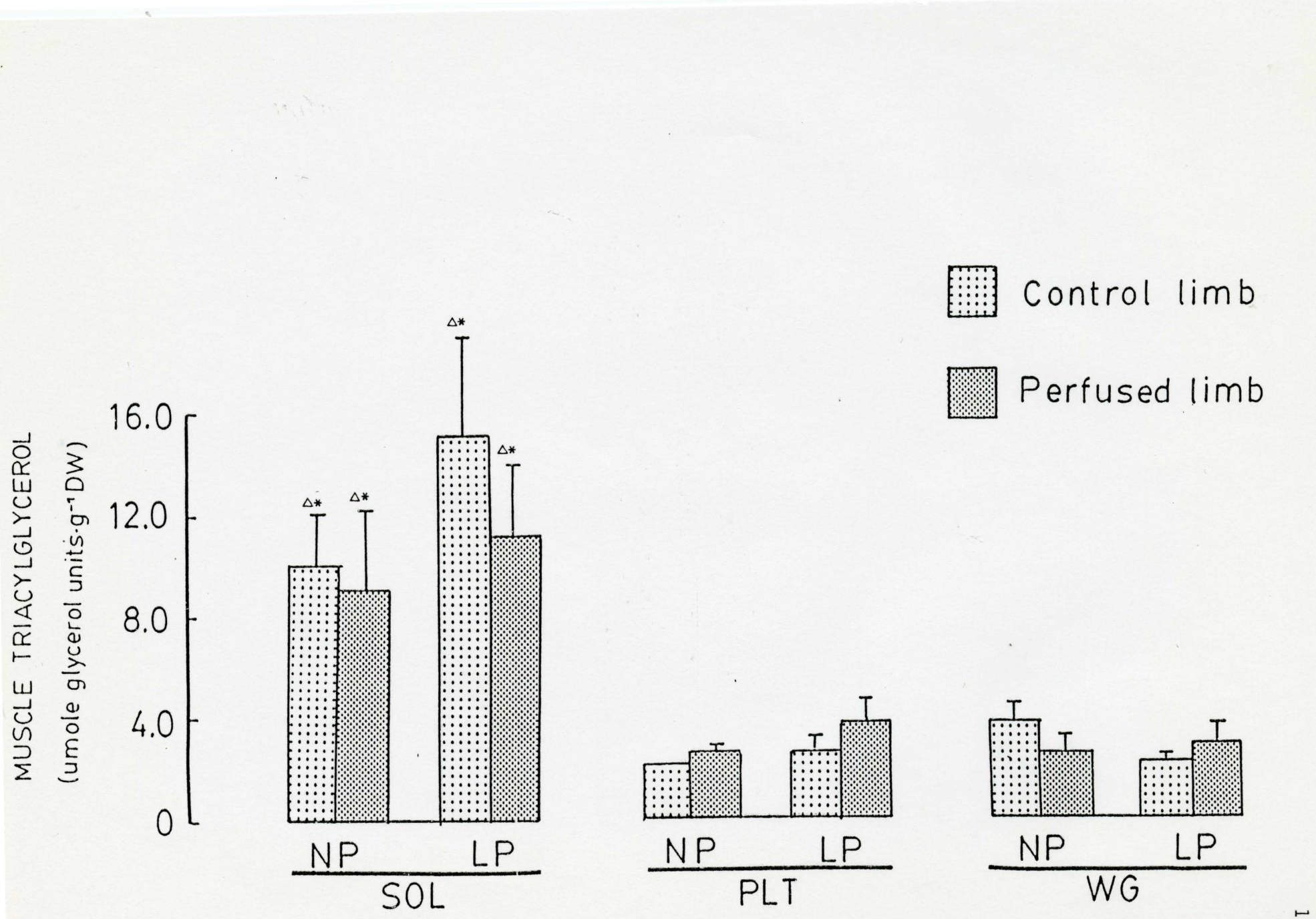
Elevated CO_2 output, muscle RQ, and glycerol release, in the absence of any change in muscle TG content, has suggested that increased TG/FFA cycling and/or increased glycerol synthesis resulted from lactacidotic perfusion. Lactate elimination via metabolic cycling within triacylglycerol pathways has been hypothesized to explain the observed disappearance of La and the increased glycerol release. Its overall contributions to La removal following high intensity work, however, may be minimal.

3.5.1 Methodological Considerations

An isolated perfused rat hindlimb model was used in the present investigation. The underlying purpose was to obtain insight into the metabolic removal patterns of La in non-working skeletal muscle following

Figure 3.4 Muscle triacylglycerol concentration in rat hindlimb muscles pre and post perfusion in lactate (LP) and normal (NP) perfusate groups. SOL = soleus, PLT = plantaris, WG = white gastrocnemius.

* Indicates significant difference between SOL vs PLT and WG for respective limb and perfuaste group.


MUSCLE TRIACYLGLYCEROL
(umole glycerol units·g⁻¹DW)
16.0
12.0
8.0
4.0
0
Control limb
Perfused limb
Δ*
NP
LP
SOL
PLT
WG

high intensity exercise, and in particular, to evaluate the involvement of La in TG/FFA substrate cycling and/or glycerol production. In this model, concentrations of exogenous substrates and circulating hormones were tightly controlled. Perfusate FFA and glycerol concentrations were maintained below the normal physiological range to minimize muscle uptake of these substrates. Lack of insulin would be expected to prevent high levels of glucose uptake, and lack of catecholamines to eliminate most, if not all, hormone-sensitive lipase activity. This would have prevented hormonal stimulation of muscle TG lipolysis and utilization of endogenous FFAs. La was therefore the most abundant substrate available to the inactive muscle of the LP group. Adaptations in skeletal muscle metabolism, as characterized by metabolic fluxes and muscle metabolite changes, could then be attributed to changes in arterial La and/or H^+ levels.

3.5.2 Percent of Lactate Removed By Glycerol Formation

To calculate the percent of La involved in the stimulated release of glycerol, previously obtained values (Chapter 2) were used for total La removed, total tissue La, and total La disappearing across the gastrocnemius-plantaris-soleus (GPS) muscles of the perfused hindlimb. These values were corrected for the relative proportion of metabolic activity occurring between 5 and 60 min of experimental perfusion. Total glycerol release was calculated as a sum of glycerol release measurements per 5 min interval. Glycerol release per 5 min interval was defined as the product of rate of glycerol release ($umole \cdot min^{-1}$) and time (5 min). Since previous calculations for La uptake, % La metabolized, and % La oxidized were restricted to metabolic activity across the GPS muscles (see Section 2.5.1), analysis of glycerol release took into account only the GPS fraction.

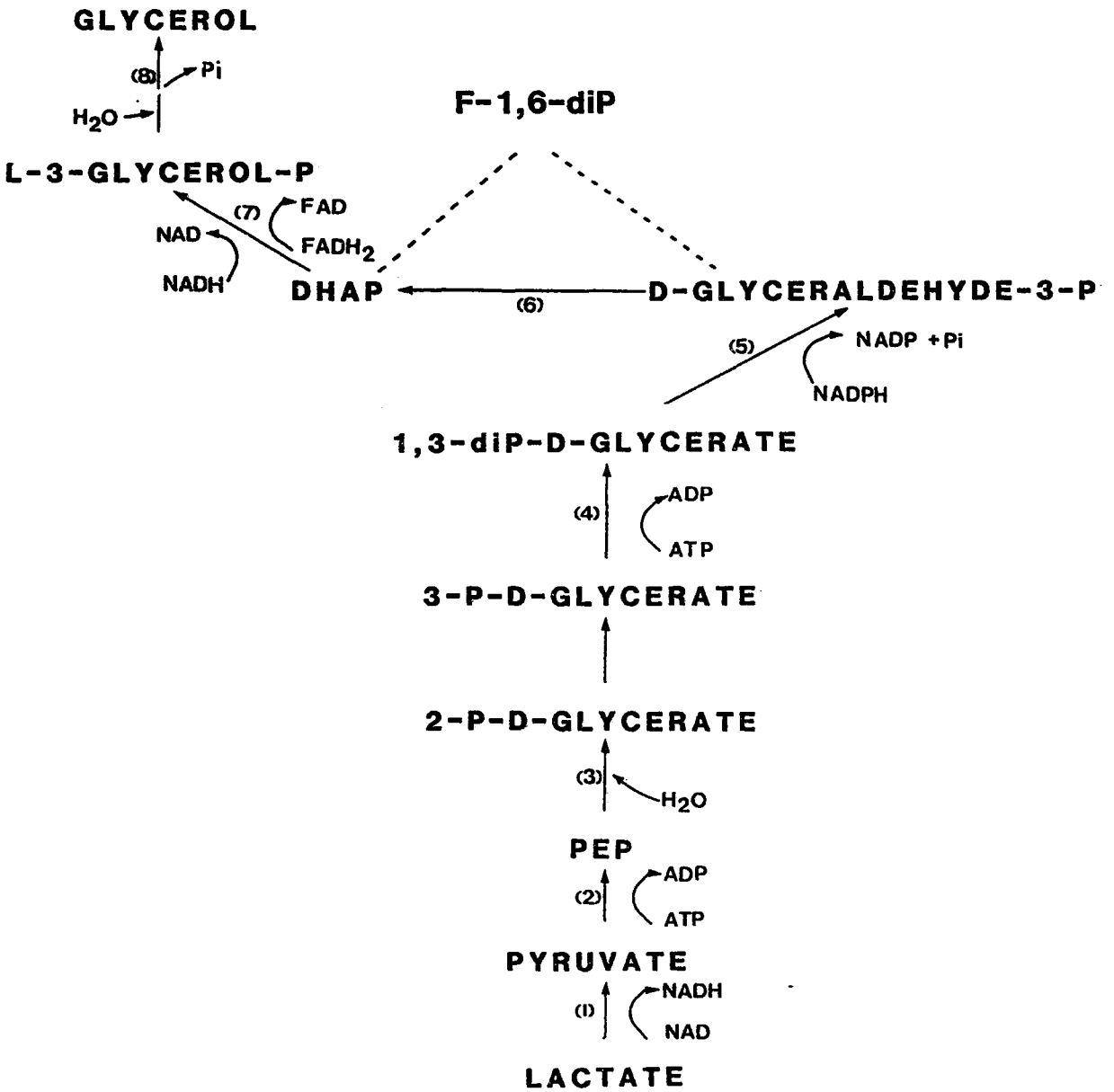
The total cumulative La removed by the GPS muscle group between 5-60 min of lactacidotic perfusion was 61.0 umoles. Of this total, 8.7 umoles (14%) remained as muscle La, while a minimum of 6.2 and a maximum of 17.2 umoles (12 and 33% respectively) was oxidized. Of the remaining 35-46 umoles of La, some may have served as a precursor for the observed glycerol release. Total cumulative release of glycerol was calculated to be 6.7 umoles for the entire hindlimb muscle group, and 2.9 umoles for the GPS portion of the hindlimb.

Based on the stoichiometry of glycerol production from glycolytic intermediates, 1 umole of La is required per umole of glycerol synthesized (McGilvrey, 1983). Therefore, the maximum amount of La removed as a precursor for glycerol synthesis, could be estimated to be 2.9 umoles. As a potential metabolic fate of La, glycerol would thus account for only 5.5% of the La removed by inactive skeletal muscle..

The metabolic pathway for La to glycerol conversion is shown in Figure 3.5. The required enzymes, ionic cofactors, and the regulatory metabolites are indicated in Table 3.1. Glycerol synthesis is an energy-requiring process. The net energy input, for conversion of 1 mole of La to 1 mole of glycerol, would be 2 moles of ATP and and 1 mole of NAD (which generates 3 mole ATP/mole NAD). Thus, total energy required, in ATP equivalents, is 5 mole ATP/mole glycerol produced.

Assuming that all glycerol was formed from La, total ATP required for the observed glycerol release would have been 14.5 umoles. Oxidation of 1 umole of La releases approximately 19 umoles of ATP (McGilvrey, 1983). Therefore, total La required (both as a precursor and as a fuel source) to account for the net glycerol released would be 3.9 umoles, representing 7.5% of the La removed by the inactive GPS muscles. This suggests that glycerol

Figure 3.5 Schematic representation of glyceroneogenic pathway. Enzymes catalyzing each reaction are shown in Table 3.1. PEP = phosphoenol pyruvate, P = phospho/phosphate, diP = diphosphate, DHAP = dihydroxyacetone phosphate, D,L = molecular configuration.
From Boehringer Mannheim Biochemical Pathways Chart.



( ) ENZYMES – See Table 3.1

Table 3.1 Enzymes, Ionic Cofactors, and Regulatory Metabolites Involved in Glyceroneogenesis

Rx	ENZYME(S)	REGULATORS - ve	REGULATORS + ve	IONIC COFACTORS
(1)	LDH	Fatty acids		Zn++
(2)	P.C.		F-1,6-diP	Mg++, K+
	PEP.C.K.		AMP	
(3)	Phospo-pyruvate hydratase	D-Phospho-La 3-P-D Glycerate		Mn++, Mg++, Zn++
	Phosph-glycero mutase		2,-3diP-glycerate	
(4)	3-Phospho-glycerate kinase			Mg++, Mn++
(5)	Glyceraldehyde-P-dehydrogenase	Thyroid hormone		
(6)	Triose-P-Isomerase			
(7)	Glycerol-3-P Dehydrogenase	F-1,6-diP		
(8)	Glycerol kinase	F-1,6-diP		Mg++

Table 3.1 From Boeringer Manheim Biochemical Pathways Chart

output by the GPS muscles of the rat hindlimb was not a major endpoint for La elimination in this study.

3.5.3 Metabolic CO_2 Production

During perfusion of inactive hindlimb muscles with an arterial lactacidosis, CO_2 output rates were elevated six-fold above control conditions. This observed increase agrees with values previously obtained in inactive forearm muscle during and following maximum cycle ergometer work (Kowalchuk, 1985). During heavy exercise, Kowalchuk (1985) found CO_2 production across the forearm increased from 5 umole·100g^{-1}·min^{-1} at rest to 50 umole·100g^{-1}·min^{-1}, a ten-fold increase. In the present study, the average CO_2 ouput over the final 40 min was 3.11 umole·min^{-1}·HQ^{-1} , equivalent to 48 umole·100g^{-1}·min^{-1}. In absolute terms, the CO_2 output values observed in the present study were similar to those found by Kowalchuk during high intensity exercise. This suggests that an exercise state, similar to that observed in inactive muscle of humans during high intensity muscular work, was induced in the lactacidotic perfused hindlimb.

Heavy exercise associated with increased La and H^+ concentrations has been found to stimulate an elevated muscle respiratory exchange ratio in active (Jones et al, 1980) and in inactive (Kowalchuk, 1985) skeletal muscle. The values noted in the present study of 1.4-1.8 over the final 40 min of LP perfusion, are somewhat higher than values reported in exercising humans during cycle ergometry at 70% maximum power output (Jones et al, 1980). In the present study CO_2 output and O_2 uptake were determined by changes in blood gases across the muscle rather than by gas exchange (RER) at the mouth. Differences between the direct measurement of muscle RQ and the indirect measurement of RER at the mouth (Jones et al, 1980) may account

for some of the discrepancies between findings.

Elevations in muscle RQ to values greater than 1.0 have been validated theoretically by linear programming models (Fell and Small, 1986, Garby and Astrup, 1987). Glycolysis and fatty acid oxidation occur simultaneously with net lipid synthesis in vivo. An RQ value representing the NET activity of all reactions could reach values between 1.0 and 2.75. Synthesis of 1 mole of fat requires the equivalent of 9 moles of La and 4 moles of O_2, with 11 moles of CO_2 being produced (McGilvrey, 1983), representing a maximum RER of 2.75. RER values measured in vivo are lower due to concurrent catabolism elsewhere in the same tissue.

Muscle RQ values greater than unity may represent net metabolic activity, however the validity of this value in the present study is questionable. If La was utilized to synthesize glycerol, the stoichiometric relationship of precursor to product would have been 1:1. Therefore, this anabolic activity would not account for the excess CO_2 released. Assuming catabolism of pure carbohydrate (ie. La), RER values would have reached 1.0. Explanation for the values obtained exceeding 1.0 include FFA synthesis and/or non-metabolic CO_2 release.

Although free fatty acid synthesis would account for excess CO_2 output, exchange of this metabolite across the muscle was not measured. Other investigators, however, have found that FFA release rates are decreased during La infusion in active (Issekutz et al, 1975, Jones et al, 1980) and in inactive (Ahlborg et al, 1975) skeletal muscle. Lipogenesis appears to be regulated primarily by hormonal status, and not by excess substrate availability, as was hypothesized. Furthermore, the present conditions do not appear to be compatible with previous reports of lipogenesis.

Lipogenesis has been reported to occur during pregnancy and progesterone treatment (Sutter-Dub et al, 1983), during post natal periods in rats (Hahn, 1986), and during periods of rapid growth (Hems et al, 1977). Where glucose or sucrose-stimulated lipogenesis has been observed, increased rates of TG synthesis have been attributed to high rates of glycerol synthesis (Hahn, 1986). Observations of lipogenesis are most common in adipose tissue (Hems et al, 1975, Granneman and Campbell, 1984, Hahn, 1986) or in hepatic tissue (Hems et al, 1975, Kalderon et al, 1983). Reports of lipid synthesis in smooth muscle (lung) can be found in the literature (Casals et al, 1983), however reports of net lipogenesis in skeletal muscle, active or inactive, have not been observed.

Under the present experimental conditions, it is not likely that FFA synthesis occured. Thus, the elevated RQ cannot be explained by excess metabolic production of CO_2, but may represent non-metabolic sources of CO_2 release. Speculations as to the origins of this net CO_2 release can be made, however direct quantification of its source was not possible.

3.5.4 Non-Metabolic CO_2 Production

It has been recognized that elevations in CO_2 output may reflect non-metabolic CO_2 release from HCO_3^- buffering of H^+ (Sahlin et al, 1978, Jones et al, 1980). Non-metabolic sources of CO_2 release, other than HCO_3^- buffering, include release of dissolved CO_2 in plasma and/or release of CO_2 from carbamino compounds in muscle (Jones, personal communication). The quantitative importance of these sources of CO_2 in the present study are not known. Thus, the explanation for the elevated CO_2 output and muscle RER in response to La perfusion of muscle remains obscure.

3.5.5 Muscle Metabolite Changes

Failure to find differences in muscle TG content following 60 min of perfusion in both LP and NP groups suggests that net TG lipolysis did not occur at rest. Under NP conditions, muscle TG may have been utilized as a metabolic fuel source, indicated by the low RQ values (0.49 - 0.79). Net TG degredation did not occur, however, indicating that TG lipolysis occured at rates equal to rates of TG esterification.

The observed O_2 uptake by the GPS fraction of the perfused rat hindlimb under NP conditions (51.5 umole) would have required the oxidation of less than 2.5 umoles of FFA. At most this would require lipolysis of 1 umole of TG, and re-esterification of 3 umole of FFA. Although not quantified in the present study, minimal concentations of albumin-bound FFA may have been sufficient to support basal FFA utilization. Intramuscular glucose may also have served as an energy substrate in the NP group (Chapter 2).

Non-significant differences between post perfusion TG content values in the LP vs NP groups may partially be attributed to the high degree of biological variability of TG concentration in rat skeletal muscle (Frayn and Maycock, 1980, Spriet et al, 1985). Muscle TG values reported in the present study (mean = 11.8, 3.3 , and 2.6 umole·g^{-1} DW in the SOL, PLT, and WG respectively) are considerably lower than those previously reported for SOL, PLT and WG (28.2 $\pm$ 5.0, 7.7 $\pm$ 0.61, 6.81 $\pm$ 1.22 umole·g^{-1} DW) (Spriet et al, 1986). Others have reported concentrations of 1.35-4.25 umole·g^{-1} WW (Frayn and Maycock, 1980), equivalent to approximately 6.0-19.0 umole·g^{-1} for dry weight values. These discrepant findings may be related to the biological variability, or possibly to methodological differences.

Percent recovery was slightly lower in the present study (89-100%) when compared to values of Spriet et al (1986) (94-100%). This may account for

some of the discrepancies between studies. Although the methods of extraction and assay procedures were the same as those used by Spriet et al (1985, 1986), as modified from Frayn and Maycock (1980), the former studies used wet muscle. In the present study, muscle TG analysis poceeded from dry tissue. Muscle lipids were extracted in chloroform/methanol, a process which may not have been as effective with non-hydrated tissue.

Differences in TG content between muscle groups was found to be significant. TG concentration in the SOL was on average 73% higher than in PLT and WG. The difference in TG content between muscle groups measured, suggests that differences were due to percentage of slow oxidative fibers. This fiber-type difference in lipid content has been observed in several investigations (Okano et al, 1980, Frayn and Maycock, 1980, Spriet et al, 1986).

3.5.6 Mechanisms of Glycerol Release

Due to the active presence of glycerol kinase in skeletal muscle, Robinson and Newsholme (1967) suggest that glycerol release should not necessarily be interpreted as TG lipolysis . It has recently been suggested that increased levels of muscle glycerol and glycerol release, found in exercised equine muscle, may be due to elevations in glycero-3-phophate (G-3-P) hydrolysis, and not to TG utilization (Snow et al, 1985). Snow et al (1985) observed increased glycerol release even following 30 min of recovery. During the same time period, little recovery of ATP or La was found suggesting that G-3-P hydrolysis and glycerol formation occurs at elevated concentrations of muscle La. This is consistent with the present findings, with glycerol release being stimulated even at muscle [La] of 35-40 umole·g^{-1} DW.

It has previously been hypothesized that La serves as a feedback mechanism to increase FFA re-esterification, thereby altering metabolic rates of the non-esterified fatty acid (Fredholm, 1971, Issekutz et al, 1975). The suggested mechanism involves La, as a H^+ donor, which alters the cellular NADH/NAD ratio, thereby shifting the dihydroxyacetone <----> G-3-P equilibrium towards G-3-P formation. The accumulating G-3-P would re-esterify intracellular FFA released by the lipolysis of various glycerides, with the net result being release of one glycerol moeity. Issekutz et al (1975), observed net glycerol release in response to La but not pyruvate infusion, confirming the role of La as a H^+ donor in stimulating glycerol release.

3.5.7 Site of Glycerol Synthesis and/or Altered Substrate Cycling

Although conversion of La to glycerol has been hypothesized to occur in inactive muscle during lactacidotic perfusion, the existance of this pathway in skeletal muscle is dependent upon the effective concentrations of key enzymes in the intramuscular compartment. Rate limiting enzymes such as glycerol kinase are found in skeletal muscle cells, but the activity of this enzyme appears to higher in adipose cells (Robinson and Newsholme, 1967).

The observed La and glycerol exchange across the hindlimb muscles may have involved metabolic activity within adjacent adipose tissue. The extent of adipose tissue metabolism in the present investigation could not accurately be determined. Previous investigators, however, have stated that rat skeletal muscle has very few interstitial adipose tissue cells (Frayn and Maycock, 1980), a factor which may explain the small degree of glycerol release from the hindlimb, and the minor importance of TG/FFA cycling to the

overall elimination of La.

3.5.8 Regulation of Triacylglycerol/Fatty Acid Substrate Cycling

Regulation of TG turnover involves control by the major enzymes for TG lipolysis and TG esterification. The lipolytic enzyme, lipoprotein lipase (LPL) has been isolated in skeletal muscle. The intracellular fraction appears to be hormone sensitive, and currently an attempt is being made to change the name of the intracellular fraction from LPL to type-L HSL (Oscai and Palmer, 1983). Type-L HSL activity appears to be stimulated by catecholamines, and thus may have played a minor role in the present model where catecholamines were absent from the perfusate. This may explain the relatively small contribution of the glycerol release pathway to La elimination (less than 7% of La metabolized).

Recent investigation using a similar rat hindlimb perfusion model revealed that in the absence of catecholamines and insulin the FFA:glycerol release ratio measured across a perfused rat hindlimb at rest was 0.37 (Spriet et al, 1986). Thus, in the resting perfused hindquarter, lipolysis and re-esterification can occur concurrently without insulin in the perfusate provided there is a substrate source for the G-3-P required for FFA re-esterification. It has been suggested that the rate of re-esterification of FFA released by lipolysis is dependent upon the amount of G-3-P available from glucose or glyceroneogenesis (Masoro, 1977).

In the present study, La cycling upwards in the glycolytic pathway, could provide the carbon skeleton for G-3-P. Previous findings (Hems et al, 1975, Bloxham et al, 1977) suggesting that 3-carbon compounds were preferred as precursors for glycerol, support this hypothesis. Therefore, one of the metabolic fates of La in inactive skeletal muscle during lactacidosis

appears to be G-3-P. Additional La may have been removed by cycling along the glyceroneogenic pathway, a pathway sharing several common intermediates with the glyconeogenic pathway.

Investigations using an isolated perfused hindlimb, with only glucose in the perfusate, have observed FFA release at rest (Van Hardeveld and Kassenar, 1977, Goodman et al, 1983). In contrast, others have found that FFA and glycerol release were inhibited by glucose infusion (Christopherson, 1983, Wolfe and Peters, 1983). The inclusion of insulin in the perfusate during rat hindlimb perfusion decreased FFA release, suggesting that insulin may enhance re-esterification and inhibit net TG lipolysis (Havel and Carlson, 1963, Goodman et al, 1983). Conflicting reports in the literature suggest a complex interaction between carbohydrate metabolism and TG/FFA substrate cycling (proposed glucose/fatty acid cycle).

In the absence of hormonal involvement, substrate availability appears to be an important regulator of resting muscle metabolism. The significance of this control mechanism under physiological conditions where regulatory hormones are available, however, may only be minimal.

Cycling between TG lipolysis and TG esterification is thought to provide control over the metabolism of non-esterified fatty acids (Hammond and Johnston,1983). Little is known, however, about the mechanisms of TG regulation in skeletal muscle. Furthermore, the integration of TG metabolism with the utilization of other substrates, which are ultimately metabolized by common energy-transducing pathways, is not well understood. The concentrations of high energy phosphates, H^+, Ca^{++}, and key metabolic intermediates (citrate, acetyl coA, and G-6-P) have been suggested to regulate the relative rates of glycolysis (Chasiotis et al, 1982) and TG utilization (Spriet et al, 1986). The present study indicates that La, a key

metabolic intermediate, plays a role in TG regulation in inactive skeletal muscle.

3.6 CONCLUSION

Significant levels of La removal, CO_2 output, and glycerol release in the absence of any net change in TG content was observed across the inactive, perfused rat hindlimb muscles under conditions of arterial lactacidosis. Interaction of La removal patterns with TG turnover appears to be linked to altered cellular NADH/NAD ratios and resultant formation of dihydroxyactetone phosphate and subsequently G-3-P. Glycerol synthesis may directly involve the La carbon skeleton. It may also reflect increased cycling of intermediates further down along the glyceroneogenic pathway. Depression of FFA turnover by acidosis along with the increased turnover of glycerol appear to have resulted in increased TG/FFA cycling rates and no net lipolysis of TGs. Direct glyceroneogenesis may only have accounted for 5-7% of the La metabolized by the inactive muscle. While the overall contributions of TG metabolism to La elimination may have reached higher proportions, this could not accurately be determined in the present investigation. Lactacidosis also stimulated increased rates of CO_2 output and HCO_3^- efflux from the inactive muscle. The majority of this gas exchange could not be accounted for. Resolution of this phenomenon requires further investigation with the use of radioisotopic tracers and direct quantification of FFA exchange across the muscle.

CHAPTER 4 EFFECTS OF LACTACIDOSIS ON IONIC FLUX ACROSS INACTIVE SKELETAL MUSCLE OF THE PERFUSED RAT HINDLIMB

4.1 ABSTRACT

The movement of strong ions (sodium (Na^+), potassium (K^+), chloride (Cl^-) and lactate (La)) across inactive skeletal muscle was investigated by using an isolated rat hindlimb perfusion model. The perfusate attempted to simulate arterial blood that would be found following intense exercise. Male Sprague-Dawley rats were perfused for 60 min with either a normal perfusate (NP) (N = 8) or a lactacidotic perfusate (LP) (N = 8). The LP was characterized by increased concentrations of La (11.0 mMol), K^+ (7.88 mMol), and hemoglobin (Hb) (16.7 $g \cdot dl^{-1}$) and a decreased pH (7.15). Arterial and venous perfusate samples were collected every 10 and 5 min respectively, and later analyzed for whole blood concentrations of Na^+, K^+, Cl^- and La. Plasma samples were analyzed immediately for pH and PCO_2, from which non-volatile H^+ could be estimated. Ionic exchange across the hindlimb muscle group was calculated from flow rate and arterio-venous concentration differences. This exchange parameter was assumed to represent ion flux from extracellular to intramuscular compartments of the inactive muscle.

In the NP perfusate group, blood electrolyte analysis revealed no significant ($p < .05$) flux for Na^+, Cl^- and non-volatile H^+ when averaged over 60 min. Net K^+ ($0.42 \pm .07$ $umole \cdot min^{-1}$) and La ($-.12 \pm .02$ $umole \cdot min^{-1}$) efflux was observed in the NP group, although the total amount of K^+ and La leaving the muscle was minimal. Increased ionic flux occurred in response to the lactacidotic perfusion. In the LP group, both La and non-volatile H^+

demonstrated a rapid influx (7.6 and 8.9 umole·min^{-1} respectively) at 5 min, but rate of influx decreased to 1.3-2.0 umole·min^{-1} for La and 2.2-4.0 umole·min^{-1} for H^+ over the final 40 min of perfusion. Na^+ and Cl^- movement occurred in an inconsistent pattern, although both ions demonstrated an average net influx between 5 and 60 min of perfusion (3.1 + .6 umole·min^{-1} for Na^+ and 1.6 $\pm$ .4 umole·min^{-1} for Cl^-). Significant inward flux of K^+ was observed, with average rate of influx being 0.4 + 0.1 umole·min^{-1} over the final 55 min of LP perfusion. These findings suggest that La is transported into inactive skeletal muscle by a number of mechanisms, including HLa diffusion, La/H^+ co-transport and possibly La/Cl^- anion exchange. These data also suggest that a number of regulatory mechanisms are activated in rat skeletal muscle to maintain muscle intracellular [H^+] and membrane potential during lactacidotic perfusion. These mechanisms include Na^+-K^+ ATPase pump activity, and Na^+/H^+ and HCO_3^-/Cl^- exchange. It may be concluded that, following high intensity exercise, total ionic exchange and not just La translocation are important in the regulation of muscle and plasma La, K^+ and non-volatile H^+ concentration by inactive skeletal muscle.

4.2 INTRODUCTION

Short term exhaustive exercise has been associated with several ionic disturbances within the exercising muscle as well as in the circulating plasma (Sjogaard, 1983, Medbo and Sejersted, 1985, Lindinger, 1987, McKelvie et al, 1987, Heigenhauser et al, 1987). Glycolytic activity increases intramuscular lactic acid production, resulting in increased muscle and plasma lactate (La) and H^+ ions (Osnes and Hermansen, 1972, Sahlin, 1978) and thus increased non-volatile (non-CO_2) acid. Net potassium (K^+) release has also been observed during high intensity exercise (Knochel et al, 1985, Lindinger et al, 1987, McKelvie et al, 1987, Heigenhauser et al, 1987) resulting in decreased intramuscular and increased plasma [K^+]. These ionic disturbances may alter glycolytic enzyme function and membrane potential which may result in muscle fatigue. Mechanisms for ionic regulation are therefore of importance in understanding strategies involved in delaying the onset of fatigue and/or improving recovery from fatigue.

Following glycolytic lactic acid production, La and H^+ efflux from active skeletal muscle may occur as an active or passive process (Hultman and Sahlin, 1980), as a single molecule (HLa) (Wolosin and Ginsberg, 1975), or as separate ions (Hultman and Sahlin, 1980, Benade and Heisler, 1978). Studies demonstrating the same time course for La and H^+ efflux confirmed the belief that La and H^+ are co-transported across across the muscle membrane (Hirche et al, 1975, Mainwood et al, 1972, Mainwood and Worsley-Brown, 1975). More recent evidence, however, indicates that La and H^+ may have different efflux rates in vitro (Heisler et al, 1973, Benade and Heisler, 1978, Heigenhauser et al, 1986), suggesting separate transport mechanisms. Others have found that efflux of La and H^+ occur in both a

stoichiometric and non-stoichiometric relationship, with the efflux kinetics changing at different time points during exercise and recovery (Sahlin, 1978, Seo, 1984). Mason et al (1986) have confirmed the existence of two separate mechanisms for La transport, one which is proton-dependent and one proton-independent. Kuret et al (1986) and Juel (1987) have provided evidence to support the hypothesis that a carrier-mediated mechanism exists for La and H^+ transport out of skeletal muscle.

Previous investigation of La and H^+ efflux from the intramuscular compartment into the surrounding extracellular fluid and eventually into blood has rigorously been investigated (Heisler et al, 1973, Hirche et al, 1975, Mainwood et al, 1972, Mainwood and Worsley-Brown, 1975, Benade and Heisler, 1978, Seo, 1984, Heigenhauser et al, 1986). Furthermore, it has been recognized that La and/or H^+ uptake by inactive tissue may facilitate efflux of these metabolites out of exercising muscle by reducing extracellular $[H^+]$ (Mainwood and Renaud, 1985, Renaud and Mainwood, 1985). Mechanisms of La and/or H^+ transport from plasma into non-exercising tissue may differ from those involved in their transport in the opposite direction, from an acidotic intramuscular compartment into blood. Little direct experimental evidence exists regarding the mechanisms of La and H^+ uptake by inactive skeletal muscle.

Mechanisms of ionic regulation of an extracellular-induced acidosis may differ from those of an intracellular-induced acidosis. Several active-mediated mechanisms have been postulated for intracellular ionic regulation and ion transport in response to an intracellular-induced acidosis. These postulated mechanisms may involve active transport either through ionic channels or by protein carriers (Mason et al, 1986, Juel, 1985). Some of the active-mediated regulatory mechanisms for skeletal muscle include Na^+/H^+ and

HCO_3^-/Cl^- exchange for proton regulation (Aickin and Thomas, 1977, Galler and Moser, 1986), La/Cl^- and La/HCO_3^- anion exchange for La transport (Mason et al, 1986, Juel, 1987), and Na^+-K^+ ATPase pump for K^+ regulation (Clausen, 1983, Clausen, 1986). It is not known, however, whether the mechanisms of intracellular ionic regulation and ion transport in response to an extracellular acid load are similar to those previously suggested for an intracellular acid load.

A more recent approach to the study of muscle H^+ regulation involves analysis of the translocation of all strong ions (Na^+, K^+, Mg^{++}, Ca^{++}, Cl^-, as well as La) across the muscle membrane. It is thought that intramuscular H^+ regulation can occur only through the exchange of strong ions between muscle and the extracellular fluid compartment (Heigenhauser et al, 1986, Lindinger, 1987). This approach has evolved from the physico-chemical view of H^+ determination, as re-introduced by Stewart (1981, 1983). Measurement of the flux of strong ions may be more important in understanding the mechanisms of ionic regulation and ion transport than attempting to measure H^+ efflux directly.

Thus, the primary purpose of this study was to investigate the possible mechanisms for La removal from blood by inactive skeletal muscle. The role of this tissue in metabolically eliminating La following exercise has been previously discussed (see Chapter 2 and Chapter 3). A secondary purpose was to determine the possible mechanisms by which inactive muscle regulates intracellular $[H^+]$ in response to the extracellular ionic disturbances observed following maximal exercise. An examination of the concurrent movement of strong ions across the muscle was carried out in an attempt to evaluate the proposed exchange mechanisms for La, Cl^-, K^+, Na^+.

4.3 METHODS

The experimental animals, surgical technique, perfusion mediums (normal (NP) and lactacidotic (LP)), sampling protocol, and statistical analyses procedures were those previously described (see Section 2.2). Additional analyses of arterial and venous whole blood samples for strong ions Na^+, K^+, and Cl^-, and plasma for base excess, were carried out for the purpose of investigating the mechanisms of La and H^+ movement across the membrane of inactive skeletal muscle when presented with a lactacid challenge.

4.3.1 Ion Flux Measurements

Arterial and venous perfusate was sampled as indicated in Figure 2.2. Briefly, arterial blood was drawn from the oxygenating resevoir every ten minutes while venous blood was collected serially in 5 min intervals during the final 10 min of the equilibration period and throughout the 60 min of experimental perfusion. The venous outflow collection was used to measure flow rate and the sample later utilized for blood electrolyte determination. Blood samples were frozen in polyethylene test tubes. Total hemolysis of red cells was achieved by thawing and refreezing perfusate samples at least 3 times.

Blood Na^+ and K^+ concentrations were measured in duplicate using ion selective electrodes (Radiometer KNA1 Sodium/Potassium Analyzer) following a 1:1 dilution with double distilled water. Analysis of the distilled water revealed that no ions were present in the water. Therefore, measured Na^+ and K^+ concentrations were doubled and these values used to calculate arterio-venous (a-v) concentration differences. Arterial and venous whole blood Cl^- concentration was measured by colourometric titration (Buchler Cutlove

Chloridometer). Ionic flux for Na^+, K^+, and Cl^- was calculated as the (a-v) concentration difference times flow rate.

4.3.2 Non-Volatile H^+ Flux Determination

The non-volatile H^+ concentration was estimated using an acid-base alignment nomogram for the determination of base excess (Lindinger et al, 1986) (see Appendix II). Plasma pH and PCO_2 were determined using a blood acid-base analyzer (Radiometer BM 3). Alignment of the measured pH value with the appropriate PCO_2 allowed determination of the non-CO_2 H^+ component (base excess) under the given conditions. A negative base excess value was taken as being representative of a positive H^+ value. H^+ flux could then be calculated as the (a-v) H^+ concentration difference times flow rate.

4.4 RESULTS

Ion flux data has only been reported between 5 and 60 min of the experimental perfusion period. Flux data from the equilibration period and from 0 min was eliminated due to previously mentioned methodological considerations (see Section 3.4). Results for the flux of Na^+, K^+, Cl^- and non-volatile H^+ are represented graphically in Figures 4.1, 4.2, 4.3, and 4.4 respectively.

4.4.1 Sodium Flux

Sodium exchange across the inactive hindlimb muscles demonstrated considerable between animal variability in both NP and LP groups. The average Na^+ flux across the inactive muscle during 60 min of NP perfusion

was -0.1 $\pm$ .5 umole·min^{-1}, whereas the average Na^+ flux across inactive muscle perfused with a lactacidotic perfusate was 3.1 $\pm$ .6 umole·min^{-1}, a value which was significantly greater than the NP group (see Figure 4.1). Post-hoc analyses comparing Na^+ flux between NP and LP groups revealed that net influx of $Na+$ in the LP group was only significantly different from the NP group at 5, 20, 55 and 60 min.

4.4.2 Potassium Flux

Analysis of K^+ flux across the inactive hindlimb indicated a net efflux (-0.42 $\pm$ .07 umole·min^{-1}) during NP perfusion, and a net influx (0.40 $\pm$ .10 umole·min^{-1}) during LP perfusion, a difference which was significant at $p < .01$ (see Figure 4.2). Post-hoc analyses revealed that differences between NP and LP groups were significant at all time points from 10-60 min. A trend towards an increased K^+ efflux and towards an increased K^+ influx occurred between 50 and 60 min in the NP and LP groups respectively.

4.4.3 Chloride Flux

Measurements of chloride movement across the inactive rat hindlimb muscles demonstrated the same degree of between animal variability as observed for Na^+ flux (see Figure 4.3). During NP perfusion, average Cl^- exchange was 0.4 $\pm$ 0.3 umole·min^{-1}, indicating that little net flux of this anion occured. In response to the LP perfusion, a net flux of Cl^- was observed. The average Cl^- influx over the 55 min of perfusion was 1.6 $\pm$ .4 umole·min^{-1}. The average flux between LP and NP groups was not statistically different. Post-hoc analyses, however, indicated that differences between NP and LP groups were significant ($p < .01$) at 10, 20, 50 and 60 min.

Figure 4.1 Sodium flux across inactive rat hindlimb in normal (NP) and lactacidotic (LP) perfusate groups.
* Indicates significant difference between groups.

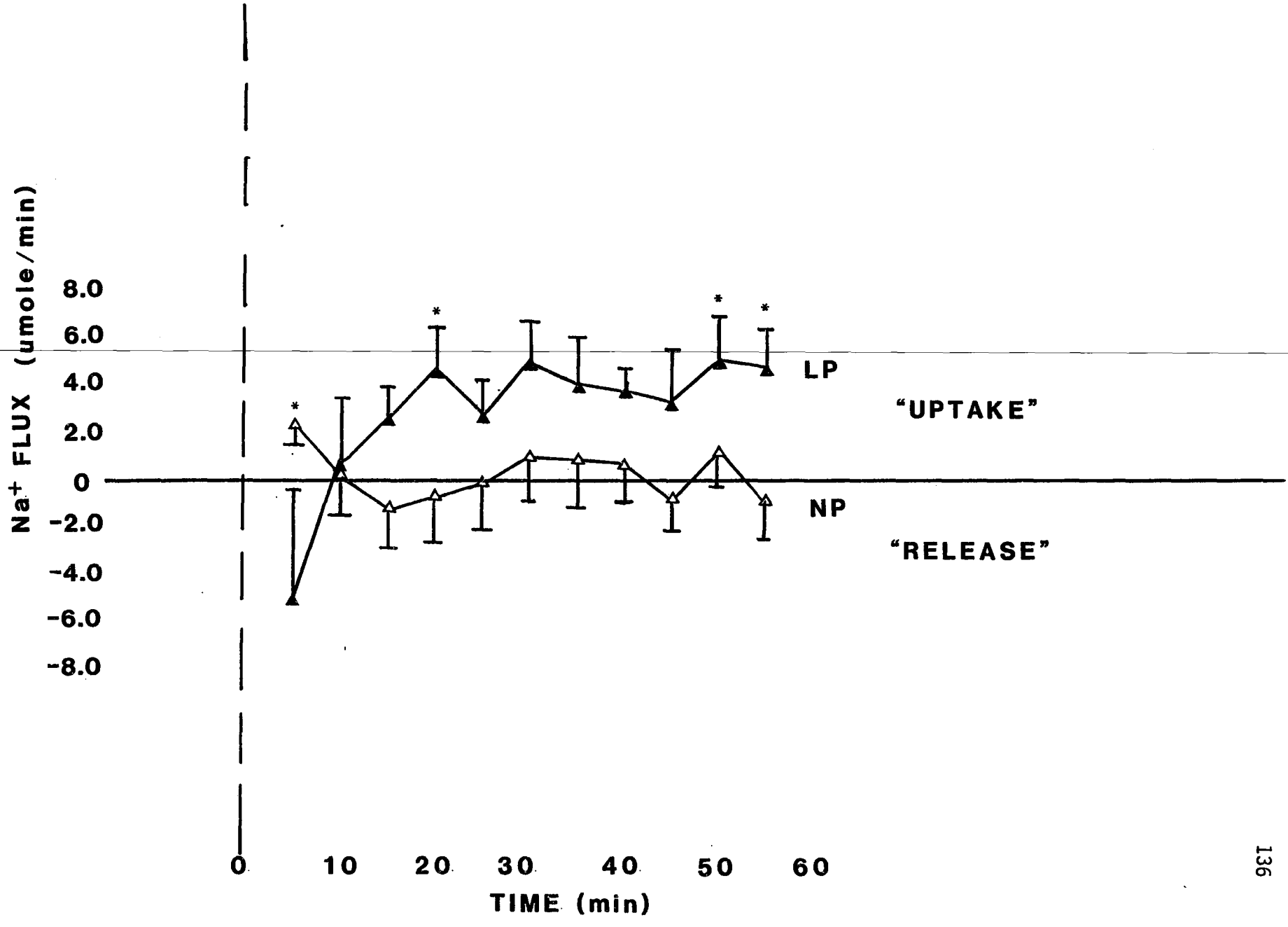

Na+ FLUX (umole/min)
8.0
6.0
4.0
2.0
0
-2.0
-4.0
-6.0
-8.0
0
10
20
30
40
50
60
TIME (min)
LP
"UPTAKE"
NP
"RELEASE"
*
*
*
*
*

Figure 4.2 Potassium flux across inactive rat hindlimb in normal (NP) and lactacidotic (LP) perfusate groups.
* Indicates significant difference between groups.

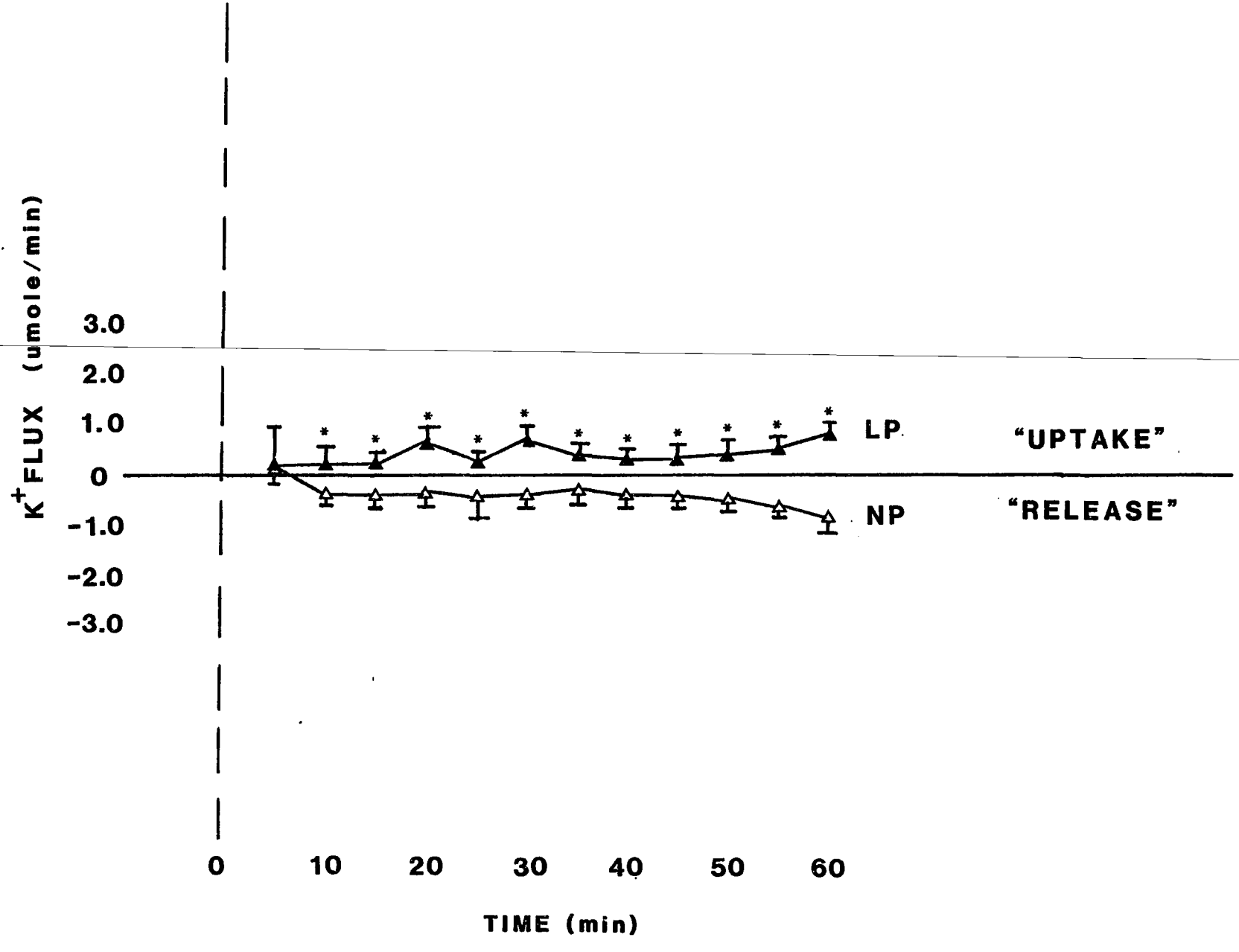
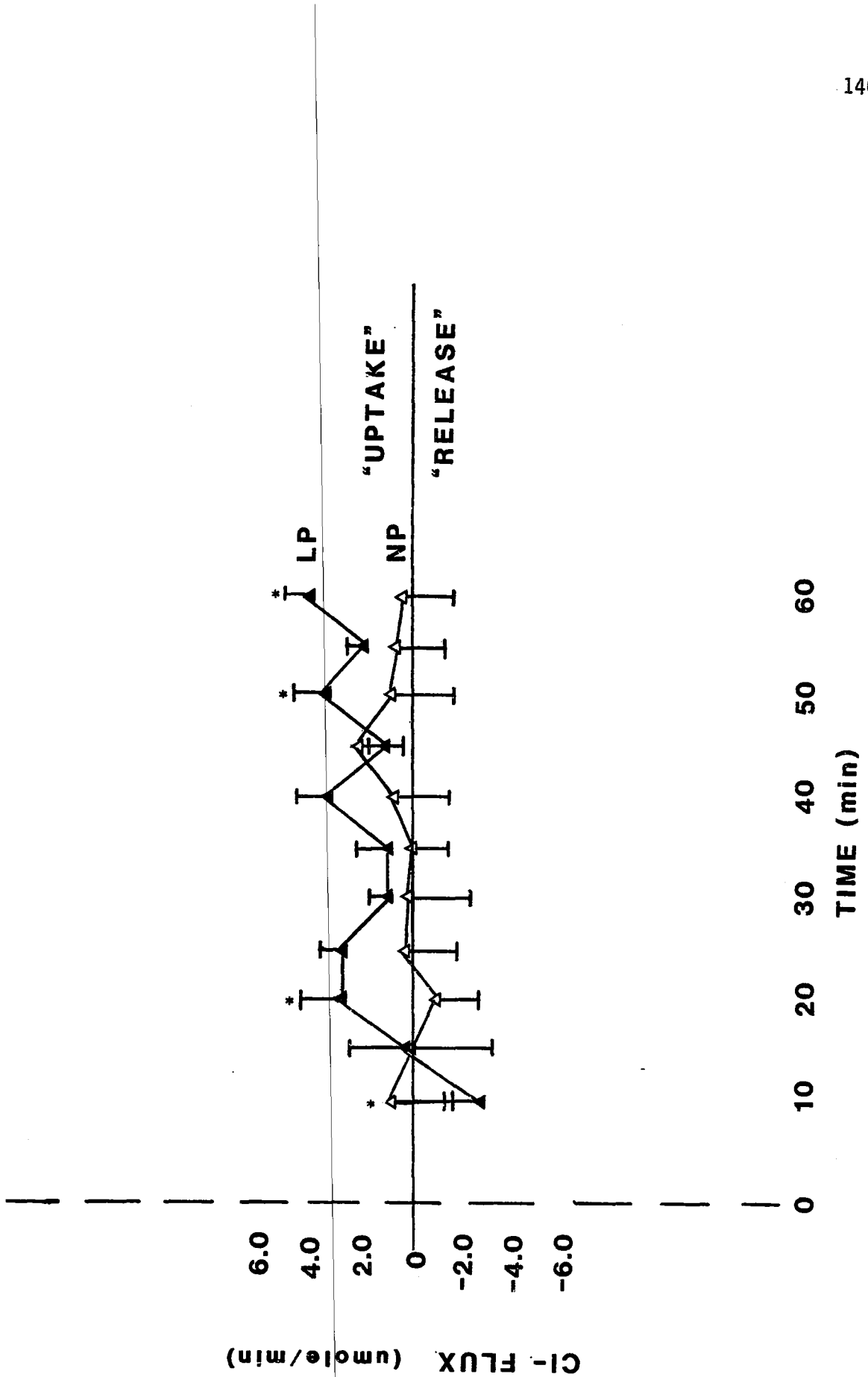

K^+ FLUX (umole/min)
3.0
2.0
1.0
0
-1.0
-2.0
-3.0
LP
NP
"UPTAKE"
"RELEASE"
0
10
20
30
40
50
60
TIME (min)

Figure 4.3 Chloride flux across inactive rat hindlimb in normal (NP) and lactacidotic (LP) perfusate groups.
* Indicates significant difference between groups.


LP
NP
"UPTAKE"
"RELEASE"
TIME (min)
0
10
20
30
40
50
60
6.0
4.0
2.0
0
-2.0
-4.0
-6.0
Cl- FLUX (umole/min)

4.4.4 Non-Volatile H^+ Flux

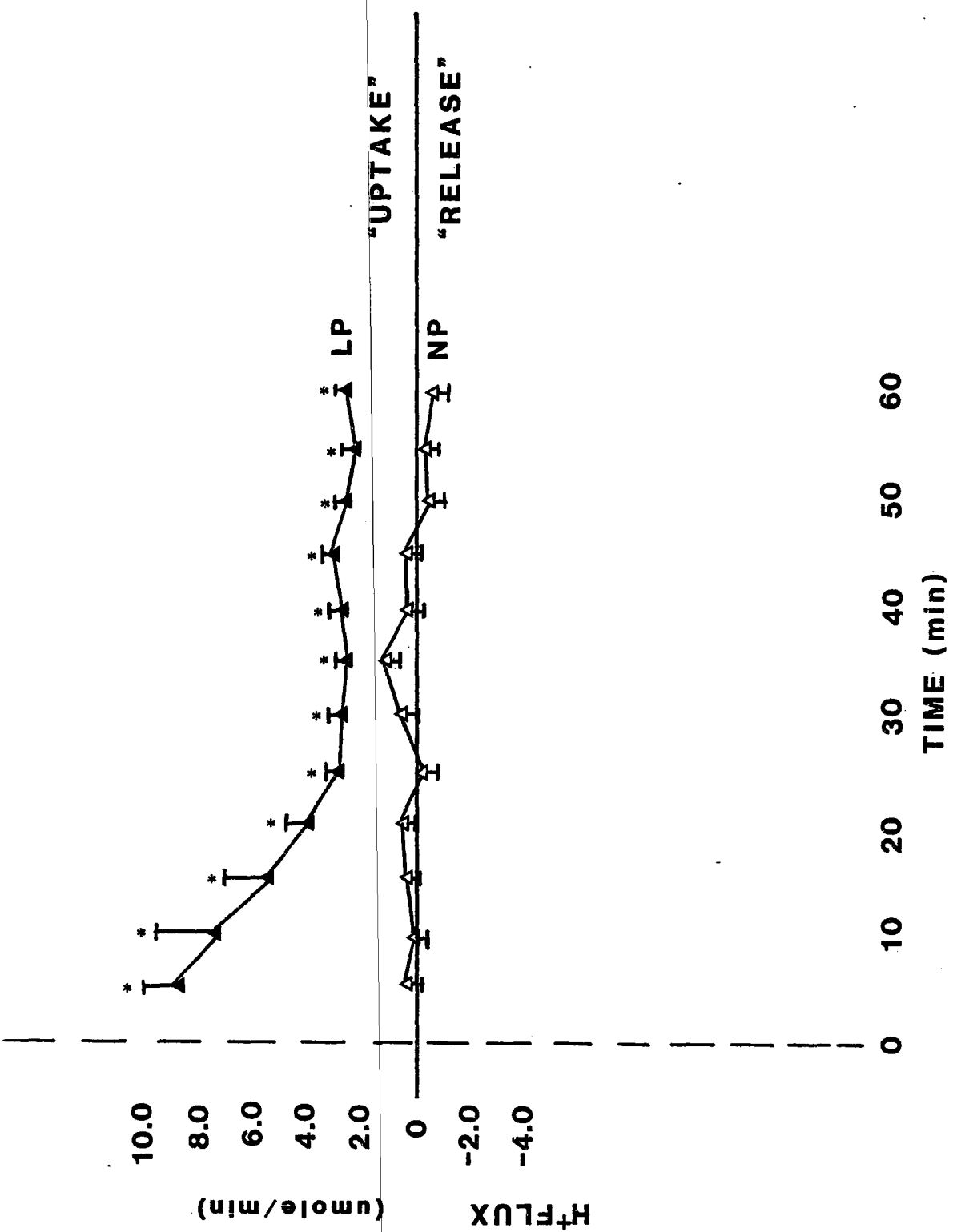
Hydrogen ion "uptake" by the inactive muscle occured at a high initial rate (7.2-8.9 umole·min^{-1}) from 5-10 min of LP perfusion. This rapid influx then reached a steady influx rate between 2.2 and 3.0 umole·min^{-1} between 25 and 60 min. The average rate of H^+ appearance was 3.9 + 0.3 umole·min^{-1} in the LP group, a value significantly higher than that of 0.06 + .12 umole·min^{-1} in the NP group.

During normal perfusion, there was essentially no net exchange of non-volatile protons, whereas perfusion with a lactacidotic medium resulted in an uptake of H^+ by the hindlimb muscles. Post-hoc analyses indicated that the rate of H^+ flux across the inactive muscle remained significantly above that of the NP at all respective time points between 5 and 60 min.

4.5 DISCUSSION

The present study has quantitatively determined the changes in strong ions (Na^+, K^+, Cl^- and La) across inactive skeletal muscle in response to perfusion with a lactacidotic medium. The latter was intended to simulate the metabolic and ionic changes found in arterial blood following high intensity exercise. The data obtained suggest that a Na^+/H^+ and HCO_3^-/Cl^- exchange mechanisms were involved in La and H^+ regulation by the inactive muscle. It has also been postulated that La movement across the membrane occurred by a number of transport mechanisms including HLa diffusion, H^+/La co-transport and possibly by La/Cl^- exchange. In addition, Na^+-K^+ ATPase activity in the sarcolema may have been increased to regulate the elevated levels of extracellular K^+. The interaction of various ionic regulatory mechanisms that may be involved in inactive skeletal muscle removal of La,

Figure 4.4 Non-volatile H + flux across inactive rat hindlimb in normal (NP) and lactacidotic (LP) perfusate groups.
* Indicates significant difference between groups.


"UPTAKE"
"RELEASE"
LP
NP
H+FLUX
(umole/min)
10.0
8.0
6.0
4.0
2.0
0
-2.0
-4.0
0
10
20
30
40
50
60
TIME (min)

non-volatile H^+ and K^+ during recovery from fatigue are discussed.

4.5.1 Methodological Considerations

The isolated perfused rat hindlimb model utilized in the present investigation allowed tightly controlled measurements of ionic flux across inactive skeletal muscle. Hormonal factors, absent from both NP and LP perfusates, may have altered the normal physiological response which would have occured in vivo. The absence of catecholamines and insulin may have altered rates of ionic movement. Catecholamines are known to enhance Na^+-K^+ ATPase function (Clausen, 1983, Clausen, 1985) while insulin has been observed to increase cell hyperpolarization by increasing the relative permeability of K^+ compared to Na^+ (Zierler et al, 1986, Wu and Zierler, 1986). Other ionic exchange mechanisms may also have been altered, although the effects of hormonal factors on these protein exchange mechanisms are not presently known. Elimination of hormonal contributions has allowed analyses of adaptations in ion flux across skeletal muscle membrane induced solely by altered strong ion concentration and/or $[H^+]$.

In addition to the strong ions measured in the present investigation, two other strong ions, Mg^{++} and Ca^{++}, make up the strong ion difference (Stewart, 1981, 1983). Mg^{++} and Ca^{++} flux, however, were not analyzed. Ca^{++} exchange across the muscle was not measured due to previous observations that little net change in whole blood $[Ca^{++}]$ occurs during exercise and/or recovery (Lindinger, personal communication). Furthermore, it is the subcellular distribution of Ca^{++} that would be of particular interest in determining the role of Ca^{++} in fatigue and recovery. Current methodology limits this type of in-depth analysis. Mg^{++} flux was not analyzed, again due to methodological limitations. Atomic absorption spectrophotometry, the most

accurate method for blood Mg^{++} determination (Lindinger, 1987), was unavailable. Previous studies have indicated that a large portion of intramuscular Mg^{++} appears to be bound to proteins or phosphate esters (Maughan and Recchia, 1985), and therefore this ion is not considered to be important in muscle ion regulation.

For the analyses of the present data, the assumption was made that calculated rates of ionic exchange across the hindlimb muscles represented net ionic movements across the muscle membrane. It has been recognized that movement of an ion must occur across the capillary as well as across the muscle cell membrane. Previous work (Hirche et al, 1975) has indicated that the muscle membrane may be the rate limiting barrier in ionic flux of La· It has therefore been assumed that the ionic composition of the extracellular compartment can be represented by the ionic composition of arterial blood supply and that arterio-venous difference represented ionic movement across the muscle fiber membrane.

Other techiques used for more precise measurements of trans-sarcolema ionic flux include ^{1}H-NMR (used by Seo, 1984), and H^{+} and La-sensitive electrode probes (used by Mainwood and Renaud, 1984, Renaud and Mainwood, 1985). These techniques were not available for the present investigation. Total tissue ion content, as measured by instrumental neutron activation analysis (Lindinger and Heigenhauser, 1987), was determined in the tissues obtained from these experiments. These values were utilized for calculation of intracellular ion concentrations, however these data will not be presented within the scope of this thesis.

4.5.2 Mechanisms of Na^{+}, K^{+} and Cl^{-} Flux

Net transmembrane movement of the inorganic ions, Na^{+}, K^{+}, and Cl^{-},

were negligible during 60 min of NP perfusion of the inactive hindlimb muscles. These findings suggest maintenance of resting muscle concentration gradients for these ions, and therefore, maintenance of a stable membrane potential. Na^+-K^+ ATPase activity is essential for the electro-chemical gradient, and for optimum muscle function (Clausen, 1986). The NP perfusion results indicate that basal activity of the Na^+-K^+ ATPase pump does occur in the present model despite lack of catecholamines and insulin in the perfusate.

During perfusion with the lactacidotic perfusate, several adaptations in net ionic movement across the inactive GPS muscle group were observed. A net influx of K^+ and La were found throughout the 60 min perfusion period, while fluctuating periods of net influx were observed for Na^+ and Cl^-. The observed patterns for Na^+ and Cl^- flux are partially attributed to the high degree of between animal variability.

Net K^+ removal from blood following exercise-induced hyperkalemia has previously been observed from measurements of reduced plasma [K^+] during recovery (Medbo and Sejersted, 1985, Knochel et al, 1985). Uptake of K^+ by previously active (McKelvie et al, 1987) and inactive (Heigenhauser et al, 1987) skeletal muscle during exercise recovery has also been observed. The present findings are in agreement with these reports. Net K^+ removal during recovery from exercise suggests that extracellular [K^+] is highly regulated by skeletal muscle in an attempt to prevent membrane hypopolarization. Several mechanisms have previously been postulated for the ionic regulation of extracellular [K^+], including a K^+/H^+ pump, a Na^+/K^+ pump, and increased membrane K^+ permeability.

Early suggestions of a K^+/H^+ exchange mechanism (Gardner et al, 1952, Waddell and Bates, 1969) have been argued against by the more rigorous

experimental observations of Aicken and Thomas (1977) which have suggested that this mechanism is not involved in muscle pH_i regulation. Increased membrane permeability to K^+, relative to that of Na^+, has been suggested to be one of the factors responsible for insulin-induced membrane hyperpolarization (Wu and Zierler, 1985). Lack of perfusate insulin in the present investigation would minimize the contributions of this mechanism to cellular K^+ regulation. Thus, the increased K^+ influx into inactive skeletal muscle would appear to be due to increased Na^+-K^+ ATPase activity. Lack of catecholamines in the perfusate would have prevented hormonal stimulation, thus the observed increase in pump activity must have been induced by the elevated extracellular [K^+] (7.88 mMol). Concentrations of Na^+ and K^+ on either side of the membrane, along with ATP supply, have been cited as key regulators of Na^+-K^+ ATPase activity (Clausen, 1986).

Observations of net Na^+ influx during LP perfusion would not be expected based on the proposed mechanism for net K^+ uptake by the inactive muscle. Sodium movement into the cell may, however, have occurred by Na^+/H^+ exchange (Aickin and Thomas, 1977, Galler and Moser, 1986). If muscle pHi regulation was activated by an increase in intracellular H^+, then Na^+/H^+ exchange would have resulted in Na^+ influx. The net inward movement of Na^+ would therefore represent the balance between the outward flux of Na^+ induced by Na^+-K^+ pump activity and the inward flux of Na^+ from Na^+/H^+ exchange.

The ionic exchange of Na^+ indicated a rapid initial efflux at 5 min, and a steadily increasing rate of influx between 15 and 60 min (see Figure 4.1). Analysis of the time course for these changes in Na^+ flux indicated that net flux may have been governed by Na^+-K^+ ATPase activity early during LP perfusion, and then by Na^+/H^+ exchange during the latter stages of

perfusion.

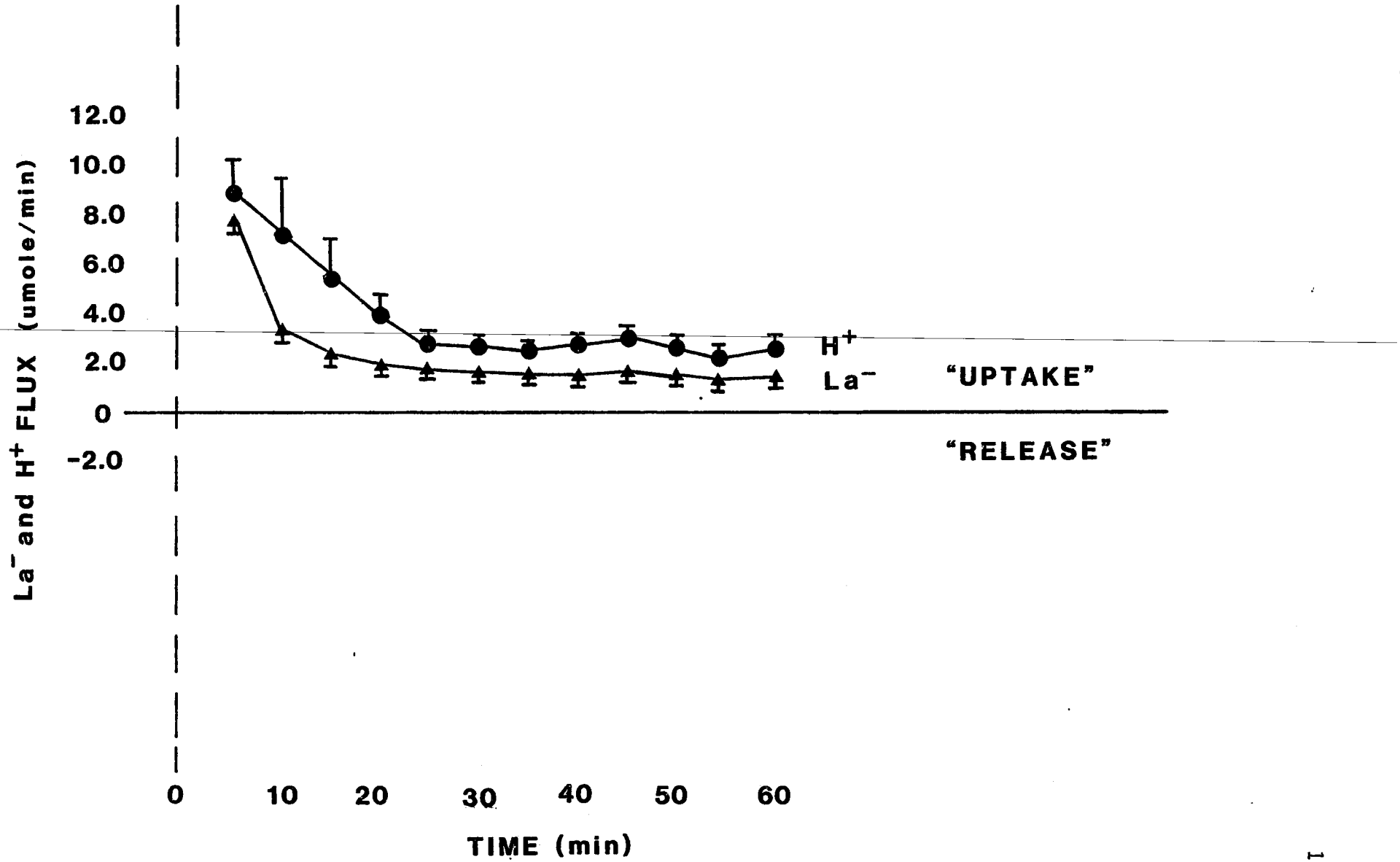
Transmembrane movement of Cl^- was increased towards the final 15 min of perfusion. Inward movement of Cl^- would be expected based on previous observations of net HCO_3^- efflux (see Chapter 3, Figure 3.3) and observations by Aickin and Thomas (1977) and Galler and Moser (1986) of a HCO_3^-/Cl^- exchange mechanism in skeletal muscle. Time course analyses for HCO_3^- efflux and Cl^- influx, however, do not coincide, with Cl^- influx being less from 0-45 min than would be predicted by its stoichiometric exchange with HCO_3^-. Outward movement of Cl^- in exchange for another anion, possibly La, may explain the decreased influx of Cl^- over the initial 45 min of LP perfusion.

4.5.3 Mechanisms of Lactate Influx

In the present study, the rapid initial uptake of both La and non-volatile H^+ by the inactive skeletal muscle (see Figure 4.5) suggested that either HLa diffusion and/or La/H^+ co-transport occurred between 5 and 55 min of perfusion. These findings are in agreement with observations of stoichiometric movement of La and H^+ out of exercising muscle (Wolosin and Ginsberg, 1975, Hirche et al, 1975, Mainwood et al, 1972, Mainwood and Worsley-Brown, 1975, Seo, 1984, Mason et al, 1986). Although the direction of transmembrane ion movement would be the reverse of that observed in exercising muscle, the stoichiometry appears to be preserved. Due to differences in electro-chemical gradients between influx and efflux across muscle fiber membranes, the similarity of the two mechanisms has been questioned.

Translocation of La out of muscle is thought to occur mainly in its ionic form due to the complete dissociation of HLa at an intramuscular pH of

Figure 4.5 Lactate and non-volatile H + flux across inactive rat himdlimb in lactacidotic perfusate group.


12.0
10.0
8.0
6.0
4.0
2.0
0
-2.0
La⁻ and H⁺ FLUX (umole/min)
H⁺
La⁻
"UPTAKE"
"RELEASE"
0
10
20
30
40
50
60
TIME (min)

6.8-7.0 or a plasma pH of 7.4. La translocation may occur by either passive or active mechanisms, however passive distribution across biological compartments with different electrical potentials can only occur when there is net energy release (Hultman and Sahlin, 1980). At a resting muscle membrane potential of 88mV, passive movement of La inward (ie. free energy release of zero) would require extracellular [La] to be 27-fold higher than intramuscular [La] (see Hultman and Sahlin, 1980 for calculations), values which normally do not occur in vivo and which were not found in the present investigation. Thus, passive inward diffusion of La did not likely occur in its ionic form.

It has been argued that passive diffusion of the ionic form of La can only occur out of the muscle. The electro-chemical differences between intramuscular and extracellular compartments would require different mechanisms for La efflux versus influx across skeletal muscle membranes. HLa diffusion, however, can occur passively in either direction (Hultman and Sahlin, 1980). The translocation of La into the inactive muscle cell, as observed in the present study, could therefore have occurred by passive diffusion of the HLa molecule but not by passive diffusion of the La ion. It may also have occurred by carrier-mediated transport of La and H^+.

Several investigators have found evidence to suggest that a carrier-mediated transport mechanism exists in skeletal muscle (Kuret et al, 1986, Mason et al, 1986, Juel, 1987). Juel (1987) recently concluded that more than 50% of the La efflux observed from mouse soleus muscle stimulated in vitro appeared to be removed by a carrier mechanism for La and H^+ co-transport. The results of the present study suggest that this active transport mechanism was activated in the reverse direction under conditions of lactacidosis.

4.5.4 Mechanisms of Non-Volatile H^+ Influx

Intramuscular pH of resting skeletal muscle has been observed in the range of 6.8-7.1 (Hultman et al, 1985, Mainwood and Renaud, 1985), and normal plasma pH approximately 7.4 (Hermansen and Osnes, 1972). During exercise these values may reach 6.4 and 6.9-7.1 for muscle and blood respectively (Hermansen and Osnes, 1972). At rest and during exercise, intramuscular [H^+] remains several orders of magnitude above extracellular [H^+], resulting in a basal concentration gradient for H^+ across the sarcolemma, with intracellular concentrations higher than extracellular.

A second skeletal muscle transmembrane H^+-gradient exists which keeps the intracellular compartment one pH unit more alkali than expected by passive distribution of H + ions according to the electrical potential of the sarcolemma (Caldwell, 1954, Aickin and Thomas, 1977). Maintenance of this transmembrane H^+-gradient has been found to be dependent on the energy state of the muscle (Hagberg, 1985), and to be oubain sensitive (Roos, 1975). These studies indicate that an ATPase pump is involved. It has been suggested (Aickin and Thomas, 1977, Galler and Moser, 1986) that a Na^+/H^+ exchange mechanism is involved and that it functions to regulate intracellular pH by continually removing H^+ from the muscle.

The transmembrane movement of non-volatile H^+ into inactive skeletal muscle therefore involves ionic flux against a concentration gradient, but along an electrical gradient for H^+. Activation of a pHi regulatory mechanism by the proposed Na^+/H^+ exchange would result in removal of non-volatile H^+ following HLa inward movement. This mechanism would also account for the observed influx of Na^+.

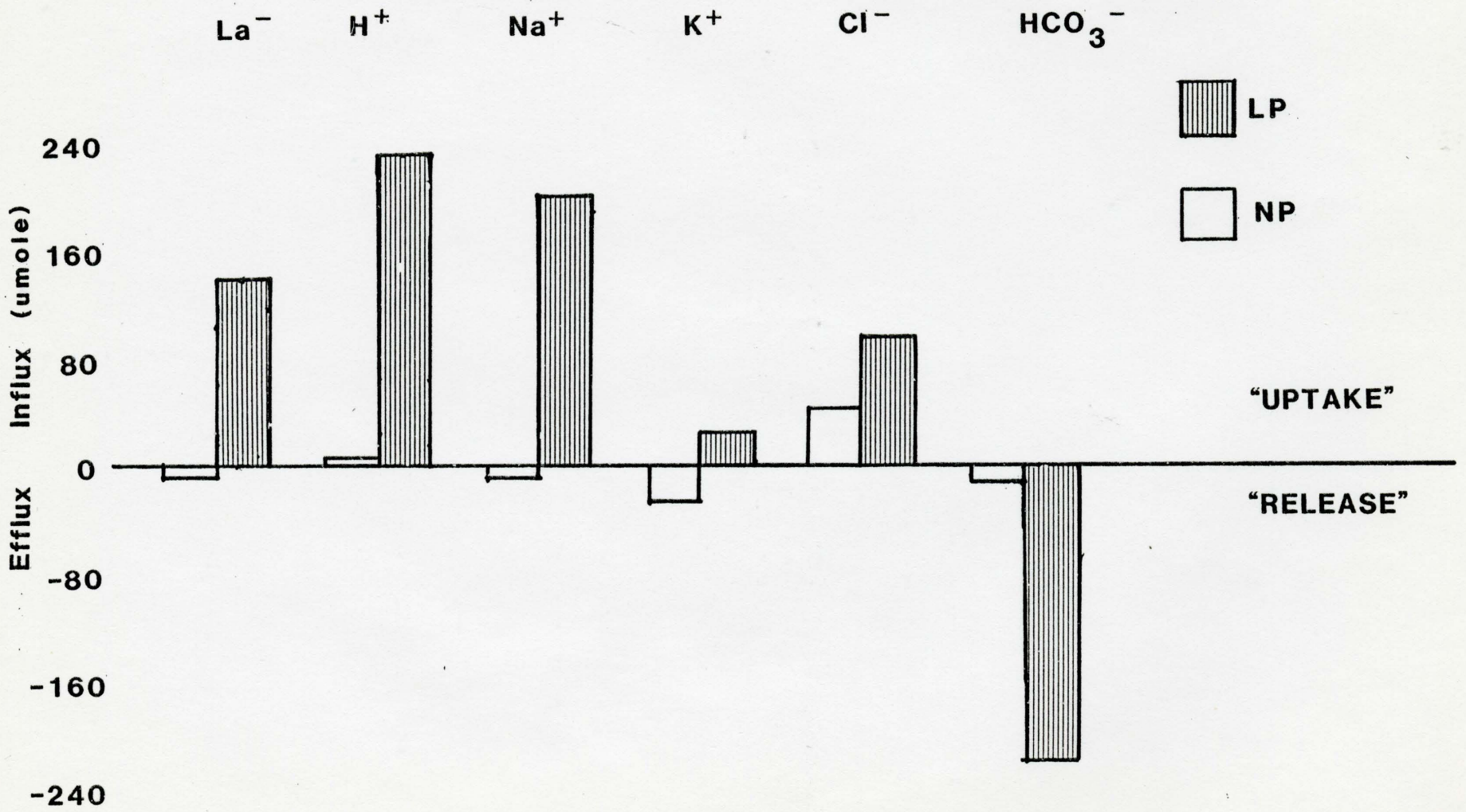
4.5.5 Ionic Regulation in Inactive Skeletal Muscle Under Lactacidotic Conditions

Investigation of ion regulation by inactive skeletal muscle under conditions simulating recovery from high intensity exercise has revealed a number of integrated adaptations in sarcolemma ion flux. In response to perfusion with a medium consisting of an 11.0 $mMol \cdot l^{-1}$ [La] and pH 7.15, a rapid inward diffusion of La (most likely as an HLa molecule) occurred. Mechanisms regulating intracellular pH, mainly Na^+/H^+ and HCO_3^-/Cl^- exchange appear to have been activated by the extracellular-induced acidosis. As a result, intracellular concentrations of Na^+ and Cl^- increased and HCO_3^- decreased. Results from muscle ion measurements have confirmed the appropriate changes for intramuscular Na^+ and HCO_3^-, but not for Cl^- (Galea et al, 1987). This again supports the proposed La/Cl^- exchange mechanism. The pattern of Cl^- flux observed (Figure 4.3) may reflect Cl^- equilibrium between the influx from Cl^-/HCO_3^- exchange, and efflux from La/Cl^- exchange.

In an attempt to regulate elevated levels of extracellular K^+, increased Na^+-K^+ ATPase pump activity and/or increased permeability to K^+ resulted in net K^+ influx. With the present muscle preparation, the first of these two adaptations most likely occured. Enhanced Na^+-K^+ ATPase activity would have helped to maintain muscle membrane potential.

From Figure 4.6, it can be seen that cumulative H^+ influx was greater than cumulative La influx. Although a large influx of non-volatile H^+ occured, calculated muscle [H^+] did not indicate a significant increase in intramuscular H^+ levels (Galea et al, 1987). This would suggest that a relatively large proportion of the non-volatile H^+ moving into the intramuscular compartment was buffered by muscle HCO_3^-. This would have resulted in elevated muscle CO_2 release, as previously observed (Chapter 3).

Figure 4.6 Cumulative ion flux for lactate (La-), non-volatile H+, sodium (Na+), potassium (K+), chloride (Cl-), and bicarbonate (HCO3-) between 5 and 60 min of lactacidotic (LP) and normal (NP) perfusion.


La⁻
H⁺
Na⁺
K⁺
Cl⁻
HCO3⁻
LP
NP
240
160
80
0
-80
-160
-240
Efflux Influx (umole)
"UPTAKE"
"RELEASE"

Previous investigations utilizing increased PCO_2 (Adler, 1965a) and decreased [HCO_3^-] (Adler, 1965b) similarly observed only minimal changes in intracellular [H^+] in response to an extracellularly-induced acidosis.

Total HCO_3^- release between 5 and 60 min of LP perfusion was 220 umole while total H^+ "uptake" during the same time period was 232 umole, indicating a 1:1 relationship between H^+ removal and HCO_3^- release. Although it cannot be concluded that all H^+ was removed via buffering by HCO_3^-, the equimolar exchange of H^+ and HCO_3- does suggest some direct or indirect relationship between the two observations. If most of the non-volatile H^+ removed by the inactive muscle was buffered by HCO3-, little H^+ (approximately 12 umole) would have remained as a free proton. Activation of a Na^+/H^+ exchange mechanism may not have been required for intracellular pH regulation. Inward flux of Na^+ would therefore have occured for other purposes.

Net influx of cations ([Na^+] + [K^+] + [unbuffered H^+]) and of anions ([La] + [Cl^-]) was 237 and 234 umoles respectively. Due to the equimolar inward movement of cations and anions, the net electrical charge of the intracellular environment, and therefore resting membrane potential, would appear to have been maintained. Thus, net inward flux of Na^+ in response to LP perfusion may have occured in order to maintain the electrical state of the muscle fiber membrane. Whether or not Na^+ influx was active-mediated or occured by passive diffusion could not be determined in the present study.

4.5.6 Ion Regulation and Acid-Base Status

Intracellular acid-base regulation by inactive skeletal muscle in response to an extracellular lactacid load must be considered when discussing removal of La and its associated H^+ ion. Current physico-chemical

approaches to the understanding of acid-base status (Stewart, 1981, Stewart 1983, Heigenhauser et al, 1986, Lindinger, 1987) attempt to integrate ionic regulation of La with other physiological strong ions, as well as weak acids and PCO_2 which exist in muscle tissue. As the present investigation has demonstrated, analysis of the various ionic changes occuring across skeletal muscle may help to explain isolated segments of an integrated system. Mechanisms of La and H^+ flux into inactive skeletal muscle should be considered along with mechanisms of intracellular H^+ regulation for a more complete understanding of intracellular ionic regulation in response to an extracellular acidosis.

According to Stewart's model for H^+ (and therefore pH) determination, proton release in any biological compartment is dependent upon the PCO_2, the total concentration of weak acids, and the net charge of all strong ions in that compartment. Therefore, H^+ may not necessarily move across a muscle membrane per se, but may only be released or consumed in the association/dissociation of other chemicals within either compartment. Apparent H^+ efflux may result from the movement of other strong ions across the muscle membrane by various exchange mechanisms. This view directly opposes the postulated Na^+/H^+ exchange mechanism (Aickin and Thomas, 1977, Galler and Moser, 1986), since the latter mechanism would imply direct H^+ translocation across a membrane barrier. Thus, controversy continues to exist regarding the precise mechanisms of muscle pH regulation, and therefore its regulation under conditions of lactacidosis.

4.6 CONCLUSION

Ionic movements across inactive skeletal muscle, as would be observed

following maximal exercise, was investigated using an isolated perfused rat hindlimb model. Perfusion of the inactive muscle was performed with a normal and a lactacidotic medium. The latter was developed to simulate metabolic and ionic changes previously observed in arterial blood following exercise. Measurement of strong ion flux across the hindlimb gave insight into the possible mechanisms of La, K^+ and non-volatile H^+ regulation by inactive skeletal muscle. From this study it has been concluded that La may be removed across inactive muscle by HLa diffusion, La/H^+ carrier-mediated transport, and/or La/Cl^- exchange. Intracellular H^+ regulation appears to have been accomplished by Na^+/H^+ and HCO_3^-/Cl^- exchange and/or HCO_3^- buffering and CO_2 release. It is hypothesized that extracellular K^+ regulation was primarily accomplished by increased Na^+-K^+ ATPase activity. The net ionic shifts suggest that a tightly regulated system exists for muscle electrical and chemical properties. Finally, it is concluded that mechanisms of La and H^+ flux, and mechanisms of intracellular [H^+] regulation following an extracellularly-induced acidosis may differ from proposed mechanisms for intramuscular regulation of an intracellular-induced acidosis.

CHAPTER 5 GENERAL SUMMARY

5.1 INTRODUCTION

Decreased muscle performance at high work intensities has primarily been attributed to lactic acid production and accumulation (Hermansen, 1981, Donaldson, 1983, Hultman and Sjoholm, 1986). Recent work has suggested that the strong ion, La, is the single most important variable in determining muscle [H^+] during and following high intensity exercise (Kowalchuk, 1985, Lindinger, 1987). Recovery from muscle fatigue appears to be dependent on extracellular [H^+] (Mainwood and Renaud, 1985, Renaud and Mainwood, 1985), and therefore must also be dependent upon extracellular [La]. It has also previously been suggested that inactive skeletal muscle plays a major role in La removal and recovery from maximal exercise (Poortmans et al, 1978, Freyschuss and Strandel, 1967, Kowalchuk, 1985). Removal of extracellular La by non-exercising tissues would facilitate La, and thus H^+, removal from working muscle, thereby contributing to recovery from a fatigued state.

The ionized form of La is both a strong ion and a metabolite. As a strong ion, La must be transported across membrane barriers and will interact in physiological solutions as governed by laws of physical chemistry (see Stewart, 1981, 1983). As a metabolite, La is a substrate that may enter various metabolic pathways. Therefore, in order to understand the mechanisms by which La is removed by inactive skeletal muscle, consideration should be given to both the physico-chemical (ionic) and biochemical (metabolic) patterns of La elimination.

Previous research in the area of La removal has focused on La and H^+ efflux or on metabolic elimination of La during and following high intensity

exercise. Hultman and Sahlin (1980), in a review on acid-base regulation, have attempted to link these aspects of La elimination. Most studies dealing with mechanisms of La removal have examined its disappearance from active skeletal muscle, and have not considered the fate of La following circulation in plasma. In-depth analyses of both ionic and metabolic aspects of La removal by inactive skeletal muscle have not previously been attempted. This thesis has examined the mechanisms by which La is transported into the intramuscular compartment and the mechanisms by which it is metabolically eliminated in inactive skeletal muscle. From this data, speculations have been made regarding the contributions of inactive muscle to decreasing extracellular [La] and [H^+], and therefore to recovery from fatigue.

5.2 RELATIONSHIP BETWEEN IONIC AND METABOLIC ELIMINATION OF LACTATE BY INACTIVE SKELETAL MUSCLE

5.2.1 Overview

The three previous chapters have dealt independently with the isolated mechanisms of La elimination. It was concluded (Chapter 2) that a large proportion (86%) of the La removed by inactive skeletal muscle of the lactacidotic perfused rat hindlimb was metabolically eliminated. Of this 86% metabolized, 12-33% could be accounted for by oxidative metabolism and 5-7% could be accounted for by the observed glycerol release (Chapter 3). It has been hypothesized that the remaining 67-88% which was unaccounted for may have been involved in metabolic cycling along the glycolytic/glyconeogenic and/or TG metabolizing pathways. Increased CO_2 output from the inactive muscle was found not to be indicative of FFA synthesis, but rather to

reflect HCO3- buffering of the increased intramuscular H^+. The various mechanisms by which La and H^+ may have been transported across the muscle membrane into the intramuscular compartment of inactive muscle has been discussed (Chapter 4). Various ionic regulatory mechanisms appear to be involved in La and H^+ transport, resulting in a net inward flux of Na^+, K^+, H^+, Cl^- and La and a net outward flux of HCO_3^-. Adaptations in intramuscular metabolic activity, as a result of these ionic disturbances, will now be considered. Conversely, alterations in ionic transport of La and H^+, as a result of metabolic elimination of La, will also be considered.

5.2.2 Effect of Ionic Disturbances on Intracellular Lactate Metabolism

Intense exercise of a 30-60 sec duration results in several intra- and extracellular electrolyte disturbances (Sjogaard, 1985, Heigenhauser et al, 1986), which have been linked to muscle fatigue. Glycolytic production of La, and the resultant decreased [SID] and increased [H^+] have also been linked to a 2 to 4-fold increase in the ratio of undissociated:dissociated weak acids (proteins) (Lindinger, 1987). Changes in the ionized state of proteins in solution (contractile, enzymatic, and structural) may result in altered enzymatic structure and therefore function.

Previous investigations have attributed altered enzymatic function during exercise to the direct effects of H^+ on protein structure (Trivedi and Danforth, 1966, Woodbury, 1974). It is currently thought that H^+ and enzymatic structure and function are co-related but that they do not necessarily reflect a cause-effect relationship. Changes in other strong ions (Na^+, K^+, La, Cl^-) in the cell may be the common factor linking altered enzyme function to [H^+] (Lindinger, 1987). In general, it has been found

that monovalent cations play important regulatory roles in enzyme structure (Mildvan, 1974), with Na^+ inhibiting and K^+ stimulating enzymatic activity within the cell. Since K^+ is one of the principal intracellular monovalent cations, with concentrations reaching up to 150 miliequivalents per liter, it is of physiological significance that K^+ is an important regulator of enzymes such as fructose-1,6-bisphosphatase (FBP) (Nakashimi and Tuboi, 1976).

In the present investigation it was found that both Na^+ and K^+ entered the inactive muscle, with net influx for Na^+ (200.6 umoles) being much greater than net K^+ influx (23.9 umoles). It has previousiy been postulated that K^+ is required for glyconeogenesis (Hultman, 1967, Patrick, 1977). Increased influx of K^+ into the inactive muscle during lactate perfusion would be expected to have a stimulatory effect on muscle glycogen synthesis. Investigations demonstrating increased muscle K^+ in association with increased muscle glycogen concentration have utilized experimental models in which initial glycogen levels were depleted prior to resynthesis. Failure to demonstrate net glycogen synthesis in the present investigation has indicated that a metabolic factor (product level) appears to have been more important in regulating glycogen synthesis than an ionic factor (cation concentration) under the conditions observed.

Potassium concentration has also been associated with glycolytic energy production. Hultman (1967) observed an association between glycogen breakdown and K^+ release from skeletal muscle. It has previously been shown that optimal K^+ concentrations are required for maximal activation of glycolytic enzymes pyruvate kinase (Boyer et al, 1943), and PFK (Paetkau and Lardy, 1967), and also of FBP, an enzyme in the glyconeogenic pathway (Hubert et al, 1970). Since both PFK and FBP are sensitive to $[K^+]$, slight

changes in intracellular K^+ will alter rates of glycolytic/glyconeogenic flux by regulating substrate cycling at the F-1,6-diP <---> F-6-P level. The causative factor for the hypothesized increased substrate cycling at the F-1,6-diP <---> F-6-P level of the glycolytic/glyconeogenic pathway may have been the increased intramuscular [K^+]. Thus, increased K^+ efflux into inactive muscle, as observed in the present investigation, may explain the fate of the La unaccounted for by oxidation or glycerol release. The elevated rate of substrate cycling may have increased the rate of carbon flux which therefore resulted in increased utilization of La.

Previous studies have linked monovalent cation concentration to the activation of key glycolytic and glyconeogenic enzymes (Mildvan, 1974, Nakashimi and Tuboi, 1976), however similar analyses have not been documented for enzymes involved in TG lipolysis and esterification. The possible effect of ionic disturbances on glycerol/FFA substrate cycling in inactive muscle is therefore unknown.

5.2.3 Effects of Metabolic Elimination of La on Ionic Transport

Elimination of La may occur via several metabolic pathways (see Figure 1.4). Metabolic elimination of La along any of these pathways represents a mechanism for permanent disposal of an acid. Since this strong ion is organic, and capable of serving as a substrate in biochemical reactions, it is the only anion that may permanently be removed from solution. Disposal of La eliminates an anion from the intracellular compartment, which in itself will alter [SID] and therefore [H^+].

Metabolic elimination of La may alter rates of ionic flux for La and its associated ionic species (Cl^-, HCO_3^-, and H^+), due to the resultant decrease in intracellular La and HLa concentration. Continual elimination of

La would help to maintain an inward concentration gradient for HLa diffusion and possibly for La/H + cotransport. Maintenance of an inward concentration gradient would ensure maximal uptake of extracellular La by inactive skeletal muscle and thus continual removal of La from the active muscle.

Increased plasma La clearance has been shown to have a positive effect on recovery from muscle fatigue. This effect has been observed during active recovery following maximal exercise (Belcastro and Bonen, 1975, Boileau et al, 1983, Dodd et al, 1984) as well as following endurance training (Donnovan and Brooks, 1983). Rate of La clearance would ultimately be self-limiting if ionic transport and metabolic utilization did not function to permanently dispose of La in peripheral tissues. It is suggested that the two procsses, ionic transport and metabolic elimination, function simultaneously to provide an optimal mechanism for La removal from plasma and therefore for recovery from fatigue.

5.2.3 Summary of the Metabolic and Ionic Relationship for Lactate Removal

Both biochemical and physico-chemical status of muscle appear to be important in determining its functional capacity. Mechanisms involved in regulating enzyme structure and function for the metabolic elimination of intramuscular La may be directly altered by ionic disturbances created by La transport. Consideration of the mechanisms for La and H^+ transport (ie. Na^+/H^+, La/Cl^- exchange) may indicate co-related movements of strong ions into the intracellular compartment of the inactive muscle. It is these co-related ionic changes that may alter enzymatic function and therefore biochemical reactions involved in La metabolism. Conversely, the regulation of extracellular ionic disturbances may be limited by the rate of metabolic

clearance of La in the intracellular space.

5.3 CONCLUSION

Both the metabolic elimination and ionic transport of La should be considered when examining mechanisms of La removal and subsequent recovery from muscle fatigue. Investigation into adaptations in carbohydrate (Chapter 2) and triacylglycerol (Chapter 3) metabolism has indicated the role of specific metabolic pathways for La elimination in inactive skeletal muscle of the rat hindlimb. Examination of concurrent ionic exchange across the hindlimb has given insight into transport mechanisms for La and H^+, and mechanisms for intracellular ionic regulation in response to an extracellular-induced acid load. The net effect of the ionic movements across the inactive hindlimb has been considered both as a result of metabolic La clearance and as a cause for the preferred metabolic pathways for La elimination.

From this investigation it can be concluded that, in inactive muscle of the isolated rat hindlimb perfused for 60 min with a lactacidotic perfusate, patterns of La uptake and metabolic elimination are different from those previously observed for active muscle. Metabolic clearance of La in the inactive muscle involves oxidative removal, glycerol release, and substrate cycling along the glycolytic/glyconeogenic and TG lipolysis/re-esterification pathways. The metabolic fates of La apper to be linked to the ionic disturbances associated with La and H^+ influx into inactive muscle.

5.4 FUTURE DIRECTIONS

Results of the current investigation have indicated that several avenues exist for subsequent research adressing the question of lactate removal by inactive skeletal muscle. Two major avenues for future investigation will be outlined, the first dealing with *in vitro* and the second dealing with *in vivo* models of investigation. Rigorous investigation using an isolated *in vitro* muscle preparation would allow tightly controlled analyses of the isolated mechanisms involved in La removal. Utilization of an intact *in vivo* animal or human model would allow further investigation into the same problem, but with greater applicability to skeletal muscle recovery following intense exercise.

Utilizing an isolated rat hindlimb model, with an experimental design and protocol similar to those used in the present investigation, more in depth analyses of the metabolic adaptations occuring in inactive skeletal muscle in response to lactacidosis could attempt to determine the metabolic fate of the La removed. Perfusate analyses for FFA and alanine may give a clearer indication of the involvement of the FFA/TG substrate cycle, and of the alanine cycle, respectively, in La elimination. Use of specifically labelled ^{14}C lactate radioisotopes would allow quantification of the metabolic turover rates and therefore give a better indication of the hypothesized flux of La carbons through the glycolytic/glyconeogenic and TG metabolic pathways.

An isolated rat hindlimb perfusion model could also be utilized to investigate La transport and metabolism by inactive muscle of varying intra- and extracellular substrate concentrations. By increasing perfusate [La], and manipulating intracellular [La], investigation into the possible

saturation of La transport and La elimination processes could be carried out. Earlier work with previously exercised muscle has indicated that La efflux from muscle appears to be saturated at a rate of 4-5 mMol·min^{-1} (Jorfeldt et al, 1978). Whether or not the transport of La into inactive muscle is a saturable process is not presently known. Furthermore, by manipulating initial glycogen concentration (ie. through stimulation and/or diet restriction regimens) the role of pre-existing muscle glycogen levels on La conversion to glycogen could be evaluated in inactive muscle. The current investigation examined the possibility of glycogen synthesis in muscle at an extracellular [La] of 11.0 mMol.l-1, and the results have lead to the conclusion that substrate supply ([La] in the perfusate) and/or initial product level (muscle glycogen) may control muscle glycogen synthesis. More rigorous investigation is needed to accept or refute the current hypothesis.

Future research with an in vitro isolated rat hindlimb muscle preparation may attempt to create a more physiological condition by addition of hormones such as catecholamines, insulin and glucagon to the perfusate. Comparison of the effects of a hormone-free lactacidotic perfusate with those of a perfusate containing catecholamines, insulin and glucagon on inactive skeletal muscle metabolism and ionic flux may give some indication of the role of the adrenergic and endocrine systems in regulating recovery from fatigue. The use of channel blocking agents, such as SITS, an anion exchange blocker and oubain, an inhibitor of Na^+-K^+ ATPase activity, could give further insight into the role of specific ion transport mechanisms in La and H^+ transport into inactive muscle.

Utilization of an in vivo model to investigate the contributions of inactive muscle to recovery from high intensity exercise would allow greater

applicability of results to an athletic performance situation. Current observations indicate that the majority of La removed by inactive skeletal muscle is eliminated by various metabolic pathways. This suggests that this tissue plays an important role in removing La, and that inactive muscle can utilize La as an energy substrate. Subsequent measurement of the performance capacity of the inactive muscle would allow evaluation of the positive effects of La uptake (increased substrate avialability for ATP recycling) relative to the negative effects of an increased acidosis.

Previous studies have indicated that light intensity work (at approximately 32-35% of maximal oxygen uptake) during recovery from maximal exercise provides optimal conditions for La clearance by working muscle (Belcastro and Bonen, 1975, Dodd et al, 1984). The speculated mechanisms for this effect include increased blood flow and therefore increased transport to and utilization of La by other sites (ie. liver, heart, inactive muscle) (Belcastro and Bonen, 1975), as well as increased La uptake and oxidation by previously active muscle (Stanley et al, 1985). Lactate removal by inactive muscle appears to be an important process that may facilitate recovery from fatigue. Since low intensity, active recovery following high intensity exercise increases metabolic demand and therefore La oxidation, low intensity work superimposed on the inactive muscle may increase the contributions of this tissue to La clearance. Knowledge of the mechansisms for La transport and metabolic elimination allows speculation as to potentially useful recovery techniques that may lead to improvements in performance where La and/or H^+ accumulation appear to be a limiting factor.

A large gap exists between knowledge of mechanisms of La transport and utilization at the cellular level and effective techniques for La clearance during or following maximal exercise. Investigations of La removal by

inactive muscle in a tightly controlled isolated preparation, such as in the present study, can provide meaningful information for improved athletic performance if certain considerations are taken into account in interpreting in vitro investigations. The in vitro experimental model may also be considered as a preliminary research tool for which basic physiological principals can be established. Follow-up investigations can be designed using an in vivo human model to test the resultant hypotheses in a more practical and applied exercise condition. Through this approach, the gap between basic physiological research and applied knowledge in the sport sciences will hopefully be narrowed.

APPENDIX I

Calculations for Correction of Metabolite and Electrolyte Concentrations for Fluid Shift

The correction factor used to normalize venous metabolite and ion concentration for a constant volume was based on the ratio of arterial to venous hemoglobin concentration ([Hb]). Hemoglobin, a protein constituent in the blood, does not cross the capillary or sarcolema membrane. Thus, any changes in Hb concentration observed is due to a shift in fluid, and not to a shift in particulate content. Correction of venous metabolite and ion concentration has been made based on the amount of fluid leaving the perfusate, as indicated by venous Hb concentration.

Two assumptions are made in this method of correction for fluid shift. The first is that the correct venous [Hb] (ie. that which would be found if no fluid shift had occured) is equivalent to arterial [Hb] (Equation 2). The second is that the amount of fluid shift alters the given metabolite or ion concentration to the same magnitude as it alters Hb concentration (Equation 3).

Theoretical development of correction factor:

$$[Hb] = \frac{Hb\ (g)}{Fluid\ Vol.\ (dl)} \qquad (1)$$

$$CORR.VEN[Hb] = ART[Hb] \qquad (2)$$

$$\frac{CORR.VEN[Hb]}{MEAS.VEN[Hb]} = \frac{CORR.VEN[x]}{MEAS.VEN[x]} \qquad (3)$$

Where: CORR.VEN [Hb] = correct venous [Hb]

ART[Hb] = arterial [Hb]

MEAS.VEN [Hb] = measured venous [Hb]

[x] = concentration of any metabolite or ion

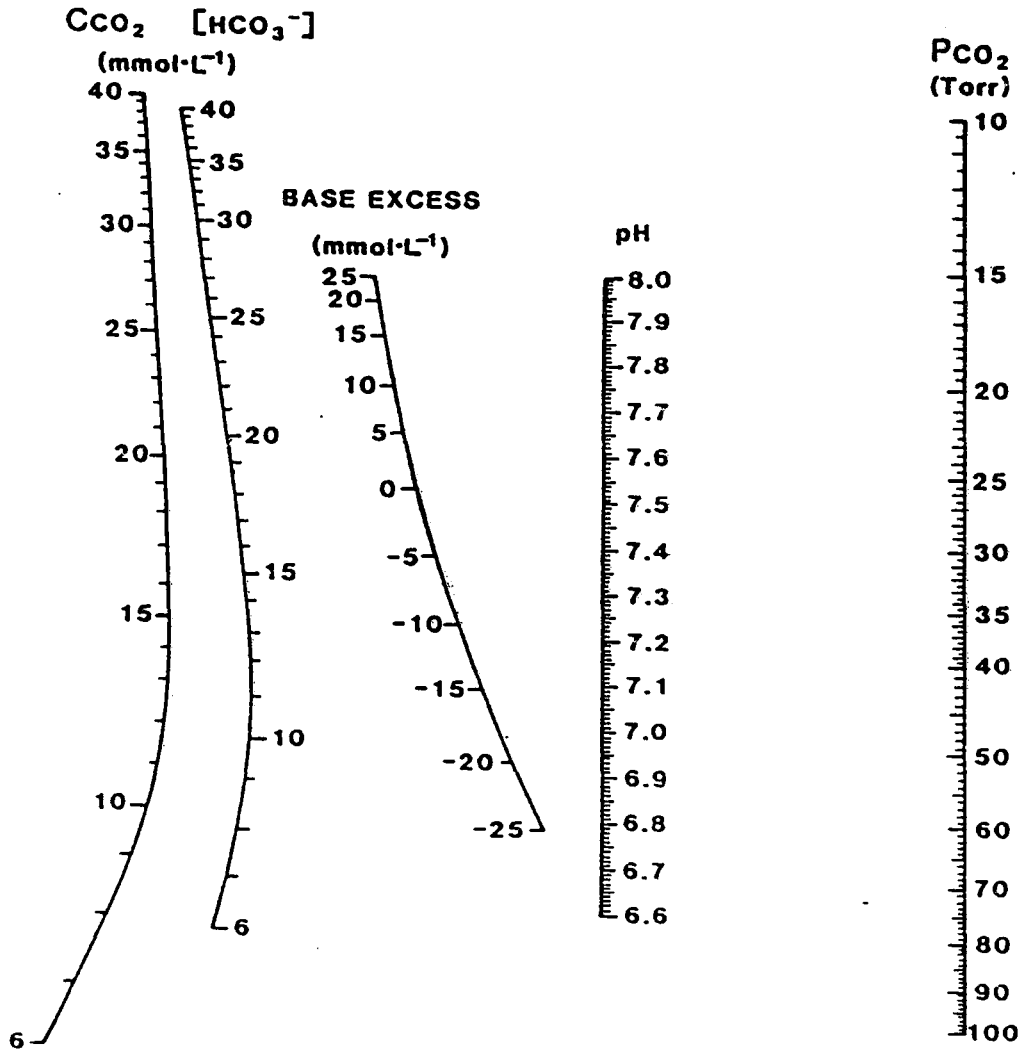
The correction fcator is arrived at by combining equations 2 and 3:

$$\frac{ART.[Hb]}{MEAS.VEN[Hb]} = \frac{CORR.VEN[Hb]}{MEAS.VEN[Hb]} \quad (4)$$

and rearranging equation 4:

$$CORR.VEN[x] = \frac{ART[Hb]}{MEAS.VEN[Hb]} * MEAS.VEN[x]$$

APPENDIX II


C_{CO_2} $[HCO_3^-]$
(mmol·L⁻¹)
40
35
30
25
20
15
10
6
40
35
30
25
20
15
10
6
BASE EXCESS
(mmol·L⁻¹)
25
20
15
10
5
0
-5
-10
-15
-20
-25
pH
8.0
7.9
7.8
7.7
7.6
7.5
7.4
7.3
7.2
7.1
7.0
6.9
6.8
6.7
6.6
P_{CO_2}
(Torr)
10
15
20
25
30
35
40
50
60
70
80
90
100

APPENDIX III

PERFUSATE ACID-BASE CHARACTERISTICS : RAW DATA

Table IIIa Venous Perfusate Acid-Base Characteristics - LP Group

	-5'	0'	5'	10'	15'	20'
pH	7.38 ±.02	7.38 ±.02	7.26 ±.03	7.13 ±.07	7.20 ±.04	7.19 ±.04
PO2 (mm Hg)	48.1 ±2.2	50.2 ±1.4	60.1 ±3.4	58.6 ±2.7	59.9 ±2.1	56.6 ±2.9
[Hb] (g.dl-1)	---	13.3 ±0.4	14.9 ±0.4	16.4 ±0.3	16.5 ±0.3	16.7 ±0.3
%O2 Saturation	80.8 ±2.0	81.9 ±1.3	84.9 ±1.6	81.6 ±1.6	81.7 ±0.5	81.7 ±0.8
PCO2 (mm Hg)	44.6 ±1.3	36.8 ±1.2	43.5 ±0.7	39.8 ±1.5	37.4 ±1.6	37.4 ±2.2
[HCO3-] (mMol)	21.6 ±0.6	21.3 ±1.1	16.6 ±1.2	13.5 ±0.9	12.6 ±0.8	12.4 ±0.8
CCO2 (mMol)	23.2 ±0.8	19.9 ±1.1	18.4 ±1.2	15.0 ±0.9	13.9 ±0.8	13.8 ±0.8
Base Excess (mMol)	0.8 ±1.2	-2.3 ±1.4	-6.5 ±1.8	-10.9 ±1.3	-11.3 ±1.6	-11.8 ±1.3
Flow rate (ml.min-1)	0.81 ±.03	0.98 ±.03	1.10 ±.07	1.03 ±.10	1.05 ±.06	1.11 ±.07

Values are mean ± standard error of the mean.
PO2 = partial pressure of oxygen
PCO2 = partial pressure of CO2
CCO2 = CO2 content

25'	30'	35'	40'	45'	50'	55'	60'
7.18 ±.03	7.17 ±.03	7.17 ±.03	7.17 ±.03	7.14 ±.03	7.16 ±.03	7.15 ±.04	7.14 ±.03
60.6 ±3.5	55.5 ±2.6	54.0 ±2.9	54.3 ±2.6	55.2 ±3.2	55.5 ±2.7	55.5 ±3.1	54.3 ±2.4
16.8 ±0.3	16.8 ±0.3	17.0 ±0.4	17.1 ±0.4	17.1 ±0.4	17.2 ±0.4	17.3 ±0.4	17.6 ±0.4
79.1 ±1.5	78.4 ±1.8	77.2 ±2.0	78.9 ±1.6	79.0 ±1.6	79.4 ±1.8	79.1 ±1.6	79.7 ±1.4
37.5 ±2.3	37.6 ±2.9	37.3 ±2.6	37.6 ±2.7	38.1 ±2.8	37.6 ±2.2	38.9 ±2.4	38.0 ±2.1
12.1 ±0.7	11.9 ±0.8	11.7 ±0.8	11.6 ±0.8	12.1 ±0.9	11.5 ±0.8	11.7 ±0.9	11.5 ±0.8
13.6 ±0.8	13.1 ±0.9	12.8 ±0.8	12.9 ±0.9	13.1 ±0.9	13.0 ±0.8	13.0 ±0.9	12.8 ±0.9
-12.5 ±1.1	-12.8 ±1.2	-13.1 ±1.1	-13.4 ±1.2	-12.9 ±1.3	-13.3 ±1.2	-13.8 ±1.2	-14.1 ±1.4
1.08 ±.06	1.08 ±.06	1.08 ±.06	1.08 ±.06	1.03 ±.07	1.01 ±.04	1.03 ±.04	1.08 ±.06

Table IIIb Venous Perfusate Acid-Base Characteristics - NP Group

	-5'	0'	5'	10'	15'	20'
pH	7.41 ±.01	7.41 ±.01	7.41 ±.01	7.41 ±.01	7.41 ±.01	7.41 ±.01
PO2 (mm Hg)	59.5 ±3.3	57.3 ±2.7	56.0 ±3.2	55.7 ±2.3	56.4 ±3.0	53.7 ±3.4
[Hb] (g.dl-1)	---	14.7 ±0.2	15.0 ±0.2	14.8 ±0.3	14.9 ±0.2	14.8 ±0.3
%O2 Saturation	80.7 ±1.1	82.2 ±0.8	77.6 ±2.5	79.6 ±1.8	79.1 ±1.1	78.7 ±1.5
PCO2 (mm Hg)	42.1 ±1.6	42.8 ±1.4	43.5 ±1.3	43.5 ±1.4	44.1 ±1.2	44.0 ±1.3
[HCO3-] (mMol)	21.5 ±0.4	21.6 ±0.4	22.2 ±0.4	22.1 ±0.4	22.3 ±0.4	22.5 ±0.4
CCO2 (mMol)	23.2 ±0.5	23.2 ±0.4	23.8 ±0.4	23.9 ±0.4	24.1 ±0.5	24.3 ±0.5
Base Excess (mMol)	2.5 ±1.3	2.0 ±0.6	1.9 ±0.5	2.1 ±0.5	2.5 ±0.6	2.5 ±0.5
Flow rate (ml.min-1)	0.97 ±.06	1.12 ±.05	0.93 ±.06	1.04 ±.04	0.96 ±.05	1.03 ±.03

Values are mean ± standard error of the mean.
PO2 = partial pressure of oxygen
PCO2 = partial pressure of CO2
CCO2 = CO2 content

25'	30'	35'	40'	45'	50'	55'	60'
7.41 ±.01	7.41 ±.01	7.41 ±.01	7.41 ±.01	7.41 ±.01	7.40 ±.01	7.40 ±.01	7.39 ±.01
54.5 ±4.4	54.5 ±4.5	54.4 ±4.3	53.7 ±3.5	54.8 ±4.0	53.1 ±3.8	54.2 ±3.5	53.6 ±3.3
14.8 ±0.2	14.9 ±0.2	14.8 ±0.2	14.9 ±0.2	15.0 ±0.2	15.0 ±0.2	15.1 ±0.1	15.2 ±0.1
78.9 ±1.6	78.7 ±1.7	78.9 ±1.8	79.7 ±1.7	79.8 ±1.6	79.3 ±1.7	79.1 ±1.6	79.7 ±1.4
42.8 ±1.0	42.9 ±1.1	44.0 ±1.2	44.8 ±1.2	44.5 ±1.2	44.5 ±0.8	44.9 ±0.7	44.7 ±1.0
21.8 ±0.5	21.9 ±0.5	22.3 ±0.6	22.4 ±0.6	22.4 ±0.6	22.3 ±0.4	22.2 ±0.5	21.9 ±0.6
23.6 ±0.6	23.8 ±0.6	24.0 ±0.7	24.2 ±0.6	24.3 ±0.6	23.9 ±0.4	24.1 ±0.4	23.8 ±0.6
2.0 ±0.8	2.0 ±0.7	2.4 ±0.8	2.5 ±0.7	2.6 ±0.7	1.9 ±0.5	2.1 ±0.6	2.0 ±0.7
1.01 ±.05	0.97 ±.04	0.98 ±.04	1.01 ±.04	1.04 ±.05	1.04 ±.07	0.99 ±.05	1.01 ±.05

Table IIIc Arterial Perfusate Acid-Base Characteristics - LP Group

	-20'	-12'	-8'	0'	10'
pH	7.41 ±.02	7.40 ±.03	7.39 ±.03	7.16 ±.04	7.15 ±.03
PO2 (mm Hg)	134.1 ±6.1	133.3 ±6.6	124.1 ±5.3	125.0 ±5.7	123.1 ±6.9
[Hb] (g.dl-1)	14.2 ±0.3	14.2 ±0.3	14.3 ±0.3	16.7 ±0.4	16.7 ±0.4
%O2 Saturation	102.1 ±0.1	102.2 ±0.1	102.1 ±0.1	101.5 ±0.2	101.4 ±0.1
PCO2 (mm Hg)	45.2 ±1.6	44.0 ±2.9	45.0 ±2.4	30.5 ±3.0	29.3 ±2.4
[HCO3-] (mMol)	22.7 ±0.9	21.8 ±0.9	22.0 ±1.4	9.3 ±1.0	8.7 ±0.9
CCO2 (mMol)	24.5 ±1.0	23.6 ±0.9	23.5 ±1.3	10.0 ±1.3	9.6 ±1.1
Base Excess (mMol)	2.9 ±1.4	1.8 ±1.4	1.6 ±1.8	-15.1 ±1.3	-16.1 ±1.2

Values are mean ± standard error of the mean.
PO2 = partial pressure of oxygen
PCO2 = partial pressure of CO2
CCO2 = CO2 content

20'	30'	40'	50'	60'
7.17 ±.04	7.16 ±.04	7.12 ±.03	7.12 ±.03	7.14 ±.03
117.5 ±5.4	114.8 ±5.5	109.8 ±6.2	110.9 ±7.3	115.1 ±5.8
16.7 ±0.4	16.7 ±0.4	16.7 ±0.4	16.7 ±0.4	16.7 ±0.4
101.1 ±0.1	100.9 ±0.1	100.6 ±0.1	100.5 ±0.1	100.5 ±0.2
29.9 ±2.3	30.4 ±2.2	30.5 ±1.9	31.5 ±2.1	31.9 ±1.7
9.3 ±0.9	9.4 ±0.8	8.8 ±0.9	9.9 ±1.0	9.5 ±0.9
10.2 ±1.0	10.3 ±1.0	9.7 ±1.0	10.2 ±1.1	10.5 ±1.0
-15.3 ±1.3	-15.4 ±1.3	-15.6 ±1.2	-15.6 ±1.3	-15.6 ±1.3

Table IIId Arterial Perfusate Acid-Base Characteristics - NP Group

	-20'	-12'	-8'	0'	10'
pH	7.45 ±.05	7.45 ±.05	7.42 ±.01	7.43 ±.01	7.43 ±.01
PO2 (mm Hg)	174.6 ±7.4	170.6 ±7.5	155.3 ±8.5	149.1 ±4.7	148.0 ±8.5
[Hb] (g.dl-1)	14.7 ±0.1	14.7 ±0.1	14.7 ±0.1	14.7 ±0.1	14.8 ±0.1
%O2 Saturation	102.6 ±0.1	102.6 ±0.1	102.5 ±0.1	102.3 ±0.1	102.2 ±0.1
PCO2 (mm Hg)	40.6 ±1.8	40.2 ±1.6	40.9 ±1.3	41.1 ±1.1	40.5 ±0.8
[HCO3-] (mMol)	21.1 ±0.5	20.4 ±0.5	21.4 ±0.4	21.6 ±0.4	21.6 ±0.4
CCO2 (mMol)	22.4 ±0.4	22.1 ±0.6	22.7 ±0.4	23.0 ±0.5	23.1 ±0.4
Base Excess (mMol)	0.8 ±0.4	0.5 ±0.6	1.3 ±0.5	2.0 ±0.6	2.0 ±0.7

Values are mean ± standard error of the mean.
PO2 = partial pressure of oxygen
PCO2 = partial pressure of CO2
CCO2 = CO2 content

20'	30'	40'	50'	60'
7.42 ±.01	7.42 ±.01	7.44 ±.01	7.45 ±.02	7.42 ±.02
134.1 ±6.4	127.7 ±7.5	133.2 ±7.8	133.4 ±4.6	130.7 ±10.4
14.8 ±0.1	14.8 ±0.1	14.8 ±0.1	14.9 ±0.1	15.0 ±0.1
102.1 ±0.1	101.9 ±0.2	101.8 ±0.2	101.8 ±0.2	101.7 ±0.2
41.1 ±0.7	41.5 ±1.7	41.3 ±1.2	39.7 ±1.5	41.3 ±2.3
21.7 ±0.6	21.8 ±0.5	22.4 ±0.6	22.2 ±0.7	21.8 ±0.7
23.1 ±0.6	23.1 ±0.6	23.5 ±0.6	23.5 ±0.5	23.4 ±0.7
2.2 ±0.8	1.5 ±0.6	2.5 ±0.8	2.5 ±0.8	2.5 ±0.9

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