REGULATION OF PYRUVATE DEHYDROGENASE ACTIVITY IN HUMAN SKELETAL MUSCLE

by

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ABSTRACT

Regulation of the flux-generating enzyme complex pyruvate dehydrogenase (PDHc) was examined in the context of its physiological function in human skeletal muscle. In the first two studies, the role of PDHc in intramuscular fuel selection and the mechanisms regulating PDHc transformation from its inactive form (PDHb) to its active form (PDHa) and PDHa activity were examined. In a third study, the role of PDHc in muscle lactate production during exercise and recovery was assessed and the factors controlling transformation to PDHa were also examined.

In the first study, 5 subjects were examined during rest and cycling exercise at 75% of $\dot{V}O_2$max after 3 days of consuming a low-carbohydrate (LCD) or high-carbohydrate (HCD) diet. In the second study, 8 subjects were examined at rest and during cycling exercise at 40% and 80% $\dot{V}O_2$max while they were infused with sodium acetate (ACE) or sodium bicarbonate (BIC). At rest, consumption of a LCD and ACE infusion increased the intramuscular acetylCoA-to-CoASH ratio and citrate, as a result of increased oxidation of available fat fuels and acetate, respectively. Elevation of the acetylCoA-to-CoASH ratio and citrate inhibited PDHa and phosphofructokinase activity, respectively. Consequently, the rates of pyruvate oxidation by PDHa and pyruvate production by glycolysis were reduced preventing the oxidation of intramuscular glucose. The resting results of these two studies were consistent with the operation of a reciprocal cycle of glucose and fat oxidation for intramuscular energy production. The events leading to glucose restriction were initiated by changes in acetylCoA accumulation which caused an increase in the acetylCoA-to-CoASH ratio and citrate concentration which induced the transformation of PDHa to PDHb and inhibition of glycolytic flux, respectively.

In contrast, during exercise at 40%, 75% and 80% $\dot{V}O_2max$, transformation from PDHb to PDHa was determined by Ca^{2+} and was not restricted in any of the conditions except the LCD condition. Lower PDHa activity and incomplete transformation to PDHa occurred in this condition, placing a restriction on intramuscular glucose utilization. However, the acetylCoA-to-CoASH ratio actually decreased in this condition, suggesting that the lower rate of transformation was independent of alterations in the acetylCoA-to-CoASH ratio. In contrast, when transformation to PDHa was not limited in any other condition there was an increase in the acetylCoA-to-CoASH ratio which should have limited transformation. Thus, during exercise, the restriction on muscle glucose oxidation occurred only after a LCD indicating that this reciprocal cycle of fuel selection and utilization was also operating during exercise and that PDHc transformation was an integral part of its operation. However, during exercise, the regulatory factors controlling PDHa differed from those that determined the activity of this enzyme at rest. Lower PDHa during exercise in the LCD condition was consistent with an increase in the PDH-kinase/PDH-phosphatase activity ratio, in the 3 day period before exercise, increasing the occupancy of monophosphate esters on both the transformation site and the inhibitory sites of the E1α components. Incomplete removal of these additional phosphate esters by PDH-phosphatase during exercise may have then resulted in lower PDHa and glucose conservation later on during exercise.

In a third study, the role of PDHa in muscle lactate production during exercise and recovery was examined and the factors controlling PDHc transformation were determined in 7 subjects. During repeated 30 second bouts of maximal isokinetic cycling exercise, complete transformation to PDHa occurred concurrently with muscle lactate accumulation and increased mitochondrial oxidation, indicating that lactate production was not dependent

on the development of tissue hypoxia. Instead, during exercise muscle lactate production resulted from a rate of glycolytic pyruvate production that was greater than PDHa activity. Conversely, during recovery, net lactate oxidation occurred as the lactate dehydrogenase equilibrium shifted more toward pyruvate production and PDHa remained partially active due to attenuation of the acetylCoA-to-CoASH ratio, reductions in the ratios of NADH-to-NAD and ATP-to-ADP and elevated concentrations of hydrogen ion and pyruvate.

The present studies extend the analysis of PDHc regulation from *in vitro* and *in situ* studies to human skeletal muscle *in vivo*. The findings of the present studies suggest that the during muscle contraction, the most important factor regulating PDHc transformation in human muscle is probably Ca^{2+}, while the other regulatory factors perform secondary roles. In contrast, during rest and recovery from maximal exercise, PDHc transformation to PDHa and PDHa activity are determined by the intramuscular ratios of acetylCoA-to-CoASH, NADH-to-NAD, ATP-to-ADP and the concentrations of hydrogen ion and pyruvate.

PERSONAL ACKNOWLEDGEMENTS

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In addition to those on my supervisory committee, I have had the good fortune to work with some other exceptional scientists throughout the course of my studies. I would like to thank Dr. Lawrence Spriet, Dr. Eric Hultman and Dr. Gitten Cederblad for the opportunity to work and learn in their laboratories. I am indebted to you for the experiences and opportunities that you provided, and for your special contributions to the present work.

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Finally, I thank my beloved Michelle, and my parents, Charles and Karen, for their unwavering support and understanding throughout. Your contributions may not be quite so tangible but they were just as important to the completion of this thesis.

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Appendix A

LIST OF SYMBOLS AND ABBREVIATIONS

ACE	acetate infusion condition
acetyl-TPP	acetyl-thiamine pyrophosphate
acetylCoA	acetyl-Coenzyme A
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BIC	bicarbonate infusion condition
°C	degrees celsius
Ca^{2+}	calcium
$CaCl_2$	calcium chloride
CO_2	carbon dioxide
Con	control condition
CS	citrate synthetase
CoASH	coenzyme A
CV	coefficient of variation
DCA	dichloroacetic acid
DPM	disintegrations per minute
DTT	dithiothreitol
E1	pyruvate decarboxylase (E.C. 1.2.4.1)
E2	dihydrolipoamide acetyltransferase (E.C. 2.3.1.12)
E3	dihydrolipoamide dehydrogenase (E.C. 1.6.4.3)
EDTA	ethylene diamine tetraacetic acid
EE	end of exercise
EGTA	ethylene glycol bis(β-aminoethyl ether)-N,N,N´,N´-tetraacetate
EX	exhaustion
Exp	experimental condition
8-AcDHL	8-S-acetyldihydrolipoate
FAD	flavin adenine dinucleotide
$FADH_2$	reduced flavin adenine dinucleotide
F-1,6-DP	fructose-1,6-diphosphate
FFA	free fatty acids
F-6-P	fructose-6-phosphate
G-1-P	glucose-1-phosphate
G-6-P	glucose-6-phosphate
HCD	high carbohydrate diet condition
H^+	hydrogen ion
HCl	hydrochloric acid
HE-TPP	hydroxyethyl-thiamine-pyrophosphate
ICDHc	isocitrate dehydrogenase complex
KCl	potassium chloride
$KHCO_3$	potassium bicarbonate

LIST OF SYMBOLS AND ABBREVIATIONS

K_2CO_3	potassium carbonate
K_2HPO_4	potassium phosphate
l	liter
LCD	low carbohydrate diet condition
LDH	lactate dehydrogenase
Liquid N_2	liquid nitrogen
Mg^{2+}	magnesium
$MgCl_2$	magnesium chloride
mCi	millicurie
min	minutes
mM	millimoles per liter
mmol	millimoles
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NaF	sodium fluoride
Na_2HPO_4	sodium phosphate
$NaHCO_3$	sodium bicarbonate
nmol	nanomoles
OGDHc	oxoglutarate dehydrogenase complex
O_2	oxygen
PCA	perchloric acid
PCr	phosphocreatine
PDHa	active form of the pyruvate dehydrogenase complex
PDHb	inactive form of the pyruvate dehydrogenase complex
PDHc	pyruvate dehydrogenase complex
PDHt	total activity of the pyruvate dehydrogenase complex
PEP	phosphoenolpyruvate
P_i	inorganic phosphate
pmol	picomoles
SDH	succinate dehydrogenase
TG	triglyceride
TGL	triglyceride lipase
TPP	thiamine pyrophosphate
μCi	microcurie
μl	microliter
μM	micromoles per liter
V_{max}	maximum enzyme activity
$\dot{V}CO_2$	rate of carbon dioxide production
$\dot{V}O_2$	rate of oxygen consumption
$\dot{V}O_2max$	maximum oxygen uptake

FORMAT AND ORGANIZATION OF THESIS

The present thesis was prepared in the "open face" format as outlined in the School of Graduate Studies Calendar, section 2, sub-section 2.7, "Theses". This thesis is comprised of three published original research papers (Chapters 2, 3 and 4) and a fourth paper that has been prepared for publication but has not yet been submitted (Appendix A). In addition, appendices B and C each contain copies of original research papers that the author collaborated on with others. Each of chapters 2, 3 and 4 are referenced as an individual papers in the other chapters of this thesis. The paper of appendix A is referred to as "Appendix A".

CONTRIBUTION TO MULTI-AUTHORED PAPERS

Chapter 2

Publication

Putman, CT, LL Spriet, E. Hultman, MI Lindinger, LC Lands, RS McKelvie, G Cederblad, NL Jones and GJF Heigenhauser. Pyruvate dehydrogenase activity and acetyl-group accumulation during exercise after different diets. *American Journal of Physiology, 265 (Endocrinology and Metabolism 28): E752-E760, 1993.*

Contributions

Experiments were conducted and samples were collected by CT Putman with the assistance of the co-authors. Special medical assistance was provided by Dr.'s RS McKelvie, LC Lands and G Cederblad who inserted and maintained brachial arterial and femoral venous lines and extracted blood samples from each of the subjects. They were also responsible for regulating glucose infusion into the antecubital vein of each subject. Special medical assistance was also provided by Dr. E Hultman and Dr. NL Jones who took muscle biopsies of the vastus lateralis and monitored arterial blood glucose for each subject, respectively.

The primary supervisor for this study was Dr. GJF Heigenhauser. This study was also supervised by Dr.'s NL Jones and LL Spriet. The data contained in this paper were generated through biochemical analysis that was completed by CT Putman with the following exception. Muscle glycogen and pyruvate, and plasma free fatty acid determinations were completed by the staff of Dr. LL Spriet's laboratory. The data were analyzed and the paper was written by CT Putman.

Chapter 3

Publication

Putman, CT, LL Spriet, E. Hultman, DJ Dyck and GJF Heigenhauser. Skeletal muscle pyruvate dehydrogenase activity during acetate infusion in humans. *American Journal of Physiology 268, (Endocrinology and Metabolism 31): E1007-E1017, 1995.*

Contributions

The experiments were conducted and samples were collected by CT Putman with the assistance of the co-authors. The primary supervisor for this study was Dr. GJF Heigenhauser. This study was also supervised by Dr. LL Spriet. PDHa and PDHt measurements were completed by CT Putman. The remaining analysis was completed by the technical staff of Dr. LL Spriet's laboratory. The data were analyzed and the paper was written by CT Putman.

CONTRIBUTION TO MULTI-AUTHORED PAPERS

Chapter 4

Publication

Putman, CT, NL Jones, LC Lands, TM Bragg, MG Hollidge-Horvat and GJF Heigenhauser. Skeletal muscle pyruvate dehydrogenase activity during maximal exercise in humans. *American Journal of Physiology, (Endocrinology and Metabolism): In Press, 1995.*

Contributions

The experiments were conducted and samples were collected by CT Putman with the assistance of Dr. GJF Heigenhauser and Dr. LC Lands, who took the biopsies from each subject. The primary supervisor for this study Dr. GJF Heigenhauser. This study was also supervised by Dr. NL Jones. Biochemical analysis was completed entirely by CT Putman. Technical assistance was provided by TM Bragg and MG Hollidge-Horvat. The data were analyzed and the paper was written by CT Putman.

Appendix A

A comparison of radiometric methods for determination of PDHa and PDHt in skeletal muscle.

Contributions

The experiments were conducted and data were generated by CT Putman. The primary supervisor for these experiments was Dr. GJF Heigenhauser. These experiments were also supervised by Dr.'s NL Jones and CJ Toews. The data were analyzed and the paper was written by CT Putman.

This study was completed to establish a reliable and sensitive method for determination of PDHa and PDHt in small skeletal muscle samples before the other studies of this thesis (Chapters 2-4 and Appendix B) were completed.

CHAPTER 1

THE PYRUVATE DEHYDROGENASE COMPLEX

1.1 Introduction

Skeletal muscle is a dynamic and highly adaptable tissue that comprises approximately 40% of the total body mass of humans. Its primary function is to generate movement, permitting purposeful interaction with the environment, a requisite of which is the capacity to draw on a variety of fuels to furnish the necessary chemical energy. This is achieved by a variety of metabolic pathways that oxidize a number of different substrates.

Despite the presence of a variety of metabolic pathways in muscle and the ability to draw on several different types of fuel, ATP remains the only chemical currency of energy transfer available to the contractile apparatus. Moreover, the available pool of ATP within skeletal muscle is small and if it were not for the combined ability of those pathways to match the rate of ATP production to ATP demand in an integrated and controlled manner, ATP stores would become depleted very rapidly. Thus, elaborate mechanisms exist to control the rate of each metabolic pathway, ensuring integrated and appropriate use of available fuels. The end result is the controlled release of chemical energy from glucose, fat and other fuels as they are broken down by a series of linked reactions within the cytosol and mitochondria to produce ATP. In skeletal muscle, enzyme regulation is the major means by which energy pathways are not only controlled to match ATP production to ATP demand but the means by which the selection of some fuels are determined.

The present chapter reviews the theoretical construct of a metabolic pathway and

basic mechanisms of regulation. This is followed by a summary of the pathways leading to intramuscular glucose oxidation and an examination of the pyruvate dehydrogenase complex (PDHc) and its regulation. In addition, the physiological importance of PDHc to energy metabolism in human and animal skeletal muscle is examined during rest and exercise. Finally, this chapter concludes with a description of the rationale for examining the regulation of PDHc in human skeletal muscle.

1.2 Theoretical Concepts in Metabolic Regulation

The theoretical construct of a metabolic pathway and the types and functions of various enzymes within a pathway have been extensively reviewed in recent years by Newsholme *et al* (77,78,79,80,81). According to the accounts of these authors there are three types of enzymes that exist within a metabolic pathway: flux-generating, near-equilibrium and pseudo-flux-generating enzymes. A pathway is initiated by a flux-generating enzyme, which also controls the overall rate at which substrate flows through a pathway, and it typically ends with either the beginning of a new pathway controlled by another flux-generating enzyme or the release of the pathway product into a physiological reservoir, where it may form the substrate for another pathway.

The flux-generating enzyme of a pathway is saturated with its substrate and is regulated by external factors other than the concentration of its substrate. Since it is saturated with its substrate at all times, alterations in activity often result in a reciprocal change in the concentration of its substrate. In addition, to control the overall rate of the pathway, its activity must be the lowest in the series of linked reactions and the reaction it catalyzes must be irreversible, requiring another separate enzyme or series of enzymes to

catalyze the reverse reaction.

The flux generating enzyme in a pathway is followed by a series of enzymes that catalyze near-equilibrium reactions, where the rate of each reaction is dependent only on changes in their respective substrate and product concentrations. The forward and reverse catalytic activity of these enzymes proceed at rates that are much greater than that of the flux generating reaction but their net activity is determined by the balance of the catalytic activity of the flux generating enzyme and the rate of pathway substrate oxidation down stream. In short, changes in the substrate concentration for one reaction in a series is communicated to the next reaction by a similar change in product formation, which is the substrate for the next reaction. Thus, the flow of pathway substrate through a series of linked near-equilibrium reactions occurs almost passively, depending only on the initial substrate concentration, which is determined by the catalytic activity of the flux-generating reaction, and the rate of oxidation or removal of the final product in the pathway.

Finally, like the flux generating enzyme, the activities of a third class of "regulatory" enzymes are also determined by external allosteric modulators. This class of enzymes has also been described by Newsholme *et al* (77,78) as "pseudo-flux-generating enzymes" but are simply referred to as regulatory enzymes, in this chapter. Unlike flux-generating enzymes, the reactions catalyzed by regulatory enzymes in a pathway are reversible and they are not saturated with their substrate. Consequently, variations in substrate and product concentrations are also very important determinants of their net catalytic activity. Coincidentally, the nature of the allosteric regulators that affect a regulatory enzyme are similar to those that regulate the flux-generating reaction of the pathway but they may differ in the sensitivity to the signal and they remain highly dependent on substrate concentration

to determine activity.

The importance of regulatory enzymes lies in their ability to co-ordinate flux through their respective pathways, assisting the flux-generating enzyme by further modulating the flow of pathway substrate. Allosteric regulation of these enzymes acts in an additive manner with changes in substrate and product concentrations. As substrate concentration increases and/or product concentration decreases, enzyme activity will also increase proportionally according to Michaelis-Menton enzyme kinetics. However, when a positive allosteric modulator is present it will also serve to increase the activity of regulatory enzymes and allow the same increase in activity and flux to be achieved but at a lower substrate concentration and/or a higher product concentration. Similarly, when an negative modulator is present in sufficient quantity to effect a decrease in activity of a regulatory enzyme, the decrease in substrate concentration and increase in product concentration, that alone would be required to reduce enzyme activity are also attenuated. In effect, extreme variations in the concentrations of pathway intermediates are minimized, allowing better regulation of cell osmolarity and improving the responsiveness of the entire pathway to changes in flux.

1.2.1 Regulation of Glucose Metabolism in Skeletal Muscle

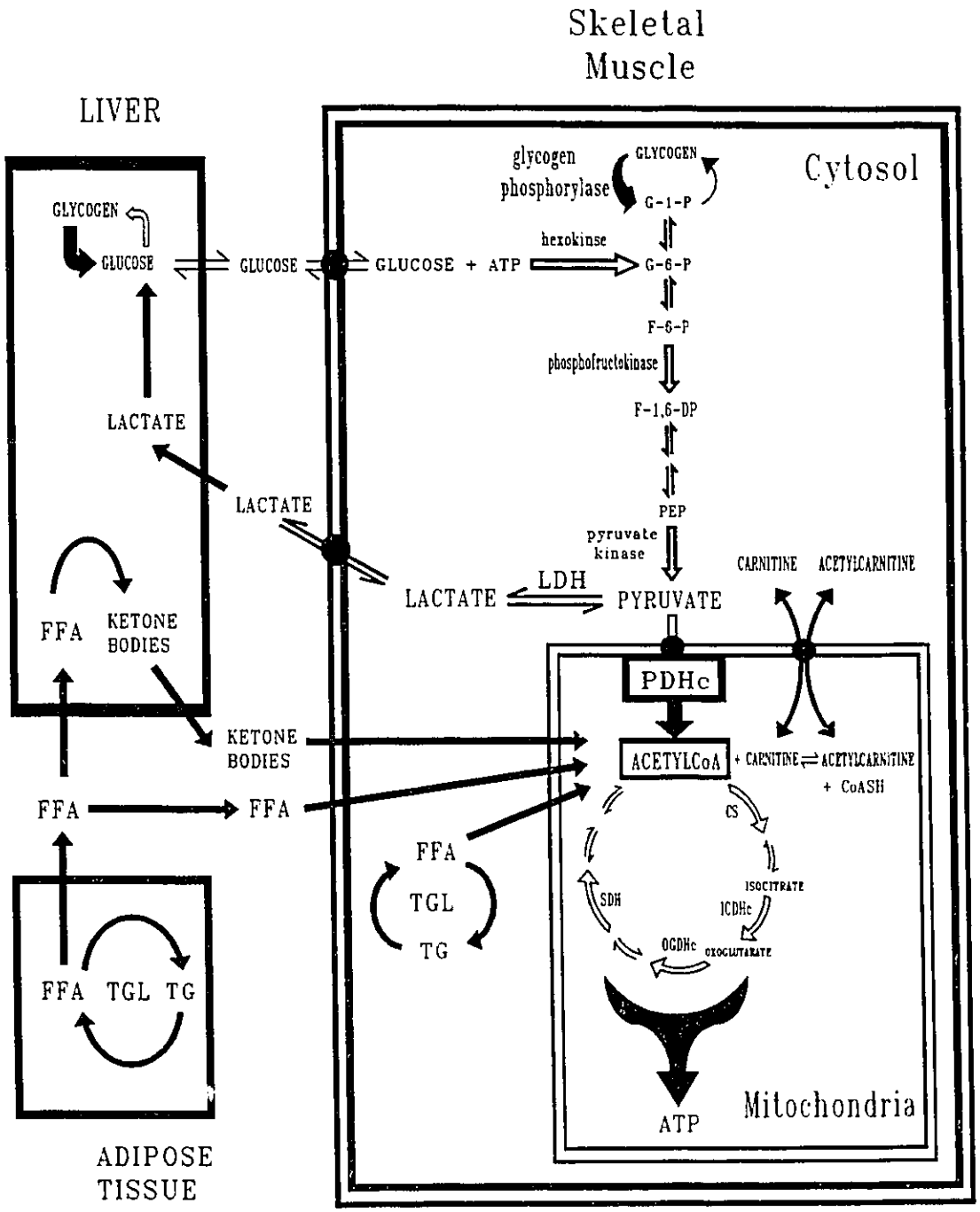
Glucose oxidation is an important source of fuel for the production of ATP in skeletal muscle. In the post-absorptive state, there are two physiological pathways leading to the oxidation of glucose to pyruvate, producing ATP in the process. A third common pathway also exists for the complete oxidation of glucose. The functional characteristics of these pathways are illustrated in Figure 1. The key regulatory steps are highlighted in bold, the reactions catalyzed by regulatory enzymes are indicated by single white arrows and the

equilibrium steps are indicated by forward and reverse arrows. The first of these pathways begins with muscle glycogen and the second begins with the release of glucose from liver glycogen stores into the blood (81). The common reactions for each of these pathways are a series of reactions known as glycolysis (81). The first pathway begins with the breakdown of muscle glycogen to glucose-1-phosphate by the flux generating enzyme glycogen phosphorylase, whose activity is increased by Ca^{2+}, AMP and P_i and decreased in their absence (12,101,102). This pathway ends in the production of pyruvate which is then converted to lactate by the near-equilibrium enzyme lactate dehydrogenase (LDH), which maintains lactate concentration 1-2 orders of magnitude above pyruvate (81). Lactate is then free to leave the muscle cell for a larger physiological reservoir of extracellular fluid. Alternatively, pyruvate can be utilized by the flux-generating enzyme of the TCA cycle.

The glycolytic pathway also has two additional regulatory enzymes that assist in the regulation of pathway flux: phosphofructokinase (PFK) and pyruvate kinase (PK) (81). In addition to being activated by their respective substrates, each is inhibited by its product and further regulated by a number of allosteric modulators which reflect the energy status of the cell. Both enzymes are inhibited by ATP and PCr and reduce flux when energy status is high (31,41,77,81,83). In addition, a number of metabolites that reflect the decreasing energy status of the cell activate these enzymes: ADP activates PK (77,81), while ADP, AMP, NH_4^+, and P_i are known to activate PFK (31,41).

The second pathway leading to glucose oxidation in skeletal muscle derives its substrate from the blood. The rate of blood glucose utilization by skeletal muscle is determined by the rate of hepatic glucose production which is ultimately regulated by the

Figure 1: The location of PDHc in intermediary metabolism. PDHc links the pathways of glycolysis and the TCA cycle. The end-product of glycolysis, pyruvate, is in equilibrium with lactate due to the catalytic activity of the equilibrium enzyme lactate dehydrogenase (LDH). LDH maintains lactate concentrations 1-2 orders of magnitude above that of pyruvate. Oxidation of alternative substrates also result in acetylCoA production and oxidation in the TCA cycle. Shown here are several possible sources which include fatty acids, ketone bodies and intramuscular triglycerides.


Skeletal
Muscle
LIVER
Cytosol
GLYCOGEN
GLUCOSE
GLUCOSE
GLUCOSE + ATP
glycogen
phosphorylase
GLYCOGEN
G-1-P
hexokinse
G-6-P
F-6-P
phosphofructokinase
F-1,6-DP
PEP
pyruvate
kinase
LACTATE
LACTATE
LACTATE
LDH
PYRUVATE
CARNITINE
ACETYLCARNITINE
FFA
KETONE
BODIES
PDHc
KETONE
BODIES
ACETYLCoA
+ CARNITINE
ACETYLCARNITINE
+ CoASH
FFA
FFA
CS
FFA
TGL
TG
ISOCITRATE
SDH
ICDHc
OGDHc
OXOGLUTARATE
FFA
TGL
TG
ATP
Mitochondria
ADIPOSE
TISSUE

catalytic rate of hepatic glycogen phosphorylase (3,8,82). In addition to the regulatory reactions previously described for glycolysis, when glucose oxidation proceeds from blood glucose there is an additional regulatory step that modulates membrane transport of glucose (64,81). While it can also be allosterically regulated it remains highly dependent on changes in blood glucose concentration. ATP production from either of these pathways alone yields comparatively little ATP: 2 ATP are produced per molecule of glucose derived from blood and 3 ATP are produced per glucose liberated from glycogen (55,81).

In contrast, complete glucose oxidation from either blood glucose reserves or from muscle glycogen yields an additional 36 ATP (55,81). The greater ATP production is achieved by a third pathway, the tricarboxylic acid (TCA) cycle. In this final pathway leading to complete glucose oxidation, acetylCoA is completely oxidized to CO_2 and H_2O resulting in the generation of NADH + H^+ and $FADH_2$ for electron transport which then drives oxidative phosphorylation and produces the additional ATP.

When glycolytically generated pyruvate is the available substrate, the flux-generating enzyme of the TCA cycle is a complex of enzymes known as the pyruvate dehydrogenase complex (PDHc). This highly regulated enzyme integrates a variety of metabolic signals, that reflect the energy status of the cell, to control the flow of glucose-derived-acetylCoA into the TCA cycle. In addition, it integrates the flow of pathway substrate from glucose and various fat fuels into the TCA cycle. By virtue of its spatial relationship with the near-equilibrium enzyme lactate dehydrogenase, which catalyzes the reversible conversion of lactate to pyruvate, PDHa activity also directly affects muscle lactate production and utilization. Four additional regulatory enzymes catalyze regulatory reactions that assist in regulating the flow of pathway substrate: citrate synthetase (CS), isocitrate dehydrogenase complex (ICDHc),

oxoglutarate dehydrogenase complex (OGDHc) and succinate dehydrogenase (SDH) (81).

1.2.2 Pyruvate Dehydrogenase as a Flux Generating Enzyme

A flux generating enzyme is fundamental to the operation of a metabolic pathway and central to energy production *in vivo.* The four criteria of a flux-generating enzyme, as described by Newsholme *et al* (78,80), are met by PDHc. The first requirement of a flux generating enzyme is that it must be non-equilibrium. This is determined by comparing the *in vivo* mass action ratio (Γ = *[acetylCoA] / [pyruvate]*) to the experimentally determined equilibrium constant for the reaction. If the former value is much less than the latter, the enzyme is said to be non-equilibrium and if the two are approximately equal the enzyme is said to be near-equilibrium. Under resting conditions in human muscle, the content of acetylCoA can vary between 12 (16,17,18) and 38 μmol·kg dry wgt.$^{-1}$ (91), depending on dietary state. Under similar conditions pyruvate content is approximately 0.5 mmol·kg dry wgt.$^{-1}$ (89,91,117,122) resulting in a mass action ratio of 24-72. During heavy and maximal exercise acetylCoA can vary from 30 to 55 μmol·kg dry wgt.$^{-1}$ (16,17,89,90,91) and pyruvate levels from 2 to 4 mmol·kg dry wgt.$^{-1}$ (89,117,122), resulting mass action ratio of 7-15. From these data it is clear that in human skeletal muscle, during rest and exercise, the mass action ratio is much less than the equilibrium constant (K'_{eq}=8.4 X 10^6) for this reaction and that the reaction catalyzed be PDHc is far removed from equilibrium *in vivo.*

The second requirement of a flux-generating enzyme is that it must be saturated with its substrate under all conditions. At rest pyruvate content is approximately 0.5 mmol·kg dry wgt.$^{-1}$ (89,122), or 166 μM, in human skeletal muscle which is approximately 5 times the K_m (36 μM) for pyruvate (128). During heavy and maximal exercise, pyruvate concentration can

increase to between 667 μM and 1333 μM in human skeletal (117), which is approximately 18-37 times the concentration necessary for half-maximal activity of isolated enzyme preparations. Thus, these data suggest that in human skeletal muscle PDHc is saturated with its substrate during exercise but may not be saturated at rest. However, the K_m of pyruvate for E1 of PDHc has not been determined under conditions that mimic the intramitochondrial milieu or the intracellular compartmentation of pyruvate. The fact that pyruvate concentration increases as PDHa increases also suggests that PDHc is, at the very least, rate limiting and is probably saturated at rest as well.

The third requirement of a flux-generating enzyme is that it be modifiable in a reversible manner by physiological concentrations of metabolites. Extensive *in vitro* study of PDHc has shown that it is allosterically regulated by a multitude of metabolic intermediates and ions that reflect the prevailing energy status and activity state of the cell. These include regulation by the ratios of acetylCoA-to-CoASH, NADH-to-NAD and ATP-to-ADP as well as regulation by Ca^{2+}, Mg^{2+} and pyruvate (see (6,100,128) for reviews). The combined effect of all these regulatory species on PDHc, creates the potential for *in vivo* flux to be precisely controlled and matched to the energy requirement of the cell. The mechanisms by which these regulatory factors control PDHc are described in detail in the following sections.

The fourth requirement of a flux-generating enzyme is that it should initiate flux to subsequent reactions of the pathway by internal regulation and provide direction to the pathway. PDHc determines the rate of pyruvate-derived-acetylCoA entry into the TCA cycle. As acetylCoA production increases, the cumulative effect results in a greater rate of enzyme activity of subsequent reactions in the pathway. The effect is transmitted to neighboring enzymes in succession and are assisted by the regulatory enzymes, CS, ICDHc, OGDHc and

SDH. As the flux generating enzyme of this pathway, PDHc has the lowest activity of all other enzymes in this cycle and, consequently, determines the maximal rate of the TCA cycle. The maximum rate the TCA cycle (2-3 mmol·min^{-1}·kg ww^{-1}), as estimated from maximum O_2 consumption, is similar to the measured total PDHc activity (PDHt) (2-3 mmol·min^{-1}·kg ww^{-1}) from muscle homogenates (16,17,18,89,90,91). Only the OGDHc has a similarly low activity (105) but the substrate concentration for this enzyme is subsaturating, leaving it dependent on changes in its substrate concentration to alter activity. In addition, both PDHc and OGDHc have similar sensitivities to external allosteric modulators such as Ca^{2+} (27,28,71,72,108), NADH-to-NAD ratio (124) and CoASH (124) which further coordinates their respective activities to ensure the smooth flow of pathway substrate.

In summary, PDHc meets all of the requirements of a flux generating enzyme in human skeletal muscle. It catalyzes a non-equilibrium reaction that is irreversible and is saturated with its substrate throughout the physiological spectra of skeletal muscle activity. It is allosterically modifiable in a reversible manner by physiological concentrations external modulators that reflect the energy status and contractile activity of skeletal muscle. Finally, it initiates and regulates the flux of pyruvate-derived-acetylCoA into the TCA cycle and determines the maximal activity of this pathway during the oxidation of glucose. Thus, these unique regulatory characteristics not only allow PDHc to finely regulate glucose oxidation but to also determine the maximal rate of oxidation by the TCA cycle.

The physiological consequences of these properties of PDHc are highlighted in its role in intramuscular fuel selection and in muscle lactate production. The importance of PDHc to ATP production becomes apparent when a balance must be struck between the oxidation of glucose and fat fuels, or during maximal exercise. When whole body glucose

reserves are low, PDHc will restrict glucose oxidation in favor of greater fat fuel utilization. Similarly, during maximal exercise PDHc places an upper limit on complete glucose oxidation, resulting in significant lactate accumulation as the rate of pyruvate production exceeds the rate of pyruvate oxidation. Consequently, the rapid depletion of whole body glucose stores is circumvented in both cases.

1.3 Structure and Function of the Pyruvate Dehydrogenase Complex

Human skeletal muscle pyruvate dehydrogenase is a multi-enzyme complex composed of three main catalytic subunits, pyruvate decarboxylase-E1 (E.C. 1.2.4.1.), dihydrolipoamide acetyltransferase-E2 (E.C. 2.3.1.12) and dihydrolipoamide dehydrogenase-E3 (E.C. 1.6.4.3.) as well as the two regulatory subunits, PDH-kinase (E.C. 2.7.1.99) and PDH-phosphatase (E.C. 3.1.3.43) (6,29,47,85,99,100,128). Located within the inner mitochondria membrane (1,85,114,128) and centrally in the chemical conversion of glucose to ATP (Figure 1) this enzyme catalyzes the oxidative decarboxylation of pyruvate to acetylCoA and is the flux generating step for entry of pyruvate derived acetylCoA into the TCA cycle.

The reaction catalyzed by PDHc can be summarized as follows;

$$\text{pyruvate} + NAD^+ + \text{CoASH} \rightarrow \text{acetylCoA} + NADH + H^+ + CO_2$$

The mechanism of catalysis has also been extensively reviewed (6,23,29,35,47,85,87,100,125,128) and is illustrated in Figure 2.

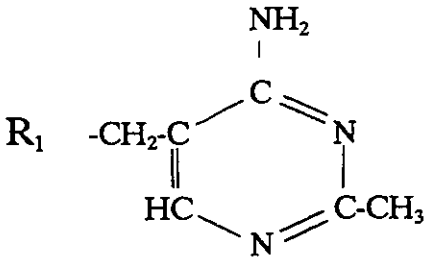
The three catalytic subunits, (E1, E2 and E3) act sequentially to remove the alpha-terminal carboxyl group from pyruvate and subsequently pass the remaining acetyl-group on

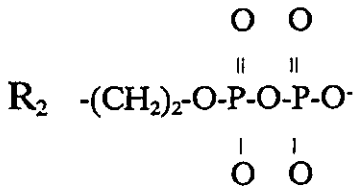
to CoASH where it is further oxidized in the TCA cycle. The mechanism, illustrated in Figure 2, begins with the binding of the second carbon of pyruvate to the second carbon of thiazole ring of thiamine pyrophosphate (TPP) which is contained on any one of the thirty E1 tetrameres of the enzyme and are in turn noncovalently bound to the edges of a pentagonal dodecahedron shaped E2 core. Ionization of thiamine pyrophosphate at physiological pH forms a highly reactive nucleophilic carbanion species that attacks the second carbon of pyruvate forming the transition state species, 1-carboxyl-2-hydroxybutyl-TPP. Through resonance stabilization decarboxylation occurs, generating CO_2 and forming hydroxyethyl-TPP (HE-TPP). HE-TPP is then converted to acetyl-TPP via resonance stabilization. Acetyl-TPP has a negative charge which acts as a nucleophile, reacting with the eletrophilic disulphide of lipoamide to form a highly reactive tetrahedral transition state species (not shown). This then results in the formation of 8-S-acetyldihydrolipoate (8-AcDHL) that extends from the E2 core atop a flexible lysine "arm" (35).

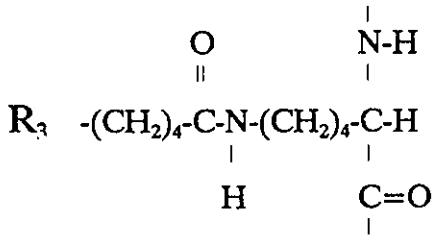
Although it is not entirely resolved, this step may occur through an alternative mechanism: 1) reduction of lipoamide to lipoic acid by HE-TPP which is converted to acetyl-TPP in the process and 2) nucleophilic attack of the sulphydryl-group of lipoic acid (carbon 8) on the acetyl-group of acetyl-TPP, forming a thioester with lipoic acid and producing 8-AcDHL (35).

Movement of 8-AcDHL atop the lipolysine arm to the second catalytic site on E2 leads to a nucleophilic attack of the sulphydryl of CoASH on the keto-carbon of 8-AcDHL, resulting in the transfer of the acetyl-group to CoASH, forming acetylCoA and reducing the sulphydryl of liopamide to lipoic acid. Lipoic acid is then oxidized, regenerating

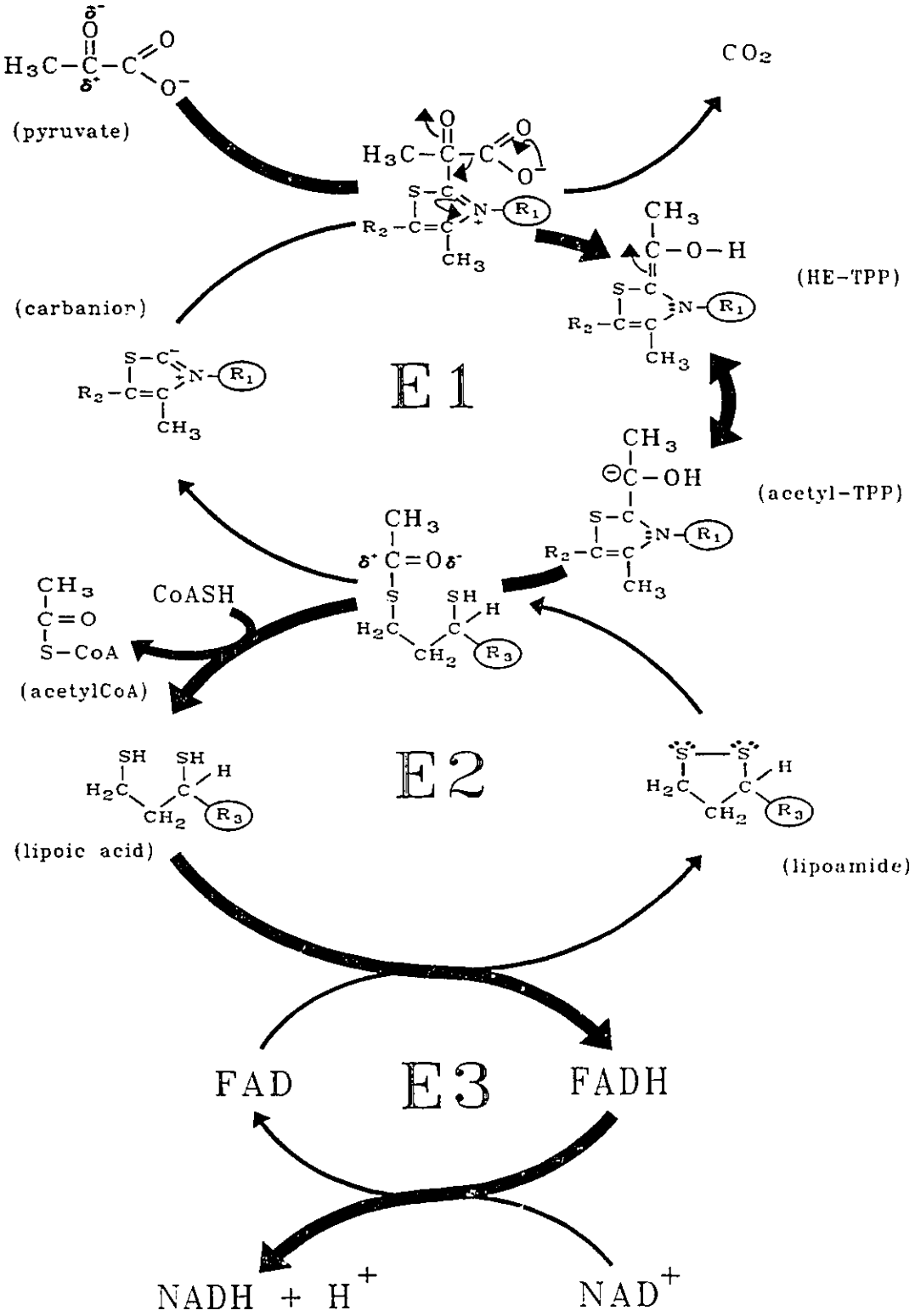
Figure 2: The mechanism of PDHa catalysis. See text for description of each step. E1 = pyruvate decarboxylase, E2 = dihydrolipoamide acetyltransferase, E3 = dihydrolipoamide dehydrogenase.







Lysine residue imbedded in the E2 subunit

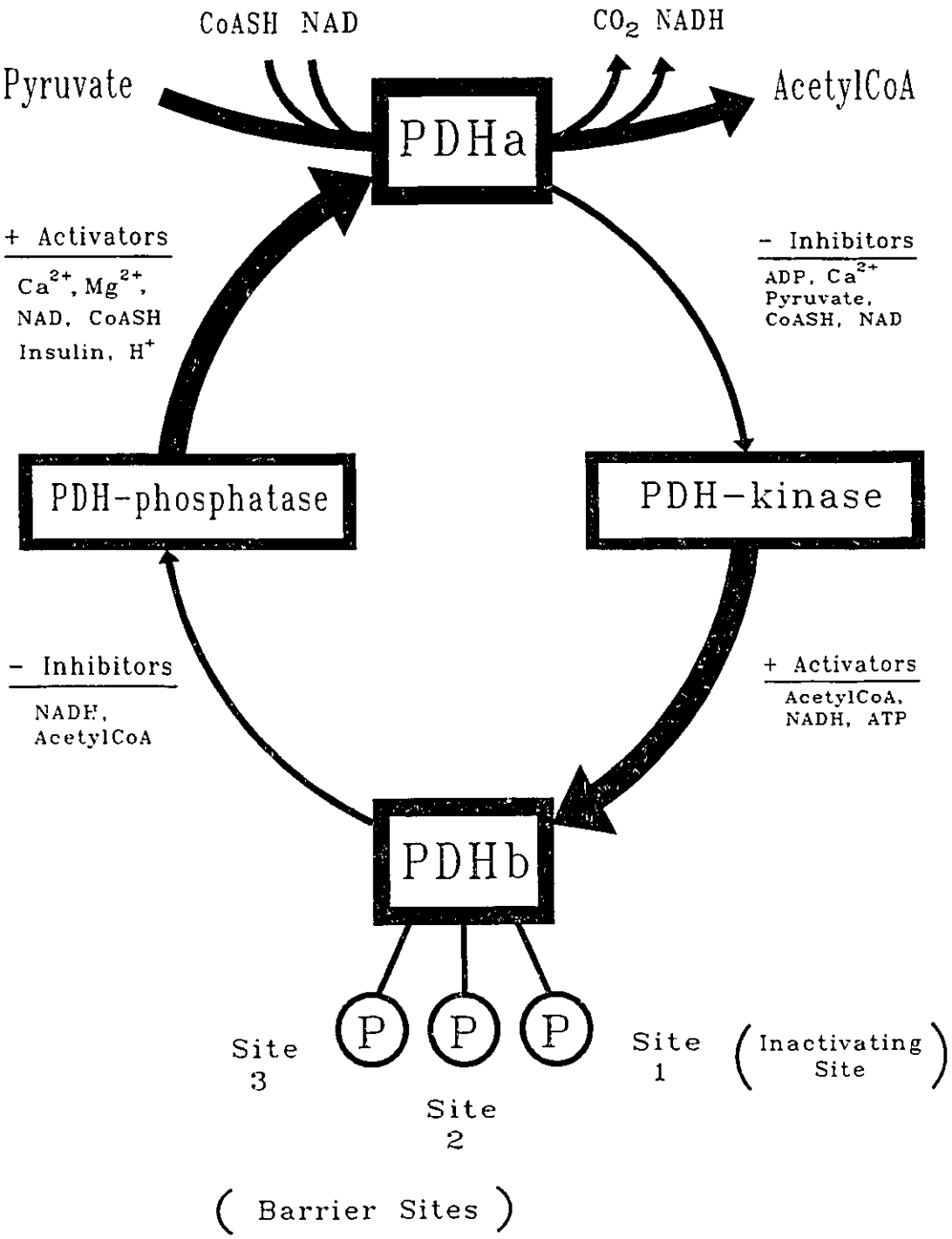

(pyruvate)
CO2
(carbanion)
(HE-TPP)
E1
(acetyl-TPP)
CoASH
(acetylCoA)
(lipoic acid)
E2
(lipoamide)
FAD
E3
FADH
NADH + H+
NAD+

the disulfide bridge of the lipoyl residue. This occurs by the action of one of the twelve FAD containing E3 subunits, generating $FADH_2$ in the process. The newly reduced flavin adenine nucleotide then passes its reducing equivalents on to NAD^+ regenerating FAD and producing 1 NADH + H^+ for oxidative phosphorylation.

A phosphorylation-dephosphorylation cycle (Figure 3) catalyzed by the regulatory subunits, PDH-kinase and PDH-phosphatase, adds an additional degree of control to this already elaborate catalytic mechanism (6,29,85,93,95,99,100,128). Both of these regulatory subunits are noncovalently bound to the E2 component but are in close proximity to the E1 component upon which they direct their catalytic activity. These regulatory subunits are further controlled by a multitude of allosteric modulators. Unlike other intracellular kinases which facilitate and in fact amplify intracellular signals, PDH-kinase reduces the activity of PDHa, transforming it to its inactive form (PDHb), by phosphorylating three distinct serine residues on the α-subunits of the E1 tetrameres (sites 1, 2 and 3). Conversely, PDH-phosphatase hydrolyses these mono-phosphate esters causing PDHc transformation from PDHb to PDHa and increasing the rate of PDHa catalysis.

Within this phosphorylation/dephosphorylation cycle is yet another level of control that determines the rate and extent of PDHc transformation. By following a sequential pattern of phosphate addition and removal on the α-subunits of the E1 tetrameres (20,36,62,93,95,99,109,110,111,112,121), the rate of PDHc transformation can be further regulated. PDHc transformation to lower or higher levels of PDHa is the result of only site 1 (inactivating site) phosphorylation and dephosphorylation, mediated by increased PDH-kinase and PDH-phosphatase activities, respectively. The remaining sites, 2 and 3, serve as barrier sites which restrict the access of PDH-phosphatase to site 1. As stimulatory signals

Figure 3: PDHc regulation by a phosphorylation and dephosphorylation cycle. PDH-phosphatase removes mono-phosphate-esters on the E1 component, transforming PDHa to a higher level and allowing the conversion of pyruvate to acetylCoA to proceed. Several allosteric modulators of PDH-phosphatase exist which increase or decrease its activity. In contrast, increased PDH-kinase activity results in phosphorylation of the E1 components, converting PDHc to its inactive " b-form ". Phosphorylation proceeds through sites 1, 2 and 3 if the activity of PDH-kinase is much greater than PDH-phosphatase for a long enough period. PDH-kinase is also allosterically modulated by a number of intracellular signals that result in either an increase or decrease in activity. At any one time the relative activities of PDH-kinase and PDH-phosphatase determine the phosphorylation state and the potential for flux through the PDHa reaction.


CoASH NAD
CO_2 NADH
Pyruvate
AcetylCoA
PDHa
+ Activators
Ca^{2+}, Mg^{2+},
NAD, CoASH
Insulin, H^+
- Inhibitors
ADP, Ca^{2+}
Pyruvate,
CoASH, NAD
PDH-phosphatase
PDH-kinase
- Inhibitors
NADH,
AcetylCoA
+ Activators
AcetylCoA,
NADH, ATP
PDHb
P
P
P
Site 3
Site 1
(Inactivating Site)
Site 2
(Barrier Sites)

are applied to PDH-kinase, phosphorylation proceeds increasing site 1 occupancy on the E1 components and transforming PDHa to PDHb and decreasing the proportion of PDHc in its active (PDHa) form. As phosphorylation proceeds further to increase site 2 and then site 3 occupancy, an ever increasing degree of inhibitory control is placed on transformation preventing conversion of PDHb back to PDHa. In effect, the additional site 2 and site 3 phosphorylations place a lock on the level of PDHa, constructing an ever more arduous barrier to dephosphorylation by PDH-phosphatase. Consequently, the activating stimuli to PDH-phosphatase must also increase in both severity and duration to first hydrolyze mono-phosphate esters on the barrier sites 3 and then 2. Thereafter, it is free to dephosphorylate site 1 and effect transformation of PDHb to PDHa.

1.4 Regulation of the Pyruvate Dehydrogenase Complex

1.4.1 PDHc Regulation by Metabolite Concentration Ratios

The mitochondrial ratios of NADH-to-NAD, acetylCoA-to-CoASH and ATP-to-ADP result from the prevailing balance of substrate supply, tissue O_2 availability and the demand for cellular ATP. Appropriately, they are also important signals influencing PDHa transformation and the rate of aerobic glucose metabolism. In this capacity they assist in coordinating the rate of aerobic glucose production with the rate of ATP utilization at the actin-myosin ATPase. The ratios of NADH-to-NAD, acetylCoA-to-CoASH and ATP-to-ADP demonstrate potent regulatory effects, contributing to the control of both PDHa transformation and flux. Alterations in these ratios have direct effects on both of the regulatory subunits PDH-kinase and PDH-phosphatase together, or on PDH-kinase alone. Therefore, they assist in determining the net phosphorylation state of PDHc and in turn the

potential for flux through the PDHa reaction. In addition, the NADH-to-NAD and acetylCoA-to-CoASH ratios may further regulate PDHa flux *in vivo* by substrate-production competition. This dual aspect of control by these ratios creates a regulatory system which not only assists in determining PDHa transformation, via the regulatory subunits, but also contributes an added measure of control by monitoring *in vivo* PDHa flux.

1.4.1.1 The NADH-to-NAD Concentration Ratio

The regulatory action of the NADH-to-NAD concentration ratio is two fold, demonstrating regulatory characteristics of both end-product inhibition (10,107) and allosteric regulation (20,61,62,63,88). The events of end-product inhibition begin as the concentration of NADH rises, displacing NAD^+ from its binding site on the E3 component of PDHc. Consequently, the flavin adenine nucleotide associated with this subunit remains primarily in a reduced state, since the catalytic steps associated with $FADH_2$ are reversible. The PDHa reaction is halted as the transfer of reducing equivalents from $FADH_2$ to the lipoyl moiety of the E2 component produces lipoic acid. Lingering molecules of acetylCoA can then react with the lipoic acid, transferring an acetyl-group to lipoic acid creating a situation were the lipolysyl arm remains primarily in a reduced state and is unavailable for further transfer of acetyl groups from TPP. Since the decarboxylation step is irreversible, the acetyl group atop the lipolysyl arm can not rejoin CO_2 to regenerate pyruvate and must remain predominately in this reduced state until NADH levels fall or NAD^+ levels rise.

A more complex level of regulation also exists for the NADH-to-NAD ratio via its direct allosteric effect on PDH-phosphatase and PDH-kinase. The allosteric effects of this ratio can be summarized as follows: NADH (20,88,92) acts by directly inhibiting PDH-

phosphatase and increasing the activity of PDH-kinase, while NAD (20,88,92) activates PDH-phosphatase and decreases the activity of PDH-kinase. Thus, an increase in this ratio has the potential to activate PDH-kinase and inhibit PDH-phosphatase, while a decrease in this ratio will inhibit PDH-kinase and activate PDH-phosphatase. These changes would then result in a decrease or increase in PDHa, respectively. When considered as a whole, alterations in this ratio appear to have the dual function of: 1) assisting to set the maximum limit for flux via alterations in PDHa transformation and 2) further regulating the actual *in vivo* PDHa flux by end-product inhibition.

1.4.1.2 The AcetylCoA-to-CoASH Concentration Ratio

Changes to the mitochondrial acetylCoA-to-CoASH ratio also affect PDHa activity and PDHa transformation in a manner similar to the NADH-to-NAD ratio. The acetylCoA-to-CoASH ratio demonstrates regulatory aspects that are consistent with end-product inhibition of PDHa and allosteric regulation of PDHa by the direct effect of this ratio on the regulatory enzymes PDH-kinase and PDH-phosphatase. It has previously been demonstrated that a high ratio of acetylCoA-to-CoASH inhibits PDHa (29,33,39,88). Additionally, it has also been suggested (29) that the mechanism by which this inhibition occurs is competition between acetylCoA and CoASH in the following manner. As acetylCoA accumulates there is a reversal of the step involving the transfer of the acetyl-group to CoASH. Consequently, a larger proportion of the lipoyl moieties atop the flexible lysine arm begin to accumulate in this acetylated state halting any further production of acetylCoA.

Allosteric regulation by the acetylCoA-to-CoASH ratio is also an important aspect of PDHa regulation and appears to operate in a similar if not identical fashion to the

NADH-to-NAD ratio. AcetylCoA inhibits PDH-phosphatase activity and increases the activity of PDH-kinase (20,61,62,88,107). Conversely, CoASH activates PDH-phosphatase and decreases the activity of PDH-kinase (20,61,62,88). Thus, the net effect of an increase in this ratio is similar to that of the NADH-to-NAD ratio: lower PDHa results from a decrease in PDH-phosphatase and increase in PDH-kinase. A decrease in this ratio has the opposite effect, resulting in greater PDHa transformation. When these two aspect of PDHa regulation by the acetylCoA-to-CoASH ratio are considered together, they also appear to have the dual function of: 1) assisting to set the maximum limit for flux via alterations in PDHa transformation and 2) further regulating the actual *in vivo* PDHa flux by end-product inhibition.

1.4.1.3 The ATP-to-ADP Concentration Ratio

Unlike the previous two ratios, the effect of the ATP-to-ADP ratio on PDHa regulation is limited to its influence on the regulatory subunit, PDH-kinase. The ATP-to-ADP ratio affects PDH-kinase activity by competition between its substrate, ATP, and its product ADP (19,61,62,63,66,107). A rise in this ratio results in an increase in PDH-kinase activity, while a decrease in the ratio results in lower PDH-kinase activity. If the stimulus for increased PDH-kinase activity is sufficiently large and remains for a sufficiently long period, PDHc will become sequentially phosphorylated on three distinct serine residues, on each of the E1 components (36,62,75,109,110,111,112,121). This process is highly dependent on the cellular ATP levels. Once all three sites have been phosphorylated it becomes increasingly difficult to remove these phosphate groups by PDH-phosphatase. Thus a more potent stimulus is required for PDH-phosphatase and/or an inhibitory stimulus must be applied to

PDH-kinase.

1.4.2 Regulation by Pyruvate and [H⁺]

As the principal substrate of PDHa, pyruvate is also an important determinant of both PDHa transformation and flux. It exerts its regulatory effects in two ways. The first is through simple substrate binding to the E1 components of PDHc, ensuring adequate substrate binding and preventing a limitation to PDHa flux. The second is allosteric regulation and inhibition of PDH-kinase directly with an estimated K_i of 0.5-2.8 mM (19,54,62), which is within the range of physiological concentrations of intramuscular pyruvate at rest (74,91) and during exercise (74,91,117). As pyruvate concentration increases during increased muscular activity the inhibitory effect of pyruvate on PDH-kinase will also increase, eventually reducing PDH-kinase activity below that of PDH-phosphatase and allowing transformation to a higher level.

Variations in the concentration of hydrogen ion may represent another important regulatory mechanism of PDHa transformation. Alterations in the intracellular pH have been shown to be important in the maintenance of proper protein function, the level of responsiveness of a protein (ie. conformational changes, reversible dissociation/association and phosphorylation) and the stability of the protein structure (116). In large multisubunit proteins such as PDHc, OGDHc and PFK these factors become increasingly important. Small changes in the pH_i by as little as one tenth of a pH unit can have a dramatic effect on the catalytic rate of such enzymes. Thus, the effect of such changes on a flux generating enzyme may assist in determining flux through a metabolic pathway.

Pearce *et al* (86) demonstrated greater PDHa in perfused rat heart during acidosis,

suggesting that acidosis alone was responsible for this effect. Several possible mechanism may be responsible which include alteration to the affinity of the regulatory metabolites for PDHc leading to greater PDH-phosphatase or lower PDH-kinase activity. Increased affinity of pyruvate for the catalytic site and/or the allosteric regulatory site on PDH-kinase may also result from alterations in pH. Alternatively, acidosis may alter the distribution of pyruvate across the mitochondrial membrane (6) such that the mitochondrial pyruvate concentration increases as cytosolic pH decreases.

During intense exercise, H^+ accumulation results primarily from the accumulation of lactate but also results from changes in other independent variables (119). The resulting acidosis can have a profound effect on the rate of muscle glucose oxidation. When the rate of lactate production exceeds the capacity for its oxidation, the accompanying acidosis will inhibit glycogen phosphorylase (31) and PFK (31) at the same time it may serve as a stimulus for greater PDHa activity (86). The physiological consequence of the relationship of H^+ with these enzymes is inhibition of lactate production at the same time the stimulus for oxidation is increased. In this way, lactate can serve as negative feedback signal on the regulatory points controlling its production at the same time it serves as a positive feed forward signal on the flux generating step which controls its oxidation and removal.

1.4.3 Regulation By Ca^{2+}

Regulation by Ca^{2+} appears to be an even more important aspect of PDHc transformation than the concentration ratios or other allosteric regulators. Release of Ca^{2+} from the sarcoplasmic reticulum not only serves to cause muscle contraction, but it also invokes the transformation of PDHa and activation other key regulatory enzymes (45,73),

increasing aerobic ATP production to meet energy demand at the actin-myosin ATPase. The effect of Ca^{2+} on PDHc transformation is to increase the activity of PDH-phosphatase and reduce PDH-kinase activity (26,27,73,115), by increasing and decreasing the affinity for their respective substrates, phosphorylated E1 and dephosphorylated E1. Calcium causes half-maximal transformation at a concentration of 0.2-0.3 μM in skeletal muscle (2,45) and 0.3-0.6 μM in heart muscle (27,28,75), which is within the range of estimates of intramitochondrial Ca^{2+} concentration for both of these tissues at rest (2,26,73). Moreover, the affinity of PDHc for Ca^{2+} is sufficiently high to ensure Ca^{2+} is present in saturating quantities during muscle contraction (2,26,73). Thus, the importance of Ca^{2+} on PDHa transformation lies in the co-ordination of ATP production from aerobic glycolysis with the rate of ATP utilization at the actin-myosin ATPase during muscle contraction.

1.5 The Physiological Importance PDHc

As a consequence of its flux generating characteristics, PDHc it thought to have two important physiological roles in human skeletal muscle. First, PDHa may play a pivotal role in the selection of intramuscular fuels, as summarized in a theory of metabolic regulation termed the " glucose-fatty acid cycle ". This theory describes the reciprocal relationship between the use of glucose and fat fuel in skeletal muscle. PDHc is thought to act as a reversible barrier to reduce the flow of pyruvate-derived acetylCoA into the TCA cycle when the availability fat fuel increases and removing it when the availability of glucose increases. The second important aspect of PDHc lies in the role of this enzyme in the control of muscle lactate concentration during exercise and recovery from exercise.

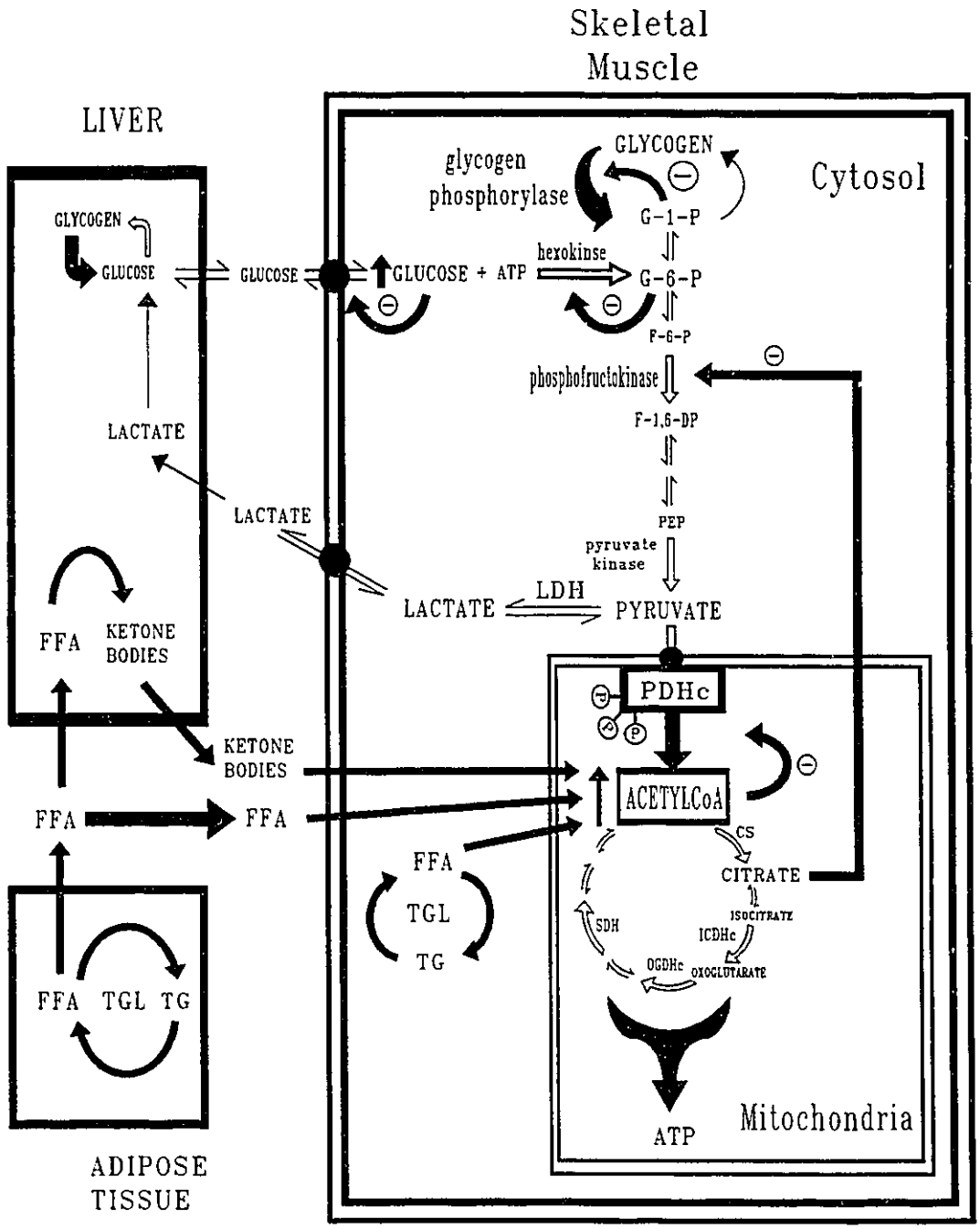
1.6 Role of PDHc in Intramuscular Fuel Selection

1.6.1 The Glucose-Fatty Acid Cycle

The glucose-fatty acid cycle was first proposed by Randle et al (96) to explain the reciprocal relationship that existed between glucose and fat oxidation by rat heart and diaphragm muscles and has been previously reviewed (76,94,95,97) (Figure 4). In this theory of metabolic regulation, it is suggested that glucose utilization by muscle is down regulated in proportion to the increased availability and utilization of exogenous and endogenous fat fuels, placing a restriction on carbohydrate utilization and conserving muscle and whole body carbohydrate reserves. Elevated fat availability and utilization restricts the oxidation of glucose and glycogen by muscle. Conversely, elevation of blood glucose increases uptake and utilization, and inhibits fat utilization. When fat fuel availability is increased, as occurs after the consumption of a high fat meal, after a period of chronic starvation or uncontrolled diabetes, fat oxidation proceeds in muscle leading to the accumulation of the end product of β-oxidation, acetylCoA. Enhanced uptake of free fatty acids (FFA) and conversion to ketones in the liver also enhances their delivery to the muscle, contributing to this mechanism of fuel selection by also contributing to the increase in muscle acetylCoA.

The sequence of events are illustrated in Figure 4. They start as the availability and utilization of alternative fat fuels, such as FFA, ketone bodies or triglycerides are increased. This may occur after: 1) consumption of a high fat meal, 2) a period of chronic starvation or 3) uncontrolled diabetes. Oxidation of these fat fuels leads to accumulation of acetylCoA and is accompanied by a corresponding decrease in CoASH, resulting in an increase in the ratio of acetylCoA-to-CoASH. In addition, a similar increase in the ratio of NADH-to-NAD also occurs as the rate at which reducing equivalents enter the mitochondria also increases.

Figure 4: The glucose-fatty acid cycle. See text for explanation.


Skeletal
Muscle
LIVER
Cytosol
glycogen
phosphorylase
GLYCOGEN
G-1-P
GLYCOGEN
GLUCOSE
GLUCOSE
GLUCOSE + ATP
hexokinse
G-6-P
F-6-P
phosphofructokinase
F-1,6-DP
LACTATE
LACTATE
PEP
pyruvate
kinase
LACTATE
LDH
PYRUVATE
FFA
KETONE
BODIES
PDHc
KETONE
BODIES
ACETYLCoA
CS
FFA
FFA
FFA
CITRATE
TGL
TG
ISOCITRATE
SDH
ICDHc
OGDHc
OXOGLUTARATE
FFA TGL TG
ATP
Mitochondria
ADIPOSE
TISSUE

The ratios of acetylCoA-to-CoASH and NADH-to-NAD inhibit PDH-phosphatase and increase the activity of PDH-kinase causing lower PDHa and inhibition of pyruvate oxidation. Consequently, glucose reserves are conserved at this first and most important site of glucose restriction. Accumulated pyruvate and lactate can then be recycled into glucose as they are transported systemically to the liver where they enter the gluconeogenic pathway.

As acetylCoA accumulates, it reacts with oxaloacetate to form citrate which, upon traversing the mitochondrial membrane, inhibits PFK in the cytosol. Newsholme *et al* (83) showed that this effect is most marked, in skeletal muscles of a variety of animal models, in the presence of physiological concentrations of ATP (7.5 mM): PFK activity was reduced to between one-fourth and one-tenth of the activity that was observed in the absence of citrate. As a consequence of this effect, glycolytic flux and glucose utilization are restricted at a second site in the pathway of glucose oxidation. An additional result of PFK inhibition is the accumulation of glucose-6-phosphate upstream, further inhibiting glucose phosphorylation by hexokinase which places a third level of restriction on glucose utilization by restricting glucose uptake. In addition, the parallel accumulation of glucose-1-phosphate also holds the potential to inhibit glycogen phosphorylase activity and conserve intramuscular glycogen stores.

The fourth level of control over this metabolic cycle extends to the hormonal level and is regulated by blood catecholamine and insulin levels. Acceleration of lipolysis in fat and muscle tissue by increased catecholamine levels increases the availability of fat fuel to muscle and sets into motion the events that culminate in glucose restriction. Conversely, an elevation of the anti-lipolytic hormone, insulin, results in increased glucose uptake and greater PDHa. The simultaneous reduction in pyruvate oxidation by PDHa and production by glycolysis by

this mechanism ensures that intramuscular lactate accumulation is kept to a minimum.

1.6.2 The Role of PDHc in Fuel Selection in Animal Models

Initial evidence for the glucose-fatty acid cycle was based on observations that starvation, diabetes and prolonged carbohydrate deprivation in rats resulted in elevation of plasma free fatty acids (FFA) and reciprocal reductions in the rates of glucose uptake, glycolysis and pyruvate oxidation by heart and diaphragm muscles (see (94,97) for reviews). This prompted further study of the relationship between the availability of exogenous fat fuel and glucose oxidation in skeletal muscle. Much of the initial work was completed by Randle and co-workers (37,38,39,40,97,98) who demonstrated increased oxidation of fat fuel resulted in decreased rates of glucose uptake and phosphorylation (38,39,98), glycolysis (37,38,39,40,41,98) and pyruvate oxidation (37,38,39,40,41,98) in heart and diaphragm muscles.

More recent studies that have examined this mechanism of fuel selection in rat skeletal muscle have shown acetylCoA accumulation (42) and a lower rate of lactate oxidation during starvation (7,42,53,69), diabetes (7,42), and during infusion of acetoacetate (7,69) and FFA (7,106). Coupled with the fact that PDHa is significantly lower in rat skeletal muscle under similar conditions during rest (30,48,52,53,68,120) and intense muscular contractions (48), suggests that this mechanism is operating in skeletal muscle and that PDHc is an integral part of intramuscular fuel selection in these models.

An additional factor is also known to contribute to glucose restriction. Using models of starvation and diabetes, Fuller et al (36) showed that prolonged elevation of fat availability and oxidation resulted in increased site 2 and site 3 phosphorylation of the α-subunits on the

E1 components of PDHc from rat skeletal muscle. Like the effects in rat (109,110,111,112) and pig heart (121), phosphorylation of these additional sites did not lower PDHa transformation further but they did place a more restrictive barrier to greater PDHa transformation, slowing the rate of transformation during repeated contractions. This suggests that PDHa could remain lower during exercise, independent of changes in the regulatory ratios. However, inhibition of transformation by this mechanism would be dependent on a prolonged period of high ratios of acetylCoA-to-CoASH and NADH-to-NAD (61,62,73) at rest before exercise to ensure a high PDH-kinase/PDH-phosphatase activity ratio (61,62).

1.6.3 The Role of PDHc in Fuel Selection in Human Muscle

Despite the large body of evidence for the operation of the glucose-fatty acid cycle in skeletal and heart muscle of several different animal models, there are qualitative and quantitative metabolic differences between these tissues and human skeletal muscle. By comparison human skeletal muscle has a lower capacity for oxidation of fat fuels, is less dependent of exogenous fuel sources and much more dependent on endogenous stores of muscle glycogen. Therefore, it remains to be determined if this reciprocal metabolic cycle exist in human skeletal muscle and if the mechanisms of operation are similar to that reported in animal models.

Studies examining the operation of the glucose-fatty acid cycle in human skeletal muscle have been very limited in scope. While several studies have examined carbohydrate utilization by skeletal muscle in the presence of increased fat fuel availability, none have provided a detailed examination of the underlying mechanisms. Previous studies have been able to demonstrate glycogen conservation (21,49,56,57,65), reduced muscle glucose uptake

(5,34,44,46,59,65,67,113,123,126,127) and reduced whole body glucose metabolism (4,59) in response to dietary carbohydrate restriction (44,56,65), diabetes mellitus (44,126), infusion of FFA/heparin (5,34,46,49,113,123,127), infusion of acetate (59,67) or after consumption of a high fat meal (4,21,34,65). Despite evidence for operation of the glucose-fatty acid cycle in human skeletal muscle, the underlying mechanisms of operation remain largely unexplored. Specifically, the presence of the initiating signal of acetylCoA accumulation and the concomitant rise in the acetylCoA-to-CoASH ratio have not been demonstrated, and the role of PDHc in fuel selection has not been characterized.

1.7 Role of PDHc in Muscle Lactate Production

Skeletal muscle lactate production during contraction has been attributed to the acceleration of glycolysis secondary to the development of tissue hypoxic (25,50,51,60). The development of tissue hypoxia is thought to result in a substrate limitation at cytochrome oxidase which in turn leads to cytosolic and mitochondrial accumulation of NADH and ADP with reciprocal decreases in NAD and ATP, respectively. As a result of the myokinase reaction, a corresponding increase in cytosolic AMP and P_i accompanies ADP accumulation. ADP, AMP and P_i then activate glycogen phosphorylase and phosphofructokinase resulting in an increased rate of glycolysis and lactate accumulation which provides an additional anaerobic source of ATP. This entire explanation of muscle lactate production during exercise is based on the assumption of tissue hypoxia.

However, it has recently been demonstrated that during exercise, under normoxic conditions, human skeletal muscle does not become hypoxic but in fact actually becomes oxidized (43). However, this point remains in dispute as researchers debate the best manner

in which to determine muscle mitochondrial O_2 concentration. Using surface fluorometry, Jobsis and Stainsby provided the first evidence that skeletal muscle actually became more oxidized in stimulated dog skeletal muscle (58,118) and that tissue O_2 was not limiting for the electron transport chain and oxidative phosphorylation. Their findings were later supported by the work of Connet *et al* (14,15) who measured myoglobin saturation and showed that O_2 was not limiting for function of the electron transport chain in stimulated dog muscle. Similar findings have been reported in human skeletal muscle during submaximal exercise (11,58). Measures of mitochondrial redox state (58) and tissue PO_2 (11) in human muscle during exercise have shown increased mitochondrial oxidation (58) and that tissue PO_2 (11) was not limiting for oxidative phosphorylation.

In contrast to these findings, others (see (60) for review) have reported the development of tissue hypoxia in human skeletal muscle during incremental exercise and in various animal models of lactate production. However, the conclusions of these studies must be viewed with skepticism, as they measured whole cell redox state which does not reflect the mitochondrial redox state or tissue O_2 availability (58,84,129), as originally assumed. Thus, there is evidence that tissue and mitochondrial O_2 levels may remain well above the critical level for the electron transport chain and cytochrome oxidase to function adequately, and that O_2 levels do not pose a limitation to aerobic glucose metabolism during exercise in normoxia. When considered together, these findings suggest that other factors control muscle lactate production in humans during exercise.

Recent studies of human PDHc place the maximal activity (PDHt) at 2-3 $mmol \cdot min^{-1} \cdot kg\ ww^{-1}$ (16,17,18,90,91) which is low compared to maximal estimates of glycogen phosphorylase activity and glycolytic pyruvate production which can exceed 70

(12,13,102,103,104) and 40 mmol·min^{-1}·kg ww^{-1} (55,70,117), respectively. This suggests that the production of muscle lactate in humans during exercise may simply be a function of widely different capacities between the ability to generate and to oxidize pyruvate, leading to lactate accumulation as the equilibrium enzyme lactate dehydrogenase (9,22,32) maintains lactate concentration 1-2 orders of magnitude above pyruvate concentration (26,24,89,91).

1.8 Rational for Studying PDHc in Human Muscle

The purpose of the present work was two fold. It was first necessary to determine a precise and sensitive means of PDHa and PDHt measurement in very small muscle samples that are typically obtained from a biopsy of human skeletal muscle. The second purpose of the present work was to assess the physiological role of PDHc in human skeletal muscle.

The first purpose was achieved by comparing available radiometric assay systems for determination of PDHa and PDHt and by characterizing their utility for analytical use in skeletal muscle (Appendix A). The second purpose of the present work was met by examining the role of PDHc in the reciprocal cycle of intramuscular fuel utilization known as the glucose-fatty acid cycle and in muscle lactate production.

The role of PDHc in the glucose-fatty acid cycle was investigated in two separate studies. In the first study, a model of dietary manipulation was employed to increase the availability of fat fuel for a prolonged period (Chapter 2). An exercise protocol of glycogen depletion followed by three days of carbohydrate deprivation and consumption of a high fat diet resulted in elevated fat fuel availability and oxidation. This approach induced a prolonged increase in blood borne fat fuel and allowed examination of intramuscular fuel utilization and PDHa at rest and during prolonged heavy exercise. In the second study

(Chapter 3), a method was employed that allowed acute manipulation of the primary signal thought to initiate this mechanism of intramuscular fuel selection. Constant infusion of acetate during rest and incremental exercise was used to acutely elevate the intramuscular acetylCoA-to-CoASH ratio and examine the effect on PDHa and intramuscular fuel use during rest and exercise.

In a third study, the role of PDHc in skeletal muscle lactate production (Chapter 4) was examined using a model of 3 repeated 30 second bouts of maximal isokinetic exercise, each separated by 4 minutes of rest recovery. This protocol induced very large and repeated changes in muscle lactate accumulation and allowed simultaneous examination of the mitochondrial redox state, lactate accumulation and the regulatory factors that determined PDHa transformation.

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CHAPTER 2

PYRUVATE DEHYDROGENASE ACTIVITY AND ACETYL-GROUP ACCUMULATION DURING EXERCISE AFTER DIFFERENT DIETS

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2.1 Abstract

Pyruvate dehydrogenase activity (PDHa) and acetyl-group accumulation were examined in human skeletal muscle at rest and during exercise after different diets. Five males cycled at 75% $\dot{V}O_2$max to exhaustion after consuming a low carbohydrate diet (LCD) for 3 d, and again 1 to 2 wk later for the same duration after consuming a high carbohydrate diet (HCD) for 3 d. Resting PDHa was lower after a LCD (0.20 ± 0.04 vs. 0.69 ± 0.05 mmol/min/kg wet weight (ww); $P < 0.05$) and coincided with a greater intramuscular acetylCoA-to-CoASH ratio, acetyl-CoA and acetylcarnitine. PDHa increased during exercise in both conditions but at a lower rate in the LCD condition compared to the HCD condition (1.46 ± 0.25 vs. 2.65 ± 0.23 mmol.min^{-1}.kg ww^{-1} at 16 min, and 1.88 ± 0.20 vs. 3.11 ± 0.14 at the end of exercise; $P < 0.05$). During exercise muscle acetyl-CoA, acetylcarnitine and the acetylCoA-to-CoASH ratio decreased in the LCD condition but increased in the HCD condition.

Under resting conditions PDHa was influenced mainly by the availability of fat or carbohydrate fuels acting through changes in the acetylCoA-to-CoASH ratio. However during exercise the activation of PDHa occurred independent of changes in the acetylCoA-to-CoASH ratio suggesting that other factors are more important.

2.2 *Introduction*

Skeletal muscle pyruvate dehydrogenase is a multi-enzyme complex (PDHc), located within the inner mitochondrial membrane. This enzyme catalyzes the oxidative decarboxylation of pyruvate to acetyl-CoA and thus controls the flux of pyruvate derived acetyl-CoA into the TCA cycle. The phosphorylation state and hence the activity of PDHc is largely regulated by the concerted action of PDH-kinase and PDH-phosphatase. Dephosphorylation of PDHc by PDH-phosphatase produces the active form (PDHa) and this transformation is dependent on the simultaneous reduction in activity of PDH-kinase. The *in vivo* activity expressed by the PDHa form is also dependent on the availability of substrates and accumulation of products (33).

The *in vivo* regulation of skeletal muscle PDHa is believed to be important to the integration of carbohydrate and fat oxidation within this tissue. The role of PDHa in this regard has been summarized in a theory of metabolic regulation proposed by Randle et al (31,32) which they termed the glucose-fatty acid cycle (G-FA). During starvation or carbohydrate deprivation PDHa is lowered when the ratio of acetyl-CoA-to-CoASH increases secondary to increased oxidation of fat fuels. The resultant physiological benefit is decreased PDHa flux and conservation of intramuscular and whole body glucose stores in the face of decreasing carbohydrate availability.

To date few studies have examined the role of skeletal muscle PDHa in intramuscular fuel selection. Henning et al (23) and Hagg et al (19) reported a lower PDHa in rat hindlimb muscles of starved and diabetic rats at rest and during direct stimulation of the sciatic nerve. Additionally, Hagg et al (19) demonstrated lower PDHa after infusion of acetoacetate. More recently, a decrease in PDHa was observed in rat skeletal muscle as a result of prolonged

moderate exercise when fuel dependence was shifted toward increased fat oxidation (14) and after 48 hr of starvation (24,36). In all of these investigations, increased oxidation of fat fuels coincided with lower PDHa. Studies of isolated intact skeletal muscle mitochondria have confirmed that inhibition of PDHa under these conditions results from the inhibitory effect of increased acetyl-CoA accumulation (1,16).

The role of PDHa in the selection of fuel in human skeletal muscle remains to be explored. The transformation of skeletal muscle PDHc to the active form during low intensity (10) and heavy aerobic exercise (10,38,39) has been established. Furthermore, previous reports examining intramuscular fuel selection in the presence of increased blood borne fat fuels, are consistent with reduced carbohydrate and increased fat oxidation at rest (2,17,20) and during heavy aerobic exercise (13,17), which should coincide with lower PDHa. Nevertheless, to date no study has examined the role of PDHa in intramuscular fuel selection in humans at rest or during heavy aerobic exercise. Therefore the primary purpose of this study was to examine intramuscular PDHa in humans at rest and during prolonged cycling at 75% of $\dot{V}O_2max$, while in a state of prolonged carbohydrate deprivation. It was hypothesized that PDHa would be lower at rest and during exercise after consuming a low carbohydrate diet (LCD) for three days, as compared to consuming a high carbohydrate diet (HCD) for three days. Muscle acetyl-CoA and CoASH accumulation were measured at rest and during heavy aerobic exercise to determine the importance of these metabolites as regulatory signals in determining PDHa in humans.

A secondary purpose of this study was to determine if acetyl-CoA and acetylcarnitine would become depleted at exhaustion and if depletion was related to fatigue in carbohydrate deprived muscle exercised to exhaustion. It has previously been demonstrated that muscle

acetyl-CoA and acetylcarnitine levels do not fall below resting levels at exhaustion (35), which suggests that acetyl-CoA depletion is not related to fatigue during prolonged aerobic exercise in subjects consuming a mixed diet. However, it is not known what effect pre-exercise depletion of muscle glycogen would have on acetyl-group accumulation at exhaustion in prolonged exercise.

2.3 Methods

2.3.1 Subjects

Five trained young males (mean ± SD, $\dot{V}O_2max$: 61.1 ± 8.1 ml kg^{-1} min^{-1}; age 22.8 ± 1.9 yr; height 181.6 ± 2.9 cm, and weight 78.3 ± 9.2 kg) participated in this study. This study had the approval of The Ethics Committee of McMaster University. Written informed consent was obtained from each subject following a presentation of the study and its attendant risks.

2.3.2 Experimental Protocol

Subjects visited the lab on two occasions separated by 1-2 wk. Prior to each visit, subjects underwent an exercise protocol to deplete intramuscular glycogen in the quadriceps muscles. This was followed by 3 d on a LCD that was composed of 46% protein, 51% fat, and 3% carbohydrate, prior to the first visit. Prior to the second visit, each subject consumed a HCD for 3 d that was composed 10% protein, 4% fat and 86% carbohydrate. A dietary record was kept for each subject.

On the morning of the experiment, the brachial artery was catheterized percutaneously with a teflon catheter (20 gauge, 3.2 cm; Parke Davis and Co.) following local

anesthesia with 0.5 ml of 2% xylocaine without epinephrine as described by Bernéus et al (6). The femoral vein was catheterized percutaneously (VC FN 7.5 Cook Co., Bloomington, IN) using the Seldinger technique, following administration of 3-4 ml of xylocaine, also by the method of Bernéus et al (6). The catheters were maintained patent with sterile non-heparinized isotonic saline.

On each visit, one thigh was prepared for needle biopsy of the vastus lateralis with incisions of the skin through to the deep fascia (four on the first visit and three on the second), under local anesthesia (2% xylocaine without epinephrine) as described by Bergström (4). Subjects exercised to exhaustion (EX) at 75% $\dot{V}O_2max$ on an electronically braked bicycle ergometer (Corival 400; Lode, Netherlands) in the LCD condition, and for the same duration (End Exercise: EE) 1-2 wk later in the HCD condition (Fig 1). Measurements of O_2 uptake and CO_2 output were made intermittently using a Quinton metabolic cart (Quinton Q-plex 1; Quinton Instrument Co., WA, USA).

A 20% sterile glucose solution was infused at EX in the LCD condition using the euglycemic clamp method of Coggan and Coyle (8). Briefly, assuming a glucose space equal to 20% of body weight, an initial bolus of 20% glucose was infused to return arterial blood glucose to the initial resting level or higher. This was maintained by the continuous glucose infusion at a rate of 6 ml/min during 6 min of rest and during 7 min of exercise at the same power output (Fig 1). Arterial blood glucose was monitored using an automatic glucose analyzer (Accu-chek II; BMC Canada) throughout the exercise period. EX was indicated when the subject could no longer maintain a pedalling frequency of 60 rpm. Arterial and femoral venous blood samples, and muscle biopsies were taken at the times indicated in Figure 1. Muscle biopsies were taken from the vastus lateralis at the times indicated in

Figure 1.

2.3.3 Muscle Sampling

Muscle biopsies were immediately frozen in liquid N_2, removed from the needle while frozen and stored in liquid N_2 until analyzed. A total of 10-35 mg was chipped from each biopsy and dissected free of blood and connective tissue, under liquid N_2 using a stereomicroscope with paired 10X wide-field eyepieces. From this, two clean 5-20 mg portions were stored separately in liquid N_2: one for the measurement of total pyruvate dehydrogenase activity (PDHt) and the other for measurement of the active fraction (PDHa). The remaining frozen muscle was freeze dried, dissected free of blood and connective tissue, and stored dry at -80°C. A portion of the powdered muscle (4-10 mg) was alkaline extracted and used for glycogen determination and the remaining powdered muscle was extracted in a solution of 0.5 M perchloric acid (PCA) and 1 mM EDTA, neutralized to pH 7.0 with 2.2 M $KHCO_3$ and stored at -80°C until analyzed for metabolites.

2.3.4 Analysis of Muscle

PDHa and total PDH activity (PDHt) were measured in muscle homogenates using the method of Constantin-Teodosiu et al (11) with the following modifications. For the measurement of PDHt, the sample was homogenized in a buffer similar to PDHa with the following differences: exclusion of 50 mM NaF, inclusion of 10 mM $CaCl_2$, 25 mM $MgCl_2$, 4 U/ml hexokinase, 10 mM glucose, 10 mM dichloroacetic acid (DCA), and 0.1% (v:v) triton X-100. No preincubation reagent was used for the measurement of PDHt, as prior preincubation at 37°C led to a loss of activity.

The assay buffer for PDHa and PDHt was slightly modified by increasing the concentration of all coenzymes: NAD^+ to 3.0 mM, CoASH to 1.0 mM, and thiamine pyrophosphate (TPP) to 1.0 mM. During the assay, 200 µl aliquots sampled at 1, 2, and 3 min intervals. The reaction was stopped by addition of each aliquot to 40 µl of 0.5 M PCA. After 5 min each aliquot was neutralized with 1.0 M K_2CO_3, centrifuged for 3 min at 15,900xG (Beckman microcentrifuge E) and the resulting supernatant was stored at -50°C until analyzed for acetyl-CoA by the method of Cederblad et al (7). Plots of acetyl-CoA as a function of time were used to determine the reaction rates.

Muscle glycogen was measured enzymatically as described by Harris et al (22). Neutralized PCA muscle extracts were analyzed for CoASH, acetyl-CoA, carnitine, acetylcarnitine (7), citrate (3), and glucose, glucose-6-phosphate (G-6-P), pyruvate and lactate (22).

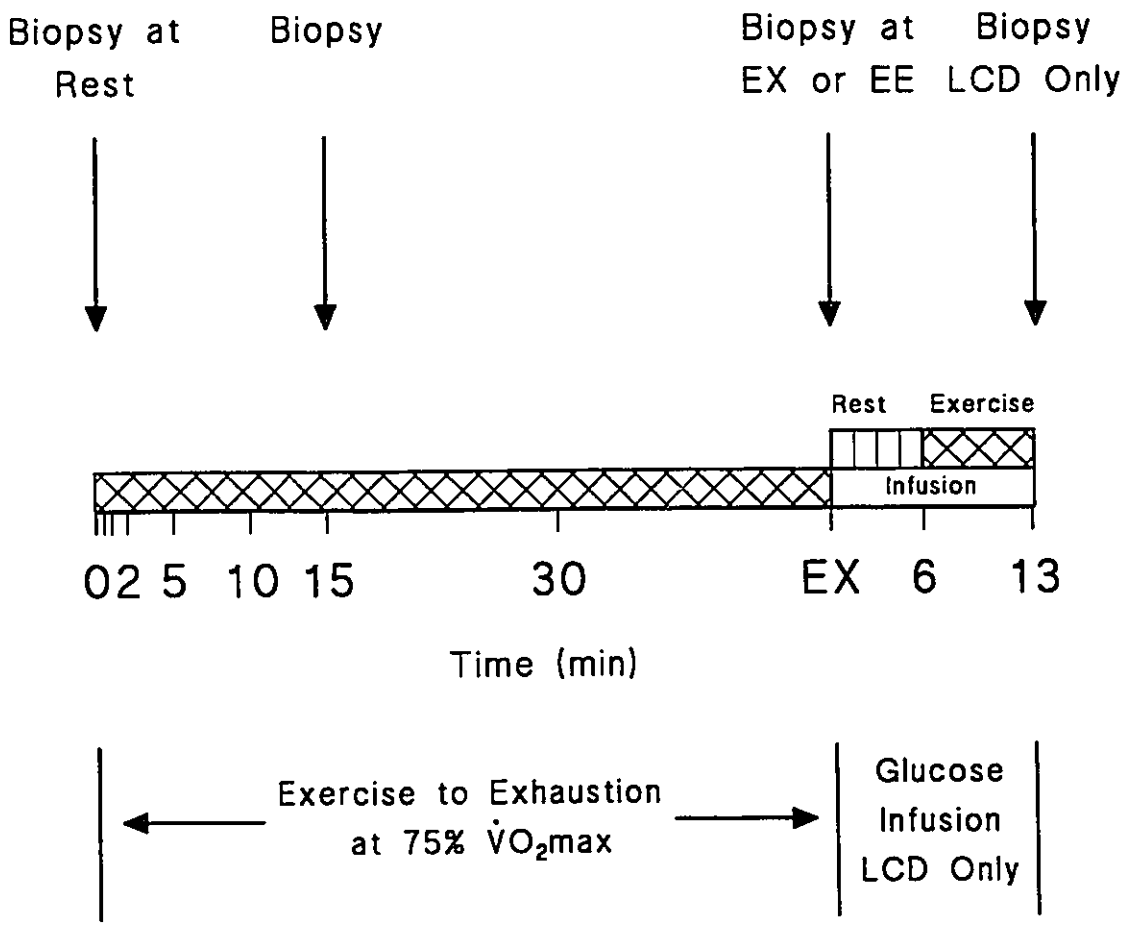
2.3.5 Blood Sampling, Blood Analysis and Calculation of Blood Flow

Blood samples (~ 6 ml) were drawn from the brachial artery and femoral vein in heparinized plastic syringes and placed on ice. One portion of each blood sample was deproteinized in two volumes of 6% PCA, stored at -20°C and subsequently analyzed for lactate, glycerol, glucose and citrate as described in Bergmeyer (3). A second portion was centrifuged at 15,900xG for 2 min and the plasma supernatant was analyzed for free fatty acids using the Wako NEFA C test kit supplied by Wako Chemical Inc. (VA, USA). Blood flow was calculated from pulmonary $\dot{V}O_2$ and arterio-venous O_2 difference (Table 1) as described by Jorfeldt and Wahren (26).

2.3.6 Statistical Analysis

Analysis of muscle metabolite data and blood substrate data were carried out by a two way analysis of variance (ANOVA) with repeated measures to compare measurements over time. When a significant F-ratio was found the Newman-Keuls post hoc test was used to compare means. Respiratory measures, arterial minus venous O_2 content difference, blood flow and muscle pyruvate content measures were compared between conditions using a paired dependent samples t-test as no comparisons were made over time. Results were considered significant at a $p<0.05$.

FIGURE 1. Experimental protocol. Arterial and venous blood samples were taken at the times indicated. Muscle biopsies were taken as indicated. EX, indicates exhaustion in the low carbohydrate diet (LCD) condition. EE, indicates the end of the exercise period in the high carbohydrate diet (HCD) condition.


Biopsy at Rest
Biopsy
Biopsy at EX or EE
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Rest
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02 5 10 15 30 EX 6 13
Time (min)
Exercise to Exhaustion at 75% $\dot{V}O_2$max
Glucose Infusion LCD Only

2.4 *Results*

2.4.1 *Exercise Performance, Respiratory Gas Exchange and Blood Flow*

Cycling time to EX in the LCD condition was 47.7 ± 5.5 min (mean ± SEM) and exercise was terminated at the same time in the HCD condition. Whole body $\dot{V}O_2$ was similar in the two conditions (Table 1), but $\dot{V}CO_2$ and RER were both lower in the LCD condition at 5 min and at steady state (Table 1). At steady state the RER was 0.80 ± 0.03 and 0.90 ± 0.02 in the LCD and HCD conditions, respectively. Ventilation ($\dot{V}_E$) was lower in the LCD condition at 5 min but did not differ between conditions during the first min or during steady state (Table 1). Estimated blood flow to the lower limbs during steady state exercise was ~13.5 and ~14.5 l/min (Table 1) in the LCD and HCD conditions, respectively. No significant differences were observed between the LCD and HCD conditions except during the first minute.

2.4.2 *Blood Metabolites*

2.4.2.1 *Glucose*

Arterial and venous samples were not corrected for fluid shifts as there was no significant difference in hemoglobin or hematocrit between the two conditions (data not shown). In the LCD condition, [glucose] was 4.47 ± 0.10 mM at rest and decreased to 3.79 ± 0.70 mM at 30 min exercise and to 2.38 ± 0.21 mM at EX (Table 2). There was a net uptake of glucose throughout exercise in LCD except for the first 5 min (Table 3). Infusion of glucose during the second exercise period following EX elevated [glucose] to 6.48 ± 0.31 mM at 5 min (Table 2). In the HCD condition, [glucose] was 5.27 ± 0.29 mM at rest and did not change throughout the exercise period (Table 2).

2.4.2.2 Lactate

Arterial [Lac^-] increased in the LCD condition from 0.50 ± 0.03 mM at rest to a maximum of 3.90 ± 0.73 mM at 5 min of exercise and continuously declined to 2.59 ± 0.81 mM at EX. In the HCD condition arterial blood [Lac^-] increased from a resting level of 0.97 ± 0.17 mM to 5.90 ± 0.69 mM by the fifth min of exercise and remained elevated throughout the exercise period (Table 2).

2.4.2.3 Free Fatty Acids and Glycerol

In the LCD condition [FFA] was 1.26 ± 0.13 mM at rest and decreased to 0.57 ± 0.04 mM at 5 min and remained at this level until increasing to 0.81 ± 0.10 mM at 30 min and 0.98 ± 0.16 mM at EX. There was a net uptake of FFA during this time (Table 3). In the HCD condition, the arterial [FFA] was 0.56 ± 0.15 mM at rest, decreased to 0.36 ± 0.11 mM at 2 min and did not change thereafter during the exercise period (Table 2). A net uptake of FFA was observed during exercise (Table 3).

Arterial [glycerol] also increased in the LCD condition during the exercise period, ~3 fold over rest at 10 min and almost 10 fold at EX (Table 2). [Glycerol] was significantly greater in the LCD condition as compared to the HCD condition, from 10 min until the end of the exercise period. There was a net release of glycerol in both conditions (Table 3).

2.4.3 Muscle Metabolites

2.4.3.1 Glycogen

Glycogen content was significantly greater in the HCD condition at all time points studied (Figure 2). In the LCD condition, glycogen content decreased from 185.0 ± 21.6

mmol glucosyl U/kg dry weight (dw) at rest, to 69.6 ± 19.6 at 16 min and to 32.4 ± 18.0 at EX and did not change during the glucose infusion period (38.5 ± 19.9 mmol/kg dw). In the HCD condition glycogen content was 655.3 ± 79.6 mmol/kg dw at rest, decreased to 489.3 ± 43.6 at 16 min and further to 253.2 ± 44.4 mmol/kg dw at the end of the exercise period. The rate of glycogen degradation during exercise was significantly greater ($p<0.01$) in the HCD condition (9.5 ± 1.08 mmol/kg dw/min) than in the LCD condition (3.5 ± 0.65 mmol/kg dw/min). During the period 16 min to EX the glycogenolytic rate was reduced to ~1.2 mmol/min/kg dw (0.3 mmol/min/kg wet weight (ww)) in the LCD condition and 7.5 mmol/min/kg dw (1.7 $mmol \cdot min^{-1} \cdot kg\ ww^{-1}$) in the HCD condition.

2.4.3.2 Glucose, Glucose-6-phosphate (G-6-P), Lactate, Pyruvate and Citrate

Muscle glucose content was lower in the LCD condition but this difference was significant only during exercise (Table 4). At 16 min exercise glucose content increased to 2.39 ± 0.37 mmol/kg dw in LCD condition and 5.32 ± 1.22 mmol/kg dw in the HCD cond Glucose content decreased to 0.69 ± 0.16 mmol/kg dw at EX in the LCD condition, but continued to rise in the HCD condition. G-6-P content was similar in the two conditions at rest but decreased during exercise in the LCD condition and increased in the HCD condition (Table 4).

Both pyruvate and lactate content were lower in the LCD condition during exercise (Table 4). Resting muscle citrate content was 40% greater after consuming a LCD for 3 d as compared to consuming a HCD for 3 d (Table 4) and rose to similar values in the two conditions during exercise. At the end of the glucose infusion period citrate content tended to be lower than at rest.

2.4.3.3 Acetyl Group Accumulation

Resting muscle acetyl-CoA content (Figure 3) was ~ ~ fold greater after consuming a LCD diet than after a HCD diet (39.3 ± 8.6 μmol/kg dw compared to 13.5 ± 2.1). In the LCD condition, cycling resulted in a significant and continued decrease throughout the exercise period and glucose infusion did not reverse this fall. Acetyl-CoA content was 17.2 ± 1.7, 16.2 ± 1.8 and 15.9 ± 1.6 μmol/kg dw at 16 min, EX and after glucose infusion, respectively. Conversely, in the HCD condition acetyl-CoA content rose during cycling to 32.2± 2.3 μmol/kg dw at 16 min and to 37.6 ± 3.1 at the end of the exercise period.

Changes in acetyl-CoA content (Figure 3) and CoASH content (Figure 3) were reciprocal only in the HCD condition. In the LCD condition, CoASH tended to decline during exercise while acetyl-CoA also decreased. CoASH content (Figure 3) was 43.4 ± 1.1 μmol/kg dw at rest, 26.6 ± 4.7 at 16 min and 37.4 ± 8.5 at EX in LCD. No change was observed after glucose infusion (31.9 ± 4.7 μmol/kg dw). In the HCD condition CoASH content was 55.3 ± 6.3 μmol/kg dw at rest, 34.3 ± 2.1 at 16 min and 30.3 ± 4.2 at EX.

The resting acetylCoA-to-CoASH ratio (Figure 4) was significantly greater in the LCD condition (0.76 ± 0.21) than in the HCD condition (0.24 ± 0.02). Thereafter, in the LCD condition, the acetylCoA-to-CoASH ratio decreased to 0.68 ± 0.17 at 16 min and to 0.47 ± 0.17 at EX and did not change during glucose infwion (0.56 ± 0.13). Conversely, in the HCD condition the acetylCoA-to-CoASH ratio rose significantly to 0.93 ± 0.11 at 16 min and to 1.26 ± 0.05 at EE.

Resting acetylcarnitine content was also higher after a LCD than after a HCD (11.1 ± 1.1 mmol/kg dw compared to 2.5 ± 0.9) (Figure 3). During exercise, changes in acetylcarnitine content (Figure 3) paralleled changes in acetyl-CoA, decreasing in the LCD

condition to 8.8 ± 0.8 mmol/kg dw at 16 min and to 6.9 ± 1.4 at EX, and increasing in the HCD condition to 12.1 ± 1.0 at 16 min and to 12.5 ± 0.8 at EE. Glucose infusion had no effect on acetylcarnitine or acetyl-CoA accumulation.

Changes in free carnitine content were reciprocal with changes in acetylcarnitine in both conditions (Figure 3). In the LCD condition, free carnitine was 8.1 ± 1.7 mmol/kg dw at rest, 7.8 ± 1.5 at 16 min and 9.9 ± 1.7 at EX. The free carnitine content remained unchanged during glucose infusion. In the HCD condition, free carnitine was 15.5 ± 1.9 at rest, 4.6 ± 0.7 at 16 min and 3.5 ± 0.8 at EE.

2.4.4 Pyruvate Dehydrogenase Complex

Resting PDHa (Figure 4) was significantly lower in the LCD condition (0.20 ± 0.04 $mmol \cdot min^{-1} \cdot kg\ ww^{-1}$) than in the HCD condition (0.69 ± 0.05 $mmol \cdot min^{-1} \cdot kg\ ww^{-1}$). During exercise the PDHa fraction increased in both conditions but at a slower rate after a LCD compared to after a HCD (ie. 1.46 ± 0.25 $mmol \cdot min^{-1} \cdot kg\ ww^{-1}$ compared to 2.65 ± 0.25 at 16 min and 1.88 ± 0.20 compared to 3.11 ± 0.14 at the end of exercise). There was no further increase in PDHa during glucose infusion: it was 2.06 ± 0.25 $mmol \cdot min^{-1} \cdot kg\ ww^{-1}$.

The total PDHc activity (PDHt) did not differ within or between conditions during exercise. However, resting values for PDHt were lower than those observed for exercise samples within each condition. In the LCD condition, PDHt was 1.50 ± 0.17, 2.20 ± 0.25, 2.37 ± 0.18 and 2.40 ± 0.24 $mmol \cdot min^{-1} \cdot kg\ ww^{-1}$ at rest, 16 min, EX and after glucose infusion, respectively. In the HCD condition, PDHt was 1.85 ± 0.13, 2.70 ± 0.19 and 2.87 ± 0.22 $mmol \cdot min^{-1} \cdot kg\ ww^{-1}$ at rest, 16 min and EE, respectively. The greater PDHt during exercise compared to rest should not be viewed as an increase in intramuscular PDHc

content during the rest-to-work transition but merely the incomplete transformation of resting samples of PDHc *in vitro*. Complete transformation of PDHc was observed in exercise samples in both the LCD and HCD conditions. This is evidenced by the fact that there were no significant differences between PDHt measures within or between conditions during exercise when complete transformation is known to occur (10).

Despite minor fluctuations in resting PDHt values, the relative pattern of PDHa inhibition observed in the LCD condition compared to the HCD condition remains unaffected. The proportion of the PDHa fraction relative to PDHt was significantly lower in the LCD condition: 14.2 ± 4.4 % at rest, 65.9 ± 9.2 % at 16 min and 80.0 ± 7.4 % at EX. Complete activation was not achieved until the glucose infusion period, where it was 90.2 ± 5.2 %. Conversely, in the HCD condition PDHa transformation was higher at rest (37.8 ± 3.2 %) and was complete after 16 min of exercise (98.0 ± 4.5 %) and at EE (109 ± 6.1 %).

TABLE 1. Oxygen uptake, carbon dioxide production, respiratory exchange ratio, ventilation, oxygen content and blood flow data during cycle ergometry at 75% $\dot{V}O_2$max following either a low carbohydrate diet (LCD) or a high carbohydrate diet (HCD) for three days.

MEASURE	CONDITION	1 min	5 min	Steady State
$\dot{V}O_2$ (l/min)	LCD	2.31 ± 0.22	3.42 ± 0.17	3.46 ± 0.16
	HCD	2.21 ± 0.20	3.35 ± 0.11	3.29 ± 0.12
$\dot{V}CO_2$ (l/min)	LCD	1.51 ± 0.13	2.81 ± 0.07†	2.76 ± 0.13†
	HCD	1.68 ± 0.18	3.16 ± 0.12	2.97 ± 0.16
RER	LCD	0.65 ± 0.01†	0.83 ± 0.03†	0.80 ± 0.03†
	HCD	0.76 ± 0.02	0.95 ± 0.03	0.90 ± 0.02
$\dot{V}_E$ (l/min)	LCD	46.0 ± 4.1	81.0 ± 4.9†	92.4 ± 2.7
	HCD	46.7 ± 5.5	87.7 ± 4.3	92.9 ± 2.6
d(A-V)O_2 Content	LCD	14.3 ± 1.3	15.9 ± 0.9	16.0 ± 0.4
	HCD	15.2 ± 0.5	16.0 ± 0.4	16.8 ± 0.2
Blood Flow (l/min)	LCD	10.1 ± 1.2	11.5 ± 2.4	14.5 ± 1.0
	HCD	9.7 ± 0.2	10.4 ± 1.3	13.5 ± 0.9

Data are means ± SEM. Sample size is n=4. †, significantly different from matched times between conditions. Steady state measures were collected or calculated between 10 min and 30 min of exercise at 75% $\dot{V}O_2$max. d(A-V)O_2 is arterial minus venous oxygen content. Leg blood flow was calculated for two legs.

TABLE 2. Arterial concer ıtion of blood borne substrates during cycle ergometry at 75% $\dot{V}O_2$max following either a low carbohydrate diet (LCD) or a high carbohydrate diet (HCD) for three days.

MEASURE	CONDITION	Rest	30 sec	1 min	2 min	5 min	10 min	15 min	30 min	EX/EE	2 min In	5 min In
Glucose (mmol/l)	LCD	4.47 0.10	4.70 0.11	4.84 0.09	4.79 0.12	4.84 0.14	4.77 0.28	4.60 0.31	3.79 *† 0.70	2.38 *† 0.21	6.96 * 0.47	6.48 * 0.31
	HCD	5.27 0.29	5.30 0.19	5.27 0.26	5.33 0.21	5.09 0.08	4.75 0.22	4.72 0.29	5.28 0.34	5.50 0.31	NA	NA
Lactate (mmol/l)	LCD	0.50 0.03	0.50 0.05	1.62 0.16	3.22 *† 0.46	3.90 *† 0.73	3.37 *† 0.91	3.42 *† 0.85	2.70 *† 0.84	2.59 *† 0.81	2.52 * 0.67	2.42 * 0.73
	HCD	0.97 0.17	1.49 0.33	2.90 * 0.16	4.79 * 0.29	5.90 * 0.69	5.75 * 0.99	5.91 * 0.99	5.97 * 1.12	5.81 * 1.24	NA	NA
FFA (mmol/l)	LCD	1.26 † 0.13	1.26 † 0.13	1.16 † 0.12	0.82 *† 0.07	0.57 *† 0.04	0.52 *† 0.07	0.57 *† 0.10	0.81 † 0.10	0.98 0.16	1.40 0.28	1.08 0.25
	HCD	0.56 0.15	0.56 0.16	0.52 0.15	0.36 * 0.11	0.31 0.10	0.33 * 0.10	0.32 * 0.11	0.37 * 0.13	0.41 0.12	NA	NA
Glycerol (μmol/l)	LCD	61.3 8.0(3)	96.0 8.6	93.5 8.6	98.8 8.3	122.0 18.4	209.5 *† 31.3	295.0 *† 43.6	485.8 *† 63.4	643.8 *† 111.1	607.8 * 8.7	589.3 * 73.8
	HCD	31.3 6.8	34.3 8.2	29.3 6.5	30.0 7.0	48.8 7.6	76.5 17.4	90.5 23.5	131.3 * 37.9	181.0 * 58.5	NA	NA

Data are means ± SEM. Sample size is n=4, except where sample size (n) is indicated in parenthesis. †, significantly different from matched times between conditions. *, significantly different from rest of the same condition. NA indicates not applicable. EX, indicates exhaustion in the LCD condition. EE, indicates end exercise in the HCD condition. In, indicates glucose infusion period in the LCD condition.

TABLE 3. Arterial-Venous concentration difference of blood borne substrates during cycle ergometry at 75% $\dot{V}O_2$max following either a low carbohydrate diet (LCD) or a high carbohydrate diet (HCD) for three days.

MEASURE	CONDITION	Rest	30 sec	1 min	2 min	5 min	10 min	15 min	30 min	EX/EE	2 min In	5 min In
Glucose (mmol/l)	LCD	0.02 0.08	0.03 0.02	0.06 0.09	-0.06 0.07	-0.05 0.06	0.16 0.08	0.19 0.13	0.28 0.06	0.16 0.08	0.23 0.24	0.38 0.18
	HCD	0.21 0.08	0.11 0.07	0.02 0.05	-0.07 0.13	0.04 0.02	0.06 0.08	-0.01 0.06	0.17 0.04	0.22 0.07	NA	NA
Lactate (mmol/l)	LCD	0.01 0.00	-0.73 *‡ 0.24	-0.69 *‡ 0.36	-0.48 0.34	-0.29 0.28	-0.08 0.09	-0.30 0.09	0.02 0.03	-0.03 0.03	0.01 0.09	0.03 0.04
	HCD	-0.09 0.03	-0.73 *‡ 0.15	-0.94 *‡ 0.23	-0.43 0.19	-0.00 0.13	-0.11 0.21	0.09 0.12	-0.05 0.06	0.01 0.10	NA	NA
FFA (mmol/l)	LCD	-0.04 0.12	0.02 0.03	0.06 0.02	0.27 0.14	0.06 0.03	0.04 0.01	0.05 0.04	0.03 0.08	0.13 0.04	-0.10 0.13	0.12 0.16
	HCD	0.11 0.06	0.03 0.02	0.07 0.01	0.02 0.02	0.02 0.01	0.05 0.02	0.03 0.01	0.01 0.05	0.01 0.02	NA	NA
Glycerol (μmol/l)	LCD	-40.5 19.9	-37.5 2.4	-4.3 3.7	-5.5 2.6	-8.5 4.3	-8.3 5.4	0.3 12.3	-33.0 16.3	-29.8 16.5	-8.8 16.4	-21.5 16.5
	HCD	-3.5 2.5	1.3 3.7	-2.5 2.4	-9.8 3.9	-3.5 1.4	-1.8 4.4	-3.3 4.0	-4.8 5.6	-7.5 3.8	NA	NA

Data are means ± SEM. Sample size is n=4. *, significantly different from rest of the same condition. ‡, significantly different from zero. †, significantly different from matched times between conditions. NA, indicates not applicable. EX, indicates exhaustion in the LCD condition. EE, indicates end exercise in the HCD condition. In, indicates infusion period in the LCD condition.

TABLE 4. Muscle metabolite content in the vastus lateralis during cycle ergometry at 75% $\dot{V}O_2$max following either a low carbohydrate diet (LCD) or a high carbohydrate diet (HCD) for three days.

MEASURE	LCD				HCD		
mmol/kg dw	Rest	16 min	Exhaustion	Infusion	Rest	16 min	End Exercise
Glucose	1.41 ± 0.27 (4)	2.39 ± 0.37 *†	0.69 ± 0.16 †	1.80 ± 0.52	1.59 ± 0.27	5.32 ± 1.22 *	7.21 ± 2.87 * (4)
G-6-P	1.54 ± 0.44 (4)	1.34 ± 0.32 †	0.75 ± 0.17 †	0.99 ± 0.30	1.32 ± 0.19	3.40 ± 0.24 *	3.05 ± 0.64 * (4)
Pyruvate	NM	0.52 ± 0.06 ††	0.62 ± 0.07 (3)	NM	NM	0.80 ± 0.10	0.86 ± 0.16 (3)
Lactate	2.4 ± 0.45 (4)	16.3 ± 5.05 *†	6.0 ± 1.07 †	5.8 ± 0.90	5.5 ± 0.96	35.4 ± 6.76 *	33.4 ± 12.65*(4)
Citrate	0.70 ± 0.10	0.93 ± 0.10	0.79 ± 0.18	0.60 ± 0.09	0.50 ± 0.08	0.91 ± 0.09 *	1.06 ± 0.16 * (4)

Data are means ± SEM. Sample size is n=5, except where sample size (n) is indicated in parenthesis. †, significantly different from matched times between conditions. *, significantly different from rest of the same condition. ††, indicates significantly different from matched times between conditions using the dependent samples paired t-test. NM indicates not measured.

FIGURE 2. Muscle glycogen content during exercise to exhaustion (EX) in the LCD condition (open symbols) and for the same duration (EE: End Exercise) in the HCD condition (closed symbols). *, indicates significantly different from rest. +, indicates significantly different from the HCD condition.

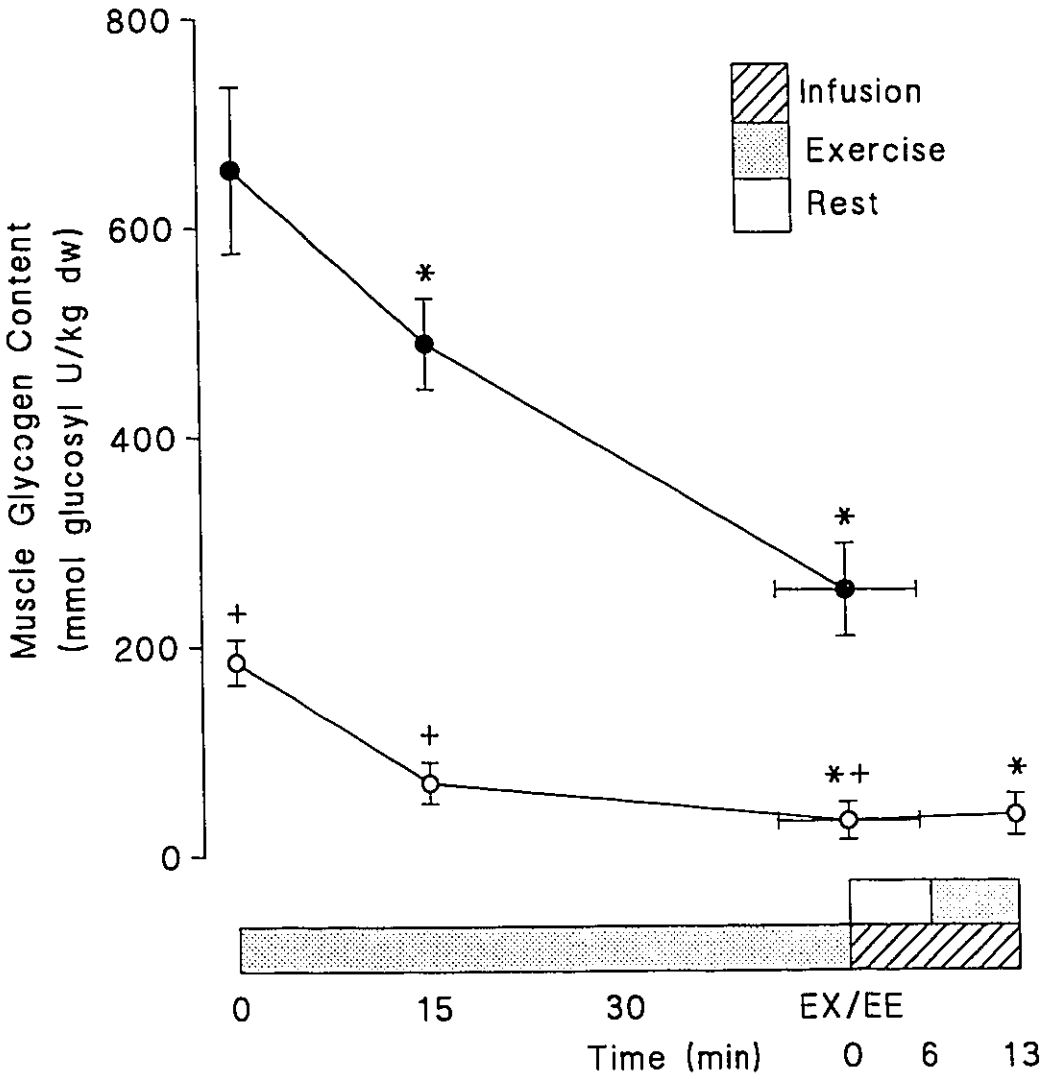

800
600
400
200
0
Muscle Glycogen Content
(mmol glucosyl U/kg dw)
Infusion
Exercise
Rest
*
*
+
+
*+
*
0
15
30
EX/EE
Time (min)
0
6
13

FIGURE 3. Muscle CoASH, acetylCoA, carnitine, and acetylcarnitine content during exercise to exhaustion (EX) in the LCD condition (open symbols) and for the same duration (EE: End Exercise) in the HCD condition (closed symbols). *, indicates significantly different from rest. +, indicates significantly different from the HCD condition.

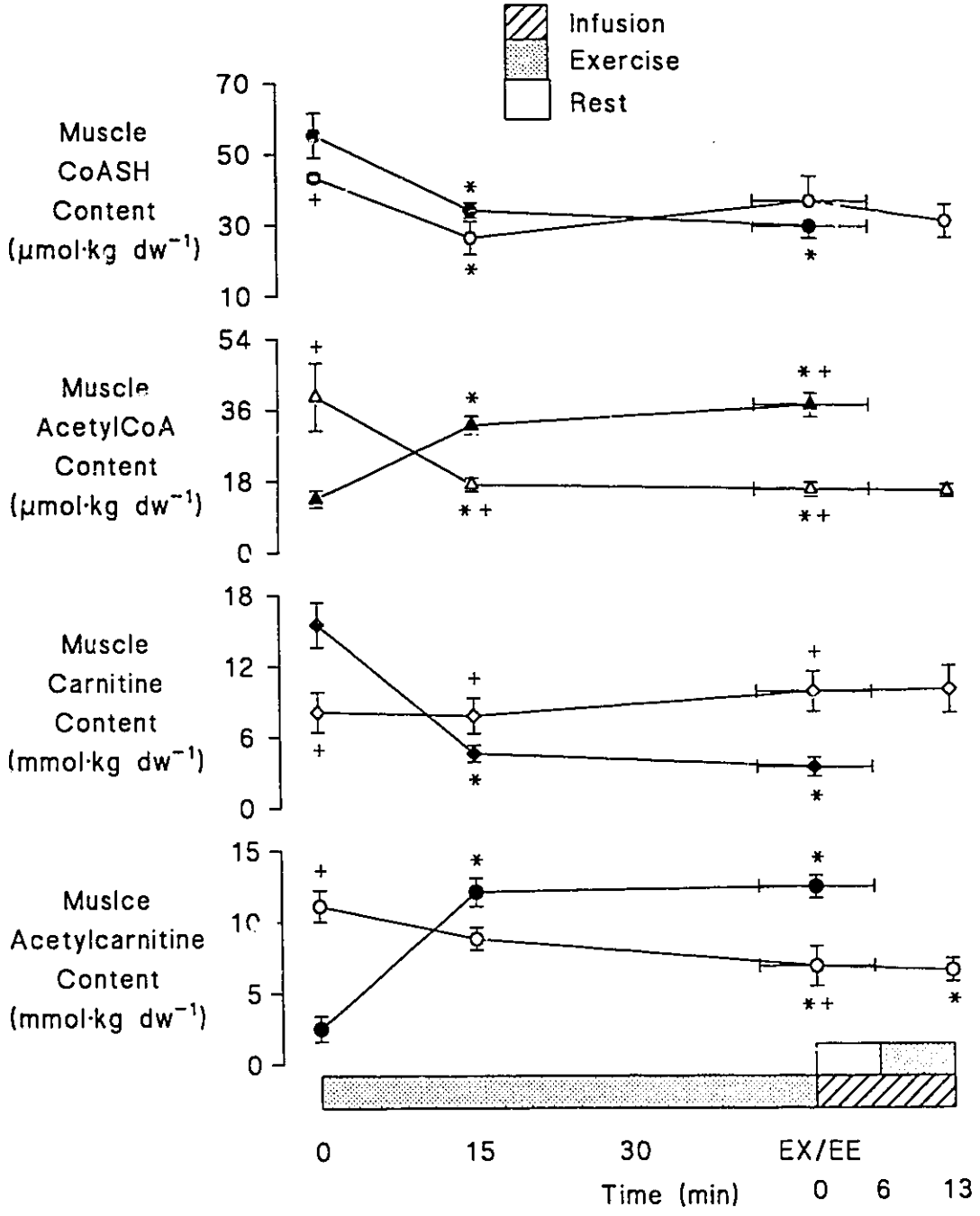
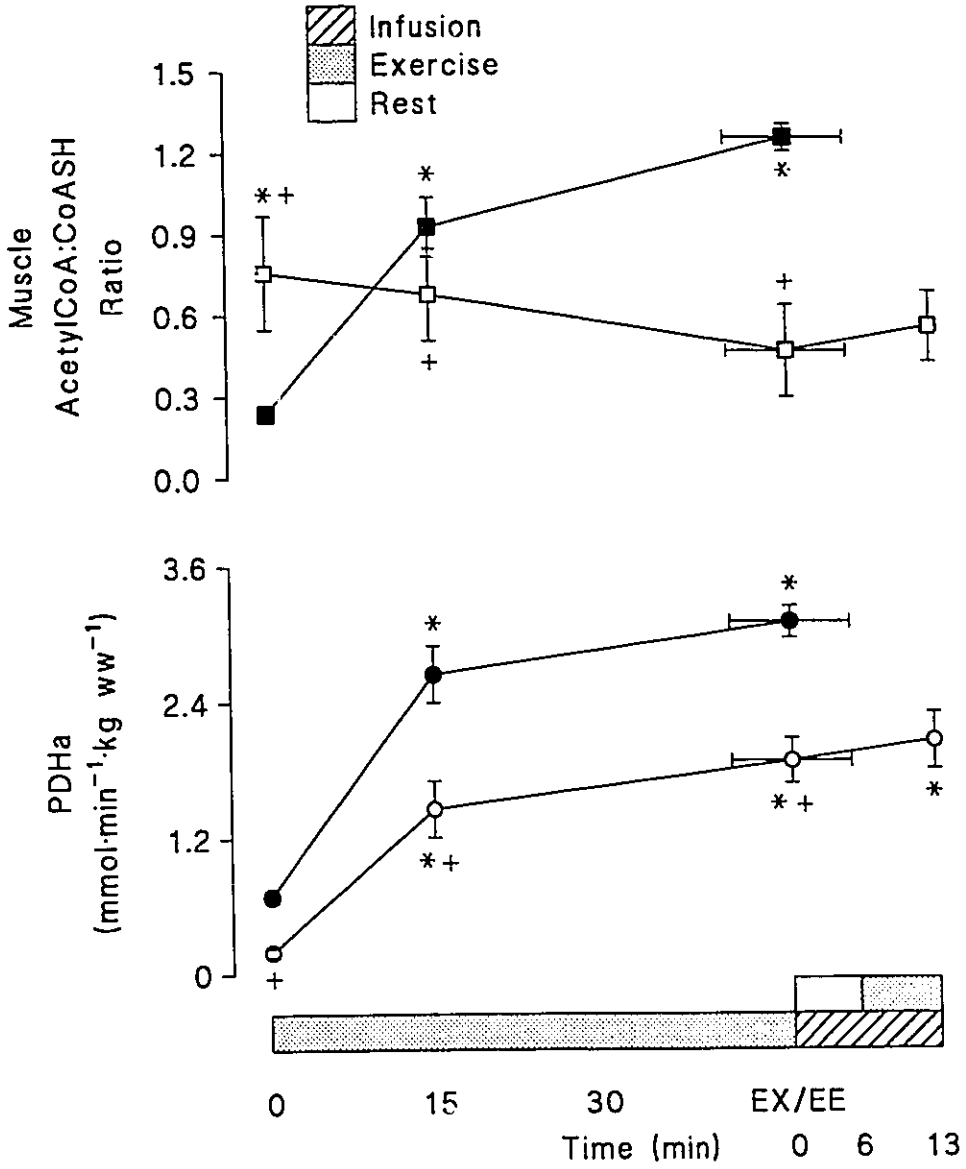

Infusion
Exercise
Rest
Muscle CoASH Content (μmol·kg dw⁻¹)
70
50
30
10
Muscle AcetylCoA Content (μmol·kg dw⁻¹)
54
36
18
0
Muscle Carnitine Content (mmol·kg dw⁻¹)
18
12
6
0
Muslce Acetylcarnitine Content (mmol·kg dw⁻¹)
15
10
5
0
0
15
30
EX/EE
Time (min)
0
6
13

FIGURE 4. Muscle pyruvate dehydrogenase activity (PDHa) and the muscle acetyl-CoA:CoASH ratio during exercise to exhaustion (EX) in the LCD condition (open symbols) and for the same duration (EE: End Exercise) in the HCD condition (closed symbols). *, indicates significantly different from rest. +, indicates significantly different from the HCD condition.


Infusion
Exercise
Rest
Muscle
AcetylCoA:CoASH
Ratio
1.5
1.2
0.9
0.6
0.3
0.0
PDHa
(mmol·min⁻¹·kg ww⁻¹)
3.6
2.4
1.2
0
0
15
30
EX/EE
Time (min)
0
6
13

2.5 DISCUSSION

In the present study, exercise and dietary alterations were used to change fuel utilization during prolonged exercise and measurements were made of some factors known to regulate the activity of the PDH complex. The pre-exercise treatment of exhaustive exercise followed by 3 d of diet manipulation was successful in reducing the intramuscular glycogen content in the LCD condition (Figure 2) and in altering the type of fuel made available to exercising muscle at rest and during exercise (Table 2). In the LCD condition these changes were similar to those reported for starvation (29), with increased delivery of FFA, and possibly ketone bodies, to muscle at rest and throughout exercise. While blood glucose did not differ between conditions at rest and for most of the exercise period, in the LCD condition glucose supply was diminished between 15 and 30 min (Table 2) and hypoglycemia coincided with exhaustion, suggesting depletion of liver glycogen (5,30). Coincidental with this was a switch in fuel availability and utilization to a greater dependence on fat in the LCD condition which is consistent with previous reports (see Coggan (9) for review).

2.5.1 PDHa

Control of PDH activity was associated with intramuscular accumulation of acetyl-CoA from the oxidation of FFA. The lower PDHa in the LCD condition at rest (Figure 4) appears to have resulted from the direct inhibitory action of excess acetyl-CoA accumulation and increase in the acetyl-CoA:CoASH ratio. An acetyl-CoA:CoASH ratio of 0.76 as observed at rest in the LCD condition, has been shown *in vitro* to result in PDHa that is 10% to 50% lower than control values (15). Accordingly, the lower PDHa observed at rest after

a LCD appears to have been due, at least in part, to the accumulation of acetyl-CoA which activated the regulatory subunit, PDH-kinase (16).

During exercise at 75% $\dot{V}O_2$max PDHa increased by 5 to 9 fold in both conditions despite different acetyl-CoA:CoSH ratios (Figure 4). PDHa increased from 0.20 at rest to 1.88 mmol·min^{-1}·kg ww^{-1} after a LCD while the acetyl-CoA:CoASH ratio decreased simultaneously from 0.76 to 0.47. After a HCD, PDHa increased from 0.69 to 3.11 and the acetyl-CoA:CoASH ratio increased simultaneously from 0.25 to 1.28. The apparent abscence of a relationship between PDHc transformation and acetyl-CoA:CoASH ratio during exercise is in contrast to the data of Erfle and Saur (15) who reported 50-60% inhibition of PDHa with similar increases in the acetyl-CoA:CoA ratios as we observed after a HCD. It can be concluded that the possible regulatory effect of the acetyl-CoA:CoASH ratio on PDHc transformation in resting muscle is overridden by other factors during exercise.

The regulation of PDHc transformation to the active form is dependent on the balance between the activating PDH-phosphatase and the inhibitory PDH-kinase. PDH-phosphatase itself is activated by increased [Ca^{2+}] during exercise which was presumably similar in the LCD and HCD conditions since the power output was identical in the two conditions. PDH-kinase activity is inhibited by ADP which may accumulate in the mitochondria during exercise but this effect is difficult to evaluate since intramitochondrial ADP concentration is unknown. Pyruvate also inhibits PDH-kinase in skeletal muscle apparently at a concentration of about 3 mM in the presence of Ca^{2+} (16), which is one to two orders of magnitude greater than the concentrations observed in this study. However, the K_i for pyruvate in other tissues (12,25,27,28) is much lower (~0.08 to 0.50 mM) and until the K_i of pyruvate is confirmed in skeletal muscle, the possibility remains that it may be

similar in magnitude to the pyruvate concentrations that we observed in our subjects. Pyruvate concentration varied between 0.03 and 0.2 mM in the LCD condition, and 0.06 and 0.3 mM in the HCD condition (calculated from pyruvate contents given in table 4, assuming an intracellular water content of 3 l per kg dw). Thus it is possible that pyruvate may exert a significant regulatory effect on PDH-kinase.

Changes in circulating insulin levels also may have had an impact on PDHc transformation. It is known that starvation and diabetes increase PDH-kinase activity (16) and that PDH-phosphatase can be activated by insulin (37). While plasma insulin was not analyzed in this study, it is known that consumption of a LCD for 3 d will decrease plasma insulin, whereas consumption of a HCD for 3 d will have the opposite effect (17). Dietary induced changes in insulin may thus explain, in part, the observed differences in PDHc transformation in resting muscle and also during the exercise period.

The measured PDHa fraction which we report here reflects the potential for flux through the PDHa reaction but in some situations may not represent the actual *in vivo* flux, since *in vivo* flux is also determined in part by the availability of substrates and accumulation of products. Our measurements of PDHa do not reflect this latter aspect of regulation. However, knowing the rate of glycogen degradation, glucose uptake and lactate production or uptake by the muscle, it is possible to calculate flux through the PDHa reaction. In the HCD condition glycogen degradation was 1.7 $mmol \cdot min^{-1} \cdot kg\ ww^{-1}$ from 16 min to EX (Figure 2). Assuming a mean arterio-venous concentration difference (mean value 15 min to EX/EE; Table 3) of 0.13 mmol/l for glucose and 0.017 mmol/l for lactate and leg blood flow of 13.5 l/min (Table 1), glucose and lactate uptake can be calculated to be 1.8 mmol/min and 0.2 mmol/min, respectively. Then, assuming a mean active muscle mass of about 10 kg it is

possible to calculate that glucose flux was 0.18 mmol·min^{-1}·kg ww^{-1} and lactate uptake was 0.02 mmol·min^{-1}·kg ww^{-1}. Conversion of these three measures to pyruvate equivalents, addition of glycogen, glucose and lactate contributions and correction for anaplerotic reactions involving pyruvate (~0.11 mmol·min^{-1}·kg ww^{-1}) (34) gives the resultant flux through the PDHa reaction (~3.7 mmol·min^{-1}·kg ww^{-1}) which was similar to the measured PDHa (3.11 mmol·min^{-1}·kg ww^{-1}) at EE.

Similar calculations in the LCD condition yield a glycogen degradation rate, glucose flux and lactate efflux of 0.27, 0.31 and 0.15 mmol·min^{-1}·kg ww^{-1}, respectively. Thus, the resultant flux through the PDHa reaction is 0.9 mmol·min^{-1}·kg ww^{-1}. This value is considerably lower than PDHa observed at EX (1.88 mmol·min^{-1}·kg ww^{-1}) and illustrates the disparity between measured PDHa and *in vivo* flux when carbohydrate supply became limiting in the LCD condition. Transformation of PDHa was in excess of flux, with flux appearing to be limited by pyruvate availability at exhaustion.

Increased [Ca^{2+}] must be an important factor regulating PDHc transformation during exercise, while differences in plasma insulin induced by the two diets could explain the lower PDHa after a LCD at rest and during exercise. Additionally, acetyl-CoA accumulation may explain the lower PDHa in resting muscle in the LCD condition, but this effect seems to be overridden by the Ca^{2+}-induced activation of PDH-phosphatase during exercise.

2.5.2 Acetyl Group Accumulation

Acetyl-groups accumulate when the formation of acetyl-CoA is greater than the rate at which it is utilized by the TCA cycle. During intense aerobic exercise acetyl-CoA is formed primarily by the PDHc reaction under normal dietary conditions (10) and the acetyl-groups

formed are transferred from acetyl-CoA to free carnitine in the cytosol (21). This transfer regenerates sufficient CoASH to maintain the activity of PDHa (10) and other CoASH-dependent intramitochondrial dehydrogenases.

In this study acetylcarnitine accumulation mirrored acetyl-CoA accumulation at rest and throughout the exercise period in both conditions (Figure 3) as has previously been reported (21,35). The LCD resulted in a large accumulation of acetyl-CoA and acetylcarnitine at rest but by 16 min of exercise acetyl-CoA had decreased from 39.3 to 17.2 μmol/kg dw and acetylcarnitine from 11.1 to 8.8 mmol/kg dw. Similar levels have been previously reported at the end of exhaustive exercise (35). However, our subjects were able to continue exercising at this new lower set point of acetyl-CoA and acetylcarnitine for an additional 32.7 min until exhaustion occurred. During this time most of the fuel utilized was derived from fat demonstrating the extent to which oxidation of fat fuels can support heavy exercise for a prolonged period. The difference in acetyl-group accumulation between the two conditions was related to substrate utilization differences after the low and high carbohydrate diets. After the LCD, increased acetyl-CoA accumulation resulted from greater oxidation of fat at rest. This had the effect of decreasing PDHa and limiting further acetyl-CoA formation from pyruvate. During exercise in LCD, the formation of acetyl-groups appeared to be lower than the amount oxidized due to an insufficient rate of fat and ketone body oxidation, and a reduction in pyruvate availability. During the first 16 min of exercise after a HCD, acetyl-group accumulation was related to the greater availability of glycogen and pyruvate, and dependent on greater PDHa, resulting in acetyl-group formation from glycogen that was greater than its rate of utilization. Thereafter, no further change in acetyl-group accumulation occurred.

Reciprocal changes in carnitine and acetylcarnitine occurred in the LCD condition but this did not occur when changes in acetyl-CoA and free CoASH were compared (Figure 3). In the LCD condition there was a decline in the sum of acetylCoA and CoASH during exercise. With increasing utilization of FFA it is possible that more of the CoASH was utilized for fatty acid activation and a portion of the total coenzyme-A may have been in the form of long chain acyl-CoA esters. This was not observed in the HCD condition during exercise where decreases in CoASH and carnitine were equal and opposite to increases in acetyl-CoA and acetylcarnitine, respectively.

During prolonged intense aerobic exercise in a state of carbohydrate deprivation (ie. LCD), glycogen utilization was slowed. From 16 min to EX, the decreasing contribution of glycogen appeared to be compensated by increased oxidation of fat fuels. Increasing the blood glucose concentration by glucose infusion did not alter acetyl-group accumulation but it was an important factor in relieving the symptoms of hypoglycemia and it allowed resumption of cycling at the same power output. Thus, the reduction of the acetyl-group pool during exercise and at EX in the LCD condition does not appear to be the eventual cause of exhaustion in the LCD condition. As the subjects were able to continue exercising when blood glucose was restored, the cause of fatigue was probably impairment of the central nervous system (CNS), secondary to the exercise induced hypoglycemia.

2.5.3 Glucose-Fatty Acid Cycle

Measurements of acetyl-CoA, citrate, PDHa and estimates of glycolytic flux enable us to comment on the existence of the classic glucose-fatty acid (G-FA) cycle in human skeletal muscle as classically proposed (31,32). At rest, the extremely high acetyl-

CoA:CoASH ratio in the LCD condition is consistent with lower PDHa (Figure 4). A 40% higher resting citrate concentration in the LCD condition (Table 4) may have also contributed to an inhibition of PFK (18). Therefore at rest, the accumulation of the putative regulators of the G-FA cycle is consistent with their involvement in the down-regulation of glycolytic flux and PDH activity in the LCD condition.

During exercise, the acetyl-CoA:CoASH ratio increased ~5 fold in the HCD condition while PDHc was completely transformed to the active state (Figure 4). Muscle glycolytic flux also increased several fold in spite of a two fold increase in citrate concentration (Table 4). According to the G-FA cycle (31), the increased acetyl-CoA:CoASH ratio should have resulted in a lower PDHa and increased citrate should have inhibited PFK and thus reduced glycolytic flux. It is clear that this did not occur and that other factors control the activity of these enzymes during heavy exercise when carbohydrate is available.

In the LCD condition, the acetyl-CoA:CoASH ratio decreased from rest to exercise, but PDHa increased at a slower rate than observed in the HCD condition (Figure 4). Citrate concentration was similar in the two conditions at 16 min and at EE/EX (Table 4) but glycolytic flux was consistently lower in the LCD condition. Our observations argue against the involvement of the acetyl-CoA:CoASH ratio and citrate in down-regulating skeletal muscle PDHa and PFK, respectively, during heavy exercise when substrate utilization shifts towards increased fat oxidation. The lower rate of glycogen utilization during the first 16 min of exercise in the LCD compared to the HCD could be explained by lower activity of phosphorylase, induced by the lower glycogen content which is clearly the reason for the differences in the later part of the exercise period.

2.5.4 Summary

In the present study the role of PDHa in the integration of carbohydrate and fat utilization was explored in human skeletal muscle at rest and during intense aerobic exercise, with carbohydrate deprivation being used to decrease carbohydrate availability and increase fat utilization. Of secondary importance was the concurrent examination of acetyl-group accumulation to establish the role of acetyl-CoA in PDHa regulation and to determine if depletion of acetyl-CoA and acetylcarnitine was related to the development of muscle fatigue. While a significant reduction in the acetyl-group pool was observed throughout exercise, complete depletion did not occur at exhaustion. Moreover, glucose replacement relieved exhaustion independent of changes in the acetyl-group pool, suggesting that the development of fatigue in these subjects was due to CNS impairment rather than peripheral metabolic impairment in the exercising muscles.

The results confirm that the functional role of PDHc, in resting muscle, is to integrate glucose and fat utilization. At rest the primary signal responsible for regulating the activation state of this enzyme, and thus controlling the rate of carbohydrate oxidation, are changes in the acetyl-CoA:CoASH ratio. In contrast to our findings at rest, during prolonged exercise at 75% of $\dot{V}O_2$max no evidence was found for regulation through the G-FA cycle as proposed by Randle et al (31,32). In the HCD condition, transformation of PDHc was complete throughout the exercise period despite an increasing acetyl-CoA:CoASH ratio that should have resulted in lower a rate of PDHa transformation than observed in the LCD condition and the opposite was true of the LCD condition.

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CHAPTER 3

SKELETAL MUSCLE PYRUVATE DEHYDROGENASE ACTIVITY DURING ACETATE INFUSION IN HUMANS

(published in Am. J. Physiol. 268 (Endocrine and Metabolism 31), E1007-E1017, 1995)

3.1 Abstract

Pyruvate dehydrogenase activity (PDHa), acetyl-group and citrate accumulation were examined in human skeletal muscle at rest and during cycling exercise while acetate availability was acutely elevated. Eight subjects were infused with 400 mmol of Na-acetate (ACE) at a constant rate during 20 min of rest, 5 min of cycling at 40% and 15 min of cycling at 80% of their maximal oxygen uptake ($\dot{V}O_2max$). Two weeks later experiments were repeated while 400 mmol of Na-bicarbonate was infused in the control condition (CON). ACE infusion increased muscle acetylCoA (17.0 ± 2.2 to 33.2 ± 4.1 μmol/kg dw), citrate and acetylcarnitine. A decline in resting PDHa during 20 min of ACE infusion (0.37± 0.08 vs. 0.16 ± 0.03 mmol·min^{-1}·kg wet wt^{-1}) coincided with an elevation in the intramuscular acetylCoA-to-CoASH ratio (0.28 ± 0.04 to 0.73 ± 0.14). After 20 min of CON infusion, resting PDHa (0.32 ± 0.06 mmol·min^{-1}·kg wet wt^{-1}) did not differ from PDHa prior to ACE infusion. During exercise, acetylCoA, citrate and the acetylCoA-to-CoASH ratio were further elevated and the differences that existed at rest were resolved. PDHa increased to the same extent in ACE and CON where it was 44-47% transformed after 5 min at 40% $\dot{V}O_2max$ and completely transformed after 15 min at 80% $\dot{V}O_2max$. During rest PDHa was regulated by

an acute increase in the availability of an alternative fat fuel acting through variations in the acetylCoA-to-CoASH ratio. Conversely, during exercise PDHa regulation was independent of variations in the acetylCoA-to-CoASH ratio and was primarily regulated by other factors. The resting data are consistent with a central role for PDHa and citrate in the regulation of the glucose-fatty acid cycle in skeletal muscle, as classically proposed. However, during exercise acetate infusion is not an effective tool to study the regulation of the glucose-fatty acid cycle in skeletal muscle.

3.2 Introduction

The pyruvate dehydrogenase complex (PDHc) is believed to play a pivotal role in the regulation of intramuscular fuels at rest and during exercise. Its function has been summarized by Randle *et al* (27,28) in a theory of metabolic regulation which they termed the glucose-fatty acid (Glc-FA) cycle and is thought to operate in the following manner: with increased availability and utilization of fat fuels a restriction is placed on intramuscular glucose utilization by the inhibition of the cytosolic enzymes hexokinase and phosphofructokinase (PFK) as well as the intramitochondrial enzyme PDHc. Randle *et al* (28) observed that it is at the level of PDHc that the effect of increased fat fuel availability was most marked and thus likely initiates intramuscular glucose restriction. Rising ratios of acetylCoA-to-CoASH and NADH-to-NAD generated from the oxidation of fat fuels inhibits PDHc transformation to its active form (PDHa), leading to the accumulation of citrate which inhibits PFK. The resultant decrease in glycolytic flux causes glucose-6-phosphate accumulation which in turn inhibits hexokinase activity, restricting further glucose uptake and glycogen utilization. Conversely, in the absence of available fat fuels the restriction on muscle

glucose oxidation is released.

Fundamental to the function of this mechanism is the inhibitory effect of acetylCoA accumulation and the rising acetylCoA-to-CoASH ratio on PDHc transformation to initiate or release the ensuing events of glucose restriction. To date, most of the information concerning the operation of this mechanism of fuel regulation in tissues and the role of PDHc therein has been limited to rat heart (13,19,20,38), rat muscle preparations (14,21) and rat muscle mitochondrial preparations (1) where evidence supports its operation as proposed (27). However, it is not known to what extent this mechanism operates in human skeletal muscle. We have recently reported lower resting PDHa in humans after 3 d of carbohydrate deprivation that resulted from a rise in the intramuscular acetylCoA-to-CoASH ratio secondary to acetylCoA accumulation (26). However, during intense aerobic exercise lower PDHa was not due to variations in the acetylCoA-to-CoASH ratio but was attributed to long term regulatory events (26). The source of the excess acetylCoA generated at rest was from the oxidation of free fatty acids and/or ketones bodies. In a subsequent study (11), we reported that Intralipid-infusion into human subjects was successful at restricting glycogen utilization in the vastus lateralis during intense aerobic cycling, independent of acetylCoA accumulation. Furthermore, the important sites of regulation were not at PDHc or PFK but rather at the level of glycogen phosphorylase (11).

Lower resting PDHa observed during carbohydrate deprivation (26) but not during Intralipid infusion (11) could be explained by the greater relative importance of short chain fatty acid anions to the generation of acetylCoA in human muscle. Indeed, in humans (17,29) and some animal models (2,18,22) the inhibitory effects on glucose utilization are most remarkable during infusion of ketone bodies (2) and acetate (18,29) or after the ingestion

of acetate generating compounds such as ethanol (17).

It is known that acetate is readily taken up by skeletal muscle and converted to acetylCoA by acetylCoA synthetase and that a significant portion of the acetate infused into humans is oxidized by skeletal muscle (35). When infused at rest this should result in intramuscular acetylCoA and citrate accumulation and an elevated acetylCoA-to-CoASH ratio, resulting in lower PDHa and PFK activity. Additionally, if greater acetylCoA and citrate accumulation could be sustained during exercise by simultaneously infusing Na-acetate, then a lower rate of PDHc transformation and PFK activity might also be observed. Thus the purpose of this study was to examine the acute effect of increasing acetyl-group accumulation on PDHa in human muscle. We hypothesized that the acetylCoA-to-CoASH ratio and citrate would be higher during Na-acetate infusion leading to lower PDHa and the potential for lower PFK activity at rest and during cycling exercise. Glycogen was also examined to determine if intramuscular glucose conservation accompanied lower PDHa during exercise.

3.3 Methods

3.3.1 Subjects

Seven males and one female (mean ± SEM, $\dot{V}O_2$max: 49.3 ± 3.4 ml · kg^{-1} · min^{-1}; age 24.9 ± 1.1 yr; height 172.6 ± 5.5 cm and weight 80.0 ± 4.8 kg) participated in this study. Approval was obtained from the Ethics Committee of McMaster University and the University of Guelph. Written informed consent was obtained from each subject following a presentation of the study protocol and its attendant risks.

3.3.2 Pre-experimental Protocol

Maximal oxygen uptake ($\dot{V}O_2max$) was determined on a cycle ergometer for each subject. On a subsequent day each subject returned for a practice ride for 15 min to confirm the experimental workloads.

3.3.3 Experimental Protocol

The antecubital or basilic vein of each arm was catheterized percutaneously with a teflon catheter (Angio-set 20 gauge, Becton-Dickinson, Clarkson, Ont., Canada). Catheters were maintained patent with a sterile non-heparinized isotonic saline solution. One thigh was prepared for needle biopsy of the vastus lateralis. Incisions of the skin through to the deep fascia were made over the vastus lateralis while under local anesthesia (2% xylocaine without epinephrine) as described by Bergström (4). Four incisions were made on one thigh during the first visit (acetate infusion) and three were made on the contralateral thigh during the second visit (control infusion).

The experimental protocol is summarized in Figure 1. During 20 min of rest and while cycling at 40% $\dot{V}O_2max$ for 5 min and 80% $\dot{V}O_2max$ for 15 min, 667 mM Na-acetate was infused into one catheter at a rate of 15 $ml \cdot min^{-1}$. Two weeks later 570 mM Na-bicarbonate was infused at a rate of 17.5 $ml \cdot min^{-1}$ during the control trial. Na-bicarbonate was chosen for control infusions to prevent variations in plasma pH between the control and experimental trials. Arterialized venous blood samples were collected from the catheter of the contralateral arm. Blood samples and muscle biopsies were taken at the times indicated in Figure 1. Prior to the each visit subjects consumed a mixed diet (mean ± SEM, carbohydrate: 49 ± 4%, fat: 31 ± 4% and protein: 20 ± 2%.) that did not differ in

composition between trials. The Na-acetate and Na-bicarbonate trials were separated by two weeks to allow sufficient time for preliminary analysis of intramuscular acetylCoA content. This was done to ensure that acetate infusion produced increased muscle acetylCoA in each subject at rest before committing them to another experiment.

The exercise intensities utilized in the present study were based on previous reports which have established that PDHc transformation to PDHa is incomplete at low intensities (6) but completely transformed and independent of the acetylCoA-to-CoASH ratio at intensities greater than 60% $\dot{V}O_2$max (8). Thus if PDHa were to be regulated by variations in the acetylCoA-to-CoASH ratio during exercise it was expected that the effect would be observable only at 40% $\dot{V}O_2$max. The high intensity workload of 80% $\dot{V}O_2$max was choosen to establish complete PDHc transformation to PDHa and to study muscle glycogen utilization.

3.3.4 Muscle Sampling

Muscle biopsies were immediately frozen in liquid N_2 and removed from the needle while frozen. Immediately 10-30 mg was chipped from each biopsy and dissected free of blood and connective tissue under liquid N_2. From this, two clean 5-15 mg portions were stored separately in liquid N_2: one for the measurement of total pyruvate dehydrogenase activity (PDHt) and the other for measurement of the active fraction (PDHa). The remaining frozen muscle was freeze dried, dissected free of blood and connective tissue, and stored dry at -80°C. A portion of the powdered muscle (4-10 mg) was alkaline extracted in duplicate and used for glycogen determination and the remaining powdered muscle was extracted in a solution of 0.5 M perchloric acid (PCA) and 1 mM EDTA, neutralized to pH 7.0 with 2.2

M $KHCO_3$ and stored at -80°C until analyzed for metabolites.

3.3.5 Analysis of Muscle

PDHa and PDHt were measured in muscle homogenates using the method of Constantin-Teodosiu *et al* (7) with the following modifications. For the measurement of PDHt, the sample was homogenized in a buffer similar to PDHa with the following differences: exclusion of 50 mM NaF, inclusion of 10 mM $CaCl_2$, 25 mM $MgCl_2$, 4 U/ml hexokinase, 10 mM glucose, 10 mM dichloroacetic acid (DCA) and 0.1% (vol/vol) triton X-100. No preincubation reagent was used for the measurement of PDHt. PDHt was taken as the highest reaction rate for a series of biopsies taken from a subject on a given day.

The assay buffer for PDHa and PDHt was modified by increasing the concentration of all coenzymes: NAD^+ to 3.0 mM, CoASH to 1.0 mM, and thiamine pyrophosphate (TPP) to 1.0 mM. During the assay, 200 µl aliquots were sampled at 1, 2, and 3 min. The reaction was stopped by addition of each aliquot to 40 µl of 0.5 M PCA. After 5 min, each aliquot was neutralized with 1.0 M K_2CO_3 and centrifuged for 3 min at 15,900xG (microcentrifuge E, Beckman Instrument Co., Mississauga, Ont., Canada). The resulting supernatant was stored at -50°C until analyzed for acetylCoA by the method of Cederblad *et al* (5). Plots of acetylCoA as a function of time were used to determine the reaction rates. Total creatine concentrations were measured in neutralized PCA extracts of PDHa and PDHt homogenates, using the enzymatic methods of Bergmeyer (3). PDHa and PDHt measures were corrected to the highest creatine content in all of biopsies taken from each subject. This was done to compensate for any contamination of the tissue homogenates due to the presence of blood or connective tissue.

Muscle glycogen was measured enzymatically as described by Harris *et al* (16). Neutralized PCA muscle extracts were analyzed for CoASH, acetylCoA, acetylcarnitine (5), citrate (3), glucose, glucose-6-phosphate (G-6-P), lactate, PCr and adenosine-triphosphate (ATP) (16). PCA extracts were corrected for total creatine.

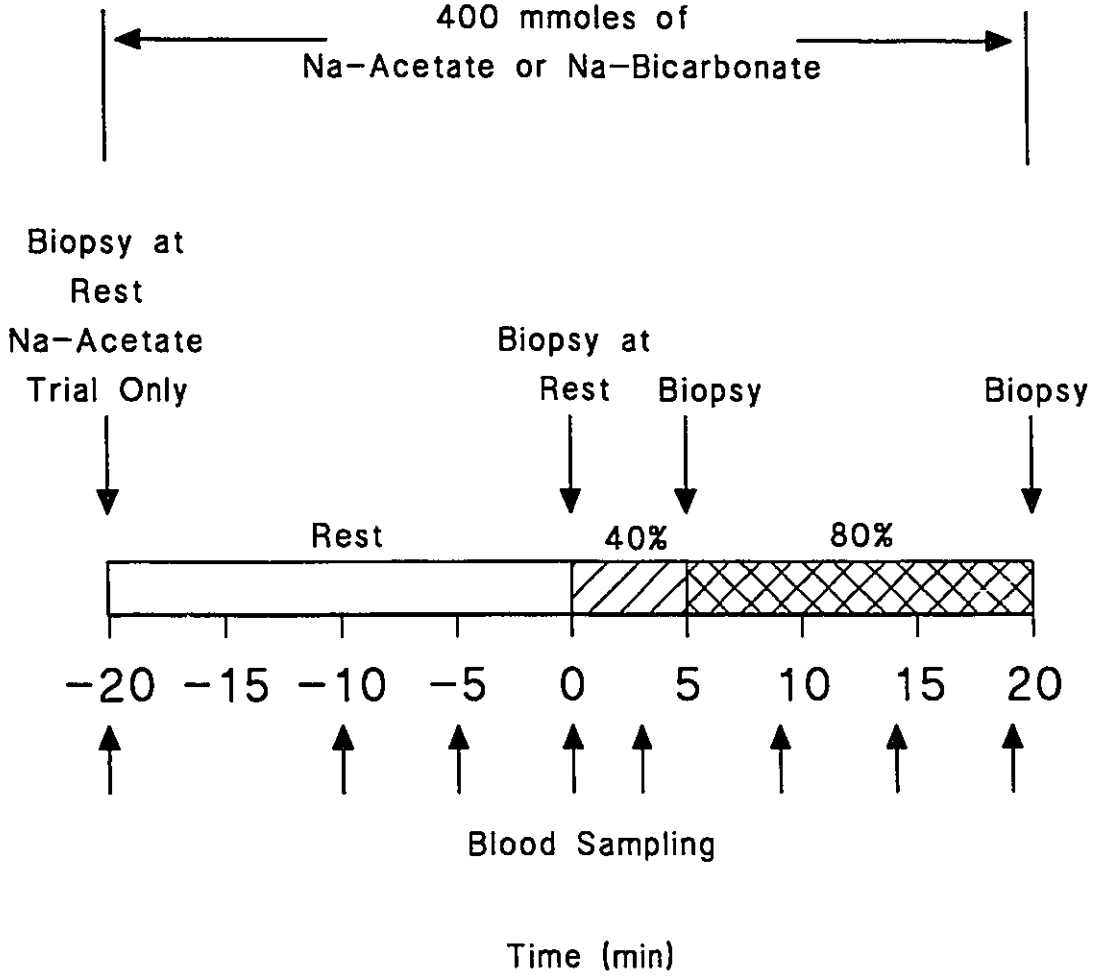
3.3.6 Blood Sampling and Analysis

Arterialized venous blood samples (~ 4-6 ml) were drawn in heparinized plastic syringes and placed on ice. One portion of each blood sample was deproteinized in two volumes of 6% PCA, stored at -20°C and subsequently analyzed for lactate, glycerol and glucose as described in Bergmeyer (3). A second portion was immediately centrifuged at 15,900xG for 2 min and the plasma supernatant was analyzed for free fatty acids (Wako NEFA C test kit; Wako Chemical Inc. Immunocrop, Montreal, Quebec, Canada) and acetate (3). A third portion of each blood sample was analyzed for plasma pH (AVL 995 Automatic Blood Gas Analyzer, Intermedico Markham Ontario), Hb and Hb-saturation (Hemoximeter OSM 3, Radiometer Copenhagen).

3.3.7 Statistical Analysis

Analysis of resting muscle metabolites, glycogen and PDHa measures were carried out by a dependent samples paired *t*-test. Exercising muscle metabolites, PDHa and blood metabolite constituents were analyzed by a two-way analysis of variance with repeated measures to compare measurements over time. When a significant F ratio was found, the Newman-Keuls post hoc test was use to compare means. Results were considered significant at $p < 0.05$.

FIGURE 1: Experimental Protocol. Arterialized venous blood samples were taken at the times indicated. 667 mM Na-acetate (experimental) was continuously infused at 15 $ml \cdot min^{-1}$. 570 mM Na-bicarbonate was continuously infused at 17.5 $ml \cdot min^{-1}$. 40% and 80% indicate cycling exercise at 40% of $\dot{V}O_2max$ and 80% $\dot{V}O_2max$, respectively.


400 mmoles of
Na-Acetate or Na-Bicarbonate
Biopsy at
Rest
Na-Acetate
Trial Only
Biopsy at
Rest
Biopsy
Biopsy
Rest
40%
80%
-20 -15 -10 -5 0 5 10 15 20
Blood Sampling
Time (min)

3.4 Results

3.4.1 Plasma Acetate Concentration, Plasma pH and $\dot{V}O_2$

Plasma acetate (Figure 2) was not detectable prior to Na-acetate infusion or during control infusions with Na-bicarbonate. Plasma acetate rose to 2.15 ± 0.24 mM after 10 min of resting infusion and no further change occurred during resting infusion or after 5 min of cycling at 40% $\dot{V}O_2$max. Acetate concentration was 2.64 ± 0.37 mM prior to the commencement of cycling at 40% $\dot{V}O_2$max, 2.59 ± 0.45 mM after 5 min and rose slightly thereafter during cycling at 80% $\dot{V}O_2$max to reach 3.79 ± 0.49 mM at 20 min. Plasma pH (Table 1) was monitored in the Na-acetate trial and in the control trial where an equal amount of Na-bicarbonate was infused. Plasma pH increased equally during Na-acetate and control infusions at rest and remained elevated over pre-infusion resting values during cycling at 40% and 80% $\dot{V}O_2$max but did not differ between the two conditions at any time. The workloads for 40% and 80% $\dot{V}O_2$max were 98 ± 11 and 207 ± 22 watts, producing oxygen uptakes of 1.4 ± 0.1 and 2.8 ± 0.3 l/min, respectively.

3.4.2 Blood Metabolites

3.4.2.1 Glucose

Blood metabolite values were not corrected for fluid shifts since there were no differences in hemoglobin (Hb) concentration between the two conditions. Hb ranged from 14.3 ± 0.2 to 15.0 ± 0.2 g/dl in the Na-acetate trial and from 13.8 ± 0.1 to 14.9 ± 0.1 g/dl in the control trial. Similarly HbO_2 saturation did not differ between conditions. Glucose (gluc) concentration ([ ]) (Figure 3) remained relatively stable between conditions, differing only at -10, 14 and 19 min. At each of these points [gluc] was greater in the Na-acetate

condition reaching 3.44 ± 0.11, 3.56 ± 0.12 and 3.48 ± 0.11 mM at -10, 14 and 19 min, respectively. In the control condition [gluc] was 3.05 ± 0.13, 3.14 ± 0.17 and 3.03 ± 0.16 mM at these same time points.

3.4.2.2 Lactate

Lactate (Lac) concentration (Figure 3) was unaffected by either Na-acetate or Na-bicarbonate infusion during rest and rose equally in both conditions during cycling. In the Na-acetate condition, [Lac] rose to 6.41 ± 0.36, 10.15 ± 0.77 and 11.39 ± 0.86 mM at 9, 14 and 19 min, respectively. In the control condition [Lac] was 5.88 ± 0.50, 9.77 ± 0.57 and 10.77 ± 0.92 mM at the same time points.

3.4.2.3 Free Fatty Acids and Glycerol

Pre-infusion plasma [FFA] (Figure 4) was 0.38 ± 0.04 and 0.29 ± 0.05 mM in the Na-acetate and control trials, respectively. There were no further changes in [FFA] during resting infusion or during cycling at 40% and 80% $\dot{V}O_2$max. At 19 min [FFA] was 0.34 ± 0.03 and 0.32 ± 0.02 mM in the Na-acetate and control conditions, respectively. Pre-infusion [glycerol] (Figure 4) was 0.019 ± 0.017 mM in the Na-acetate trial and 0.018 ± 0.011 mM in the control condition. Like plasma [FFA], [glycerol] was unaltered during infusion at rest or during cycling at 40% $\dot{V}O_2$max. During cycling at 80% $\dot{V}O_2$max [glycerol] rose continuously to 0.029 ± 0.020 and 0.023 ± 0.019 mM at 9 min in the Na-acetate and control conditions, respectively. By 19 min [glycerol] had increased further to 0.048 ± 0.020 in the Na-acetate trial and to 0.044 ± 0.04 mM in the control trial, where they were significantly greater than all previous values within their respective conditions.

3.4.3 Muscle Metabolites

3.4.3.1 Glycogen

Glycogen content (Table 2) did not differ between the Na-acetate and control trials at rest or after 20 min of cycling. In the Na-acetate trial, 234.4 ± 20.7 mmol glycosyl U/kg dw was used while 220.0 ± 22.1 mmol glycosyl U/kg dw was used in the control trial. This corresponded to glycogen utilization rates of 11.7 ± 1.0 and 11.0 ± 1.1 mmol glycosyl U·kg $dw^{-1}·min^{-1}$ in the Na-acetate and control trials, respectively.

3.4.3.2 Glucose-6-Phosphate, Lactate, Citrate, ATP and PCr

G-6-P did not increase after infusion of Na-acetate and increased to the same extent in both conditions as a result of cycling at 40% and 80% $\dot{V}O_2max$ (Table 2). Similarly, lactate increased as a result of cycling but there were no differences between conditions. In contrast to the other metabolites examined, citrate accumulated as a result of Na-acetate infusion at rest (Figure 5). Citrate also accumulated during cycling at 40% and 80% $\dot{V}O_2max$ but there were no differences between conditions. PCr content (Table 2) was unaffected by Na-acetate infusion and decreased equally in both conditions during cycling exercise. ATP content did not change throughout infusion or during cycling exercise and was not different between the Na-acetate and control trials.

3.4.3.3 Acetyl-Group Accumulation

AcetylCoA content rose approximately two fold as a result of Na-acetate infusion (Figure 5). It was 17.0 ± 2.2 μmol/kg dw at rest prior to infusion and increased to 33.2 ± 4.1 after 20 min of infusion. Na-bicarbonate infusion did not alter resting acetylCoA content

(18.1 ± 1.7 μmol/kg dw). Five min of cycling at 40% $\dot{V}O_2$max and an additional 15 min at 80% $\dot{V}O_2$max further increased muscle acetylCoA content, but differences between the conditions were resolved. Upon completion of cycling at 20 min, acetylCoA content had increased to 56.4 ± 3.4 μmo/kg dw in the Na-acetate trial and 57.3 ± 4.4 in the control condition.

Changes in free coenzyme-A (CoASH) content were equal in magnitude but opposite in direction to those observed for acetylCoA (Figure 6). Pre-infusion resting CoASH was 64.1 ± 5.7 μmol/kg dw in the Na-acetate trial and decreased to 50.9 ± 4.8 at 0 min. Post-infusion resting CoASH content in the control trial (65.4 ± 4.8 μmol/kg dw) was not different from the pre-infusion value of the Na-acetate trial but it was greater than the post-infusion value of this trial. Differences between the two conditions were resolved during cycling reaching 26.8 ± 3.5 μmol/kg dw in the Na-acetate trial and 24.8 ± 2.8 in the control condition, at 20 min.

Variations in acetylcarnitine content paralleled changes in acetylCoA but acetylcarnitine content remained greater during Na-acetate infusion at each time point (Figure 6). Acetylcarnitine increased from 5.1 ± 1.3 mmol/kg dw to 12.3 ± 1.8 after Na-acetate infusion, did not change after 40% $\dot{V}O_2$max and increased further to 19.1 ± 1.2 after 15 min at 80% $\dot{V}O_2$max. In the control condition it was 3.3 ± 0.9, 6.0 ± 0.6 and 13.1 ± 1.1 mmol/kg dw at 0, 5 and 20 min, respectively.

3.4.4 Pyruvate Dehydrogenase Complex and the AcetylCoA-to-CoASH Ratio

3.4.4.1 Rest

Resting PDHa (Figure 7) decreased from 0.37 ± 0.08 mmol·min^{-1}·kg wet wt^{-1} to 0.16

± 0.03 after 20 min of Na-acetate infusion and coincided with an ~2.6 fold rise in the acetylCoA-to-CoASH ratio. The acetylCoA-to-CoASH ratio was 0.28 ± 0.04 before and 0.73 ± 0.14 after 20 min of Na-acetate infusion. Resting PDHa in the control condition was 0.32 ± 0.06 mmol·min^{-1}·kg wet wt^{-1} and did not differ from the pre-infusion PDHa value in the Na-acetate trial. It was, however, greater than the post-infusion PDHa (0 min) in the Na-acetate trial.

3.4.4.2 Exercise

During cycling exercise PDHa did not differ between conditions at 40% or 80% $\dot{V}O_2$max (Figure 7). At 40% $\dot{V}O_2$max PDHa was 1.04 ± 0.19 mmol·min^{-1}·kg wet wt^{-1} in the Na-acetate trial and 1.28 ± 0.24 in the control condition. At 80% $\dot{V}O_2$max (20 min) PDHa had increased to 2.41 ± 0.09 in the control condition and 1.93 ± 0.17 in the Na-acetate trial. Total PDH activity was 2.31 ± 0.14 in the Na-acetate trial and 2.69 ± 0.10 in the control trial.

Differences in the acetylCoA-to-CoASH ratio that occurred between conditions during resting infusion were resolved during exercise. After 5 min of cycling at 40% $\dot{V}O_2$max this ratio was 0.77 ± 0.10 in Na-acetate trial and 0.92 ± 0.17 in the control trial. At 80% $\dot{V}O_2$max it further increased to 2.65 ± 0.62 in the Na-acetate trial and 2.63 ± 0.44 in the control trial. These changes had no apparent effect on PDHa transformation.

Determination of PDHc transformation as a per cent of total mirrored variations in PDHa. Prior to Na-acetate infusion PDHc transformation was 16.3 ± 3.1% and after 20 min of infusion decreased to 7.0 ± 1.2%. In the control trial, PDHc transformation was 12.3 ± 2.4% after resting infusion and was the same as the resting pre-infusion value of the Na-

acetate trial but was greater than the resting post-infusion value of the Na-acetate trial. After 5 min of cycling at 40% $\dot{V}O_2$max PDHc transformation was 44.4 ± 7.4% in the Na-acetate trial and 47.3 ± 8.4% in the control trial. After another 15 min at 80% $\dot{V}O_2$max it rose significantly again to 85.0 ± 8.5% and 90.2 ± 4.3% in the Na-acetate and control trials, respectively. These values were not different from 100% and represented complete transformation of PDHc to PDHa.

FIGURE 2: Concentration of plasma acetate during 20 min of Na-acetate infusion at rest (open box), during cycling exercise at 40% of $\dot{V}O_2$max (single cross hatch box) and during cycling exercise at 80% of $\dot{V}O_2$max (double cross hatch box). Data are means ± SEM. *, indicates significantly different from post-infusion rest value. +, indicates significantly different from 40% $\dot{V}O_2$max value.

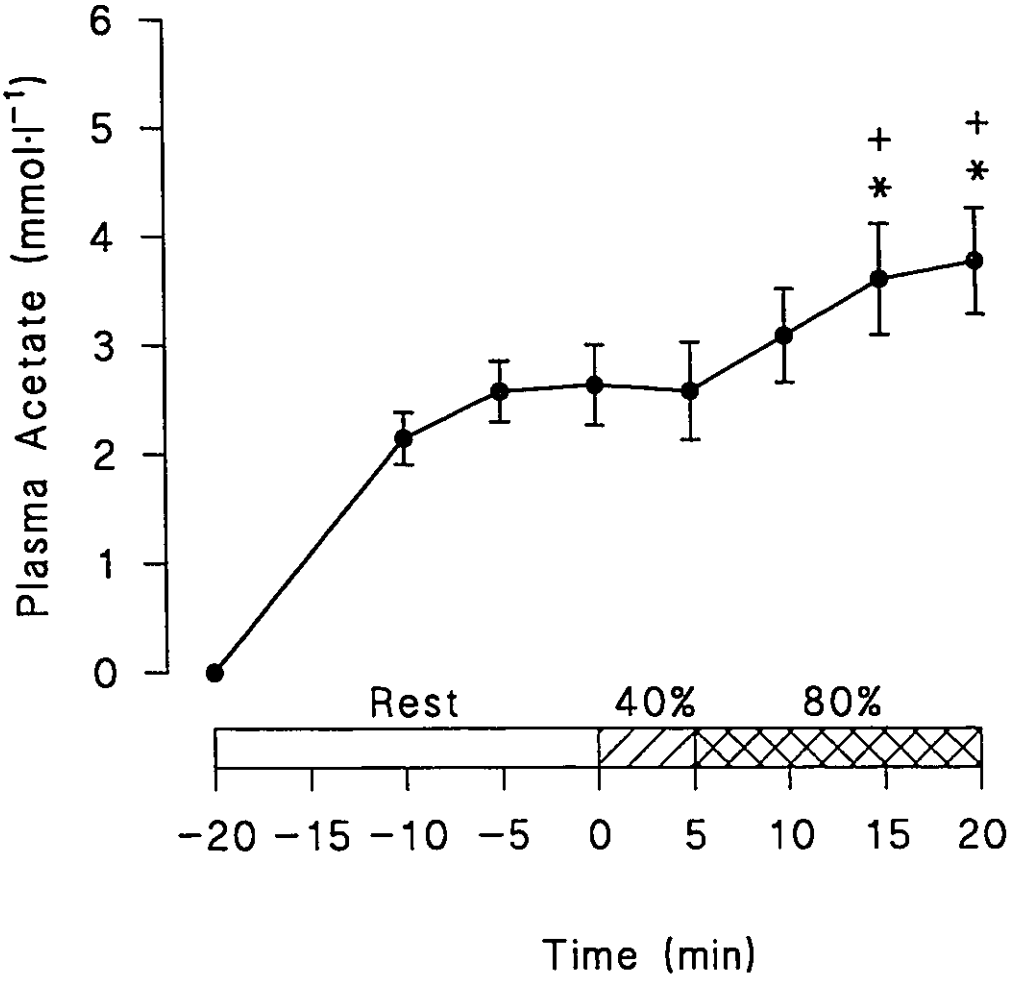

Plasma Acetate (mmol·l⁻¹)
6
5
4
3
2
1
0
+
*
+
*
Rest
40%
80%
−20 −15 −10 −5 0 5 10 15 20
Time (min)

FIGURE 3: Concentration of glucose and lactate in arterialized-venous blood samples at rest (open box), during cycling exercise at 40% of $\dot{V}O_2max$ (single cross hatch box) and during cycling exercise at 80% of $\dot{V}O_2max$ (double cross hatch box) while Na-Acetate (—— ●——) or Na-Bicarbonate (– — O — –) was infused. Data are means ± SEM. *, indicates significantly different from resting value prior to Na-acetate infusion. +, indicates significantly different between conditions at matched times. ‡, indicates significantly different from post-infusion rest value within a condition.

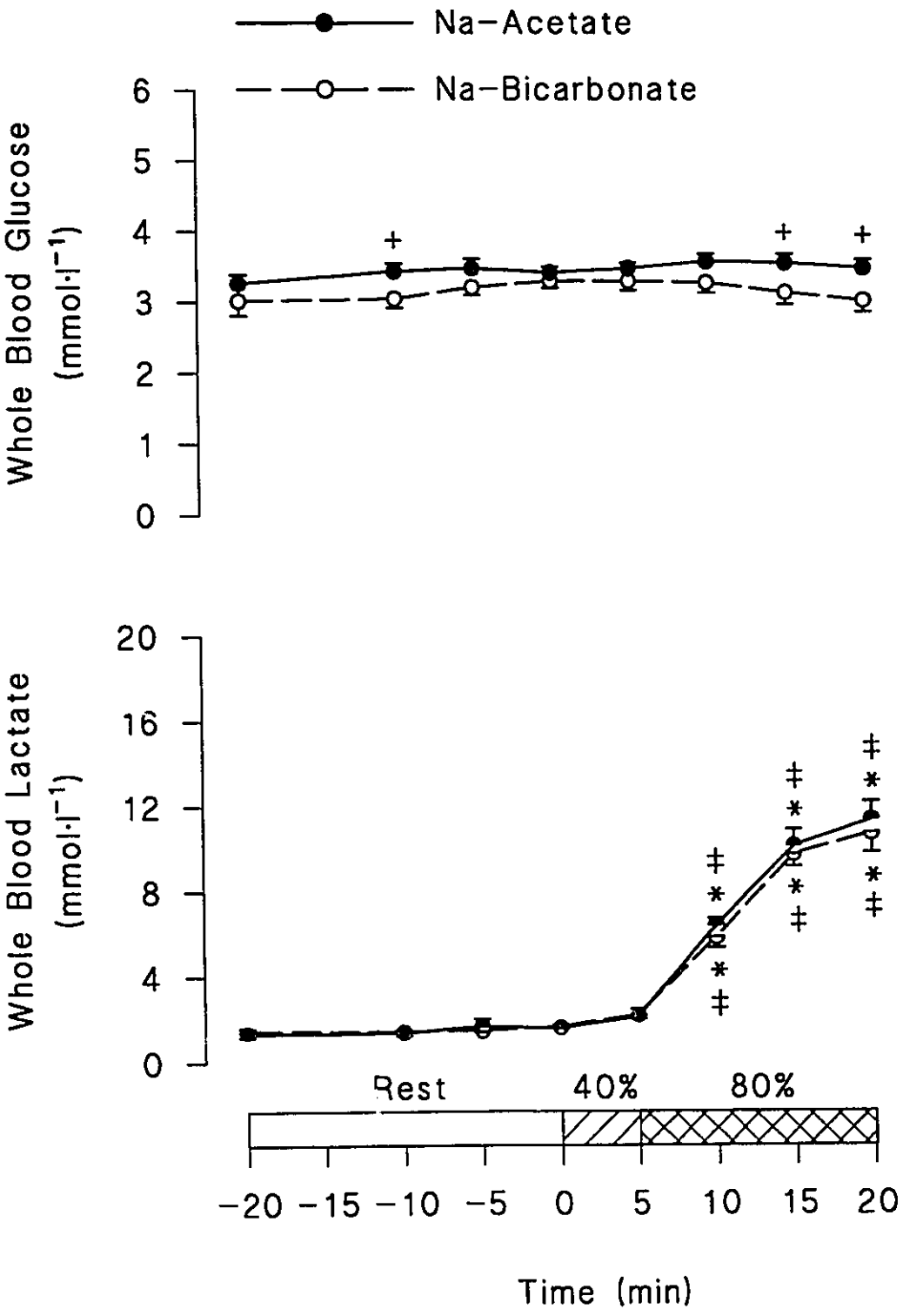

Na-Acetate
Na-Bicarbonate
Whole Blood Glucose (mmol·l⁻¹)
6
5
4
3
2
1
0
+
Whole Blood Lactate (mmol·l⁻¹)
20
16
12
8
4
0
‡
*
Rest
40%
80%
−20 −15 −10 −5 0 5 10 15 20
Time (min)

FIGURE 4: Concentration of FFA and glycerol in arterialized-venous blood samples at rest (open box), during cycling exercise at 40% of $\dot{V}O_2max$ (single cross hatch box) and during cycling exercise at 80% of $\dot{V}O_2max$ (double cross hatch box) while Na-Acetate (—— ●——) or Na-Bicarbonate (– — O — –) was infused. Data are means ± SEM. *, indicates significantly different from resting value prior to Na-acetate infusion. +, indicates significantly different between conditions at matched times. ‡, indicates significantly different from post-infusion rest value within a condition.

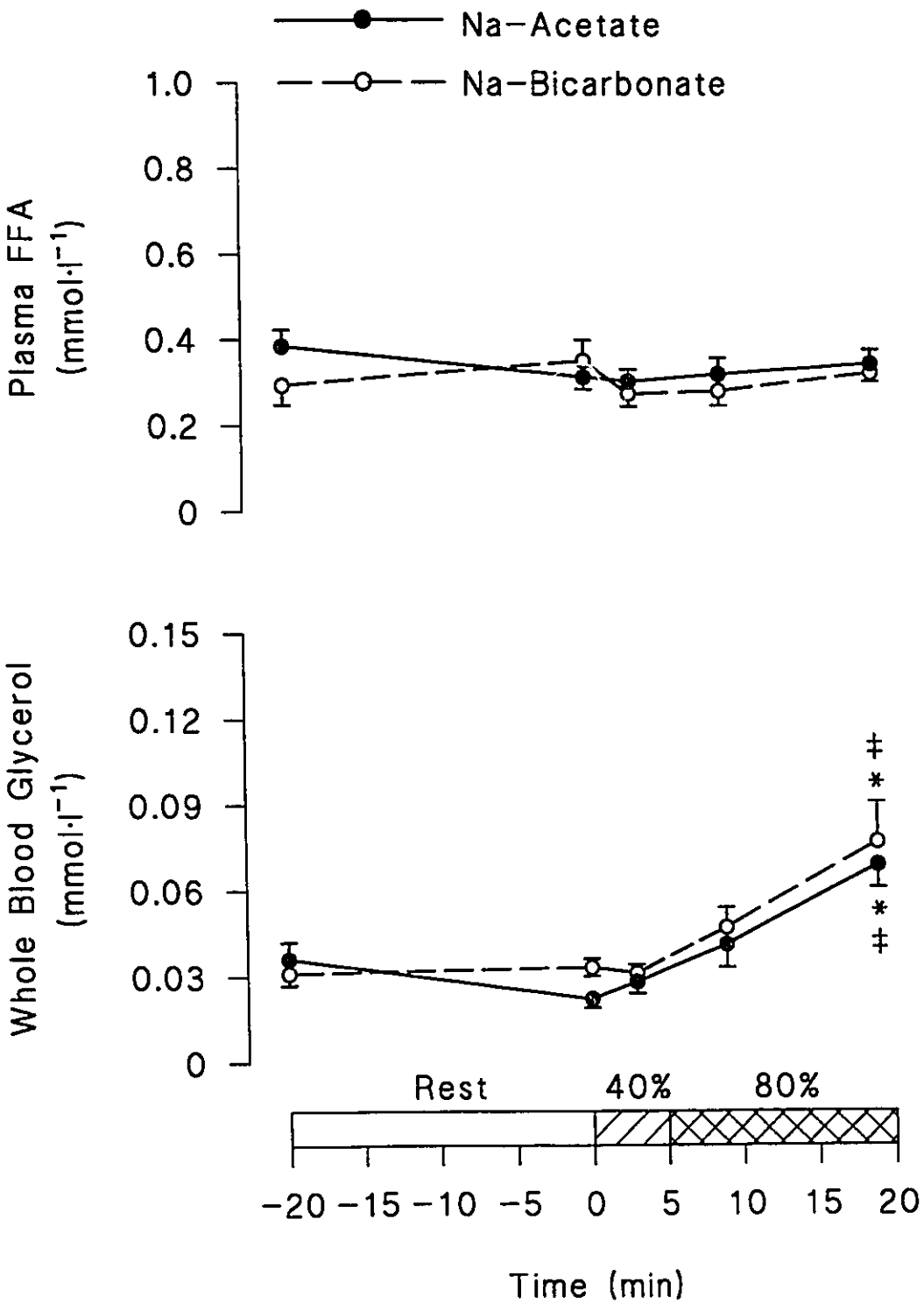

Na-Acetate
Na-Bicarbonate
Plasma FFA
(mmol·l⁻¹)
1.0
0.8
0.6
0.4
0.2
0
Whole Blood Glycerol
(mmol·l⁻¹)
0.15
0.12
0.09
0.06
0.03
0
‡
*
*
‡
Rest
40%
80%
-20 -15 -10 -5 0 5 10 15 20
Time (min)

FIGURE 5: Muscle acetylCoA and citrate content at rest (open box), during cycling exercise at 40% of $\dot{V}O_2max$ (single cross hatch box) and during cycling exercise at 80% of $\dot{V}O_2max$ (double cross hatch box) while Na-Acetate (—— ●——) or Na-Bicarbonate (— — O — —) was infused. Data are means ± SEM. *, indicates significantly different from resting value prior to Na-acetate infusion. +, indicates significantly different between conditions at matched times. ‡, indicates significantly different from post-infusion rest value within a condition.

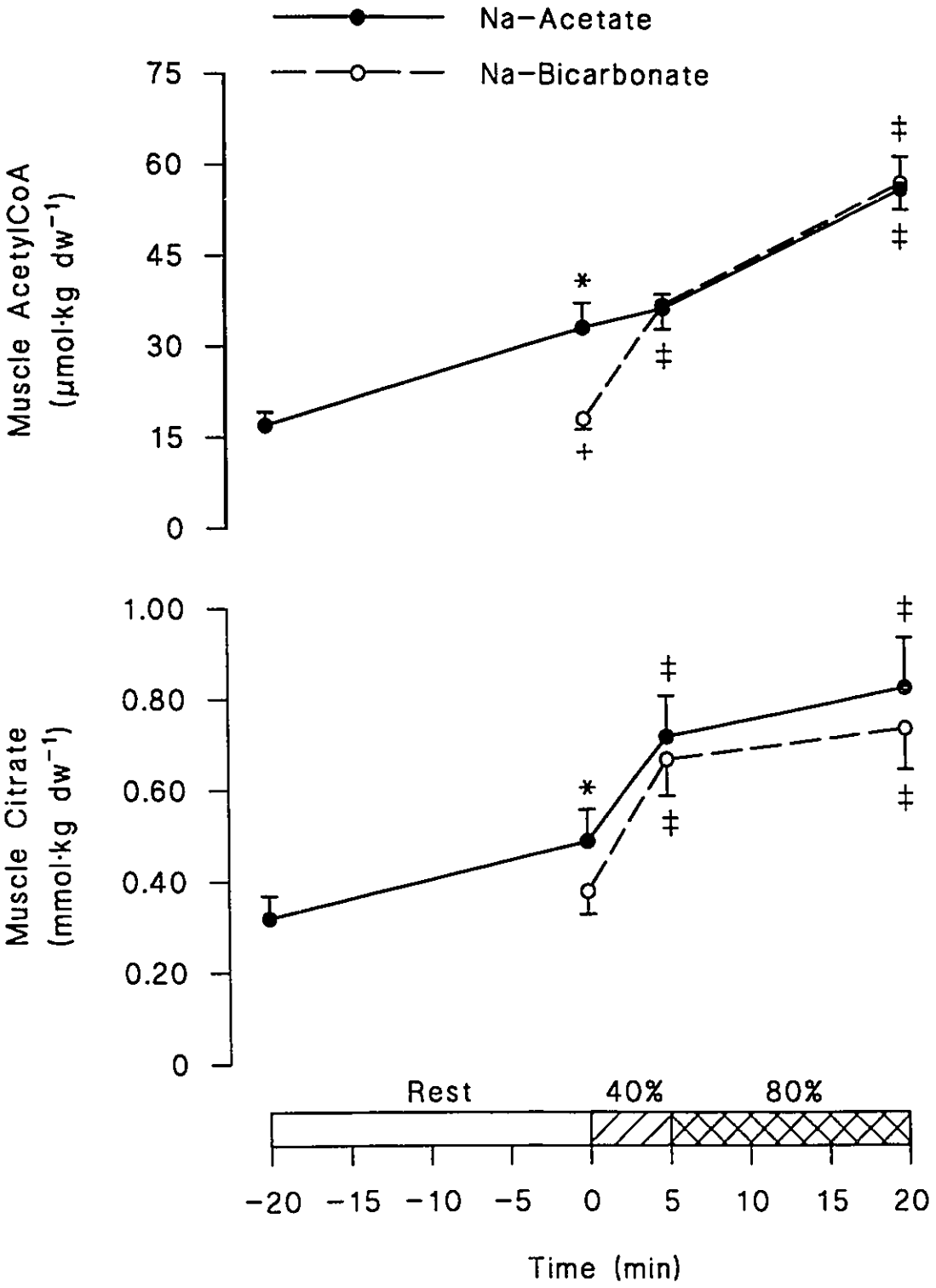

Na–Acetate
Na–Bicarbonate
Muscle AcetylCoA
(μmol·kg dw⁻¹)
75
60
45
30
15
0
Muscle Citrate
(mmol·kg dw⁻¹)
1.00
0.80
0.60
0.40
0.20
0
Rest
40%
80%
−20 −15 −10 −5 0 5 10 15 20
Time (min)

FIGURE 6: Muscle CoASH and acetylcarnitine content at rest (open box), during cycling exercise at 40% of $\dot{V}O_2max$ (single cross hatch box) and during cycling exercise at 80% of $\dot{V}O_2max$ (double cross hatch box) while Na-Acetate (—— ●——) or Na-Bicarbonate (– — O — –) was infused. Data are means ± SEM. *, indicates significantly different from resting value prior to Na-acetate infusion. +, indicates significantly different between conditions at matched times. ‡, indicates significantly different from post-infusion rest value within a condition.

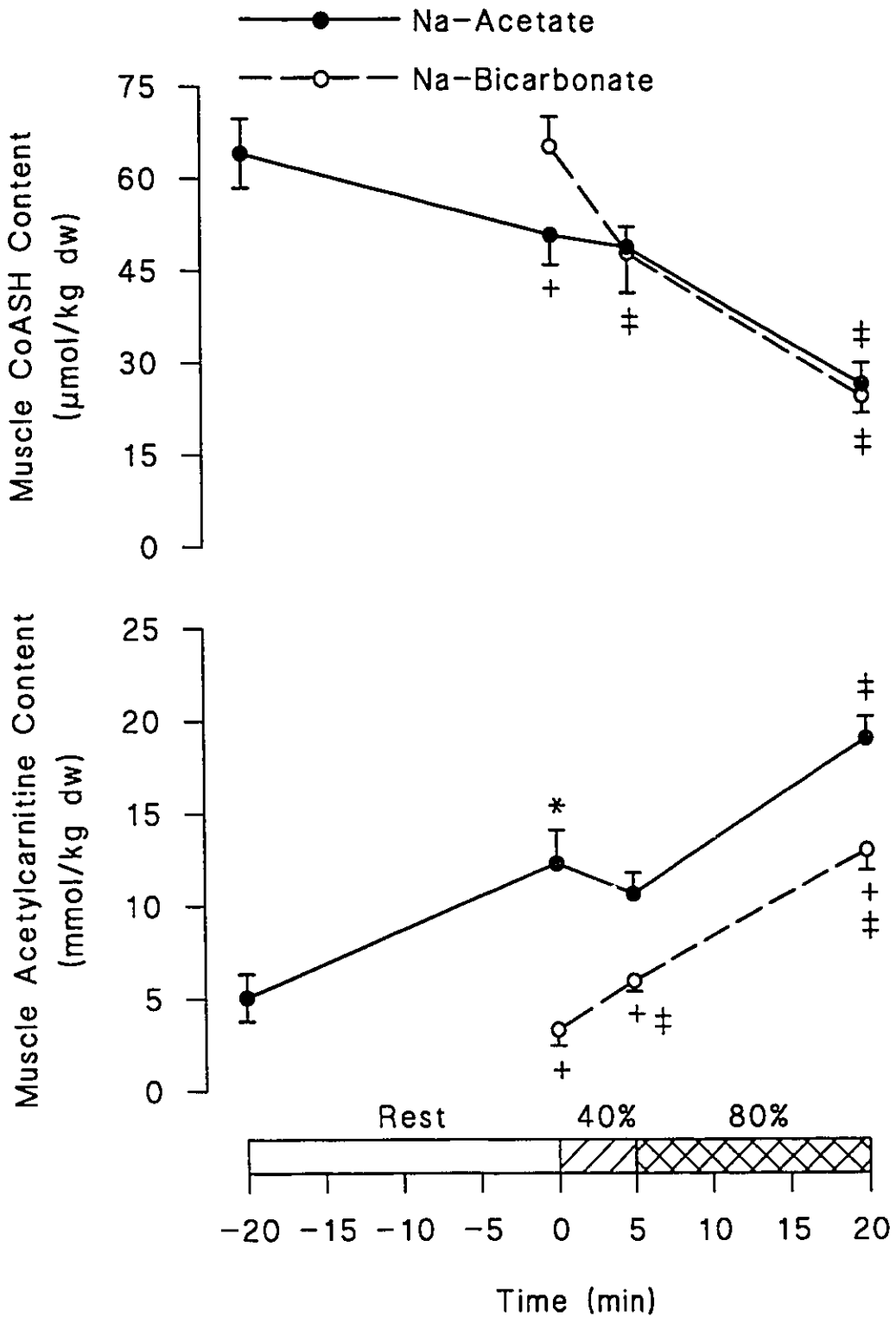

Na-Acetate
Na-Bicarbonate
Muscle CoASH Content
(µmol/kg dw)
75
60
45
30
15
0
Muscle Acetylcarnitine Content
(mmol/kg dw)
25
20
15
10
5
0
Rest
40%
80%
-20 -15 -10 -5 0 5 10 15 20
Time (min)

FIGURE 7: Muscle acetylCoA-to-CoASH ratio and pyruvate dehydrogenase activity (PDHa) at rest (open box), during cycling exercise at 40% of $\dot{V}O_2$max (single cross hatch box) and during cycling exercise at 80% of $\dot{V}O_2$max (double cross hatch box) while Na-Acetate (—— ●——) or Na-Bicarbonate (– — O — –) was infused. Data are means ± SEM. *, indicates significantly different from resting value prior to Na-acetate infusion. +, indicates significantly different between conditions at matched times. ‡, indicates significantly different from post-infusion rest value within a condition.

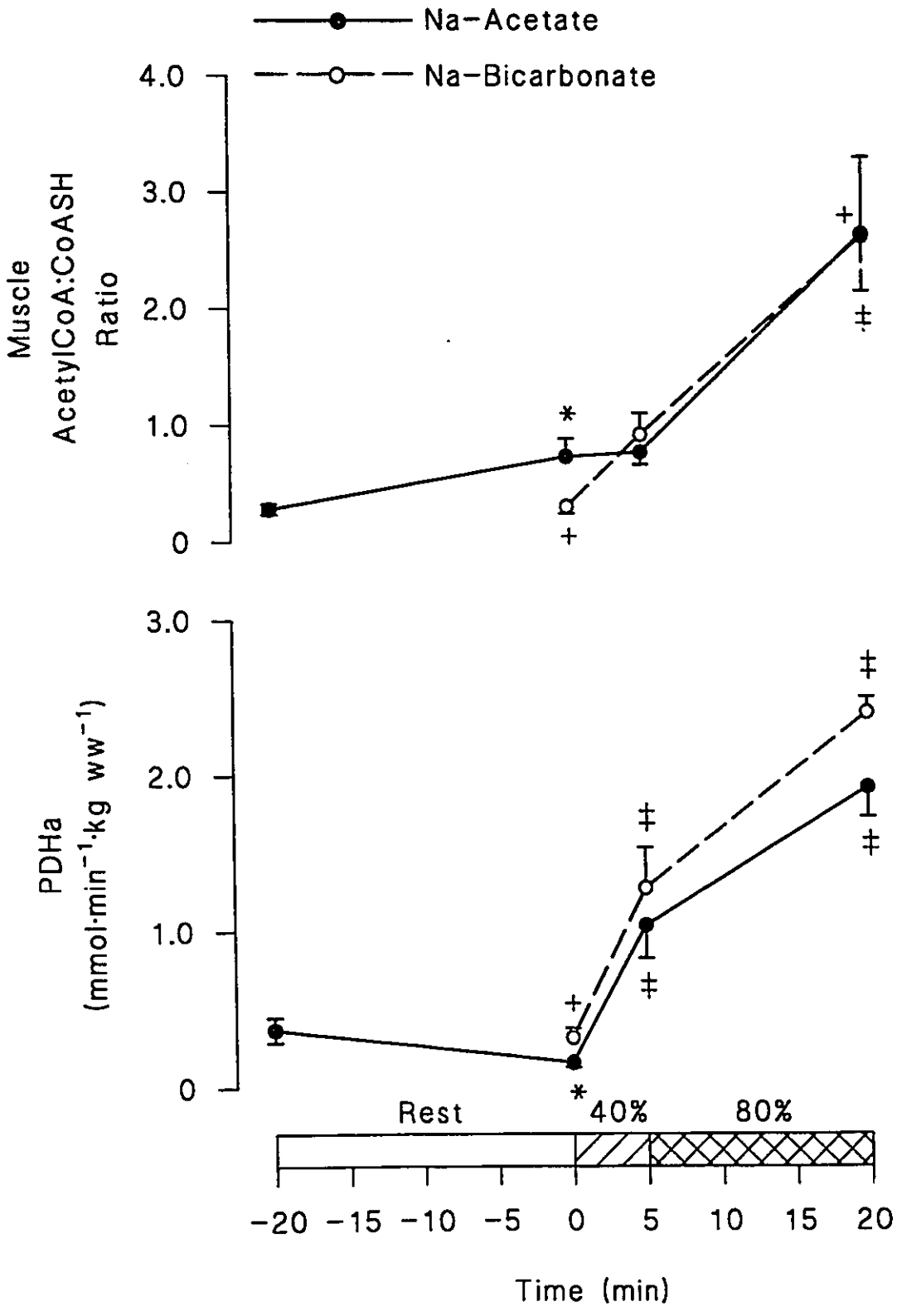

Na-Acetate
Na-Bicarbonate
Muscle Acetyl CoA:CoASH Ratio
4.0
3.0
2.0
1.0
0
PDHa (mmol·min⁻¹·kg ww⁻¹)
3.0
2.0
1.0
0
Rest
40%
80%
-20 -15 -10 -5 0 5 10 15 20
Time (min)

Table 1: Plasma pH in arterialized venous blood samples at rest and during cycle ergometry at 40% of $\dot{V}O_2max$ and at 80% of $\dot{V}O_2max$ during infusion of Na-Acetate or Na-Bicarbonate.

Measure	Condition	-20 min	-10 min	-5 min	0 min	3 min	9 min	14 min	19 min
Plasma pH	Acetate	7.369±0.007	7.413±0.007*	7.435±0.006*	7.452±0.009*	7.443±0.010*	7.425±0.009*	7.412±0.012*	7.421± 0.014*
	HCO_3^-	7.354±0.013	7.396±0.012*	7.412±0.009*	7.428±0.011*	7.413±0.011*	7.421±0.011*	7.434±0.010*	7.445±0.012*

Data are means ± SEM (n = 7). NM, indicates not measured. *, indicates significantly different from pre-infusion resting values.

Table 2. Muscle metabolite content in vastus lateralis at rest and during cycle ergometry at 40% $\dot{V}O_2$max and 80% $\dot{V}O_2$max during infusion of Na-acetate or Na-bicarbonate.

Measure, mmol/kg dry wt	Na-Acetate Infusion				Na-Bicarbonate Infusion		
	Rest		Exercise		Rest	Exercise	
	Pre-Infusion	Post-Infusion	40% $\dot{V}O_2$max	80% $\dot{V}O_2$max	Post-Infusion	40% $\dot{V}O_2$max	80% $\dot{V}O_2$max
Glycogen	NM	519.8±31.8	NM	285.3±24.6‡	540.8±36.6	NM	320.8±43.1‡
Glucose-6-phosphate	1.46±0.40 (5)	1.36±0.35 (5)	2.76±0.67 (5)	5.43±0.90 (5)‡	1.91±0.44 (5)	2.41±0.59 (5)	4.75±0.95 (5)‡
Lactate	3.2±0.8 (6)	3.6±0.6 (5)	5.4±0.6	64.4±5.0‡	4.1±1.2 (7)	8.6±1.5	56.9±8.0‡
PCr	84.2±1.7 (6)	87.2±2.0 (4)	75.1±2.4‡	26.8±2.3‡	86.6±2.7	79.8±2.3	31.5±4.8‡
ATP	27.30±0.57 (6)	26.28±0.63 (4)	27.01±1.02	23.89±1.28	26.01±0.80	26.29±0.93	23.05±2.40

Data are means ± SEM. n = 8 except where indicated in parentheses. NM, indicates not measured. *, indicates significantly different from pre-acetate infusion. ‡,indicates significantly different from post-infusion rest.

3.5 DISCUSSION

The present study examined the effect of Na-acetate infusion on PDHc transformation and substrate utilization during rest and exercise. Na-acetate infusion elevated the plasma acetate concentration to saturating levels for skeletal muscle (29,35). At rest this resulted in elevation of muscle citrate, acetylCoA and the acetlyCoA-to-CoASH ratio. It also coincided with lower PDHa which is consistant with the events that initiate intramuscular glucose restriction. Conversely, during exercise acetate infusion did not elevate muscle acetylCoA, citrate or the acetylCoA-to-CoASH ratio beyond that observed in the control condition. No differences existed between conditions during exercise in either PDHa or glycogen utilization. The results of this study illustrate that resting PDHa decreases when the acetylCoA-to-CoASH ratio is acutely increased which is consistent with the operation of the classical Glc-FA cycle. However, the acute stimulus was insufficient to initiate the ensuing events of intramuscular carbohydrate restriction during exercise.

3.5.1 Glucose-Fatty Acid Cycle

Acetate infusion allowed direct manipulation of the key putative regulators of the glucose-fatty acid cycle. The effects of elevated skeletal muscle acetylCoA and citrate contents on carbohydrate metabolism were examined independent of increased fat availability. Acetate infusion was successful in elevating muscle acetylCoA and citrate at rest but during moderate and intense exercise acetylCoA and citrate contents were similar in the two trials.

At rest acetate infusion increased muscle acetylCoA by 2 fold, the acetylCoA-to-CoASH ratio by 2.6 fold, citrate by 1.5 fold and decreased PDHa by 2.3 fold. The increases

in acetylCoA and the acetylCoA-to-CoASH ratio were consistent with these metabolites' putative inhibitory effect on PDHc transformation to PDHa as predicted in the classic regulatory scheme of the glucose-fatty acid cycle. The increase in resting citrate content was also consistent with the possibility that it downregulates PFK activity. However, there was no accumulation of G-6-P following acetate infusion as predicted if PFK and PDHa activities decreased. Unfortunately, we were not able to measure resting rates of muscle glycolytic flux and glucose oxidation in an attempt to compare them to the measured changes in the proposed regulatory parameters of the glucose-fatty acid cycle.

In summary, the majority of the resting data was consistent with the operation of the glucose-fatty acid cycle as classically proposed by Randle et al. (27,28). However during exercise, acetate infusion did not produce an increase in muscle acetylCoA and citrate content above those produced by exercise alone. Therefore, we were unable to test whether the glucose-fatty acid cycle exists or is regulated as proposed in exercising skeletal muscle.

3.5.2 PDHa Regulation

PDHc is a multi-enzyme mitochondrial complex which catalyzes the irreversible oxidative decarboxylation of pyruvate to acetylCoA and regulates the entry of pathway substrate from carbohydrate sources into the TCA cycle (37). It is primarily composed of multiple copies of the three main catalytic subunits; pyruvate decarboxylase (EC 1.2.4.1; E1), lipoate acetyltransferase (EC 2.3.1.12; E2) and dihydrolipoyol dehydrogenase (EC 1.6.4.3; E3) (37). Also closely associated with the three main subunits are multiple copies of the regulatory enzymes PDH-phosphatase (E.C. 3.1.3.43) and PDH-kinase (E.C. 2.7.1.99) which regulate the phosphorylation state of the E1 component (37) and consequently determine

the extent of PDHc transformation to PDHa. PDH-phosphatase hydrolyzes monophosphate esters from specific serine residues at 3 distinct sites on the E1 components leading to complete transformation to PDHa, while PDH-kinase re-esterifies these sites sequentially 1 through 3, leading to reversal of transformation, varying degrees of inactivation and resistance to reactivation (12,32). Thus, the concerted action of these two regulatory subunits determines PDHa and therefore the potential for pathway substrate flux into the TCA cycle from carbohydrate sources.

The activities of PDH-kinase and PDH-phosphatase are allosterically regulated by cations and metabolites present in the internal cellular environment, which reflect the prevailing tissue activity state, substrate availability and hormonal influences (37). PDH-phosphatase is primarily activated by Ca^{2+} (24) while PDH-kinase is stimulated by NADH (10,25), acetylCoA (10,19), a rising acetylCoA-to-CoASH ratio (10) and ATP (19). It is also inhibited by pyruvate (12), NAD^{+} (10,25), CoASH (10,19), and ADP (19). In the present study we examined the role of PDHc in intramuscular fuel selection with emphasis on manipulating the acetylCoA-to-CoASH ratio which is believed to be the primary physiological regulator of PDH-kinase and thus an important determinant of PDHa.

3.5.2.1 Rest

Resting acetate infusion elevated the acetylCoA-to-CoASH ratio 2.6 fold which caused a concomitant 2.3 fold decrease in PDHc transformation. This was similar to a previous report from our laboratory (26) where subjects demonstrated a 3.2 fold increase in the acetylCoA-to-CoASH ratio and a 3.5 fold decrease in PDHc transformation, after 3 d of consuming a low carbohydrate diet (LCD). Lower resting PDHa observed in the present

study could be explained by a synchronous increase in PDH-kinase and decrease in PDH-phosphatase activities leading to site 1 phosphorylation of the E1 component, which is known to rapidly yet reversibly reduce PDHc transformation (12,32). Furthermore, site 1 phosphorylation has been shown to directly correlate with PDHa in rat muscle (12) and other tissues (32,34). Thus a higher acetylCoA-to-CoASH ratio after acetate infusion may have activated PDH-kinase (10,20) in this manner causing lower PDHa in our subjects.

While it is not possible to directly determine the effect that variations in the mitochondrial ATP-to-ADP ratio may have had on PDHc transformation, lack of changes in muscle PCr content between -20 and 0 min of Na-acetate infusion suggests that no differences existed in this ratio (36) and thus did not affect PDHc transformation. While it is also not possible to determine what effect the NADH-to-NAD ratio may have had on PDHc transformation in the present study, it has previously been demonstrated that the NADH-to-NAD ratio does not affect PDHc transformation in human muscle during activity (9) but it may at rest.

3.5.2.2 Exercise

During the transition from rest to exercise there was a dissociation between PDHa and the acetylCoA-to-CoASH ratio as first reported by first by Constantin-Teodosiu *et al* (6,8,9) and later by our laboratory (26). At 40% $\dot{V}O_2$max PDHa and the acetylCoA-to-CoASH ratio increased to the same extent in the two conditions. In the Na-acetate trial PDHa increased ~6.5 fold to achieve the same degree of transformation observed in the control condition. Upon increasing the power output to 80% $\dot{V}O_2$max, PDHa was completely transformed in both trials, while the acetylCoA-to-CoASH ratio also increased equally in both

conditions.

Transformation to PDHa during the rest-to-work transition was primarily mediated by an increase in Ca^{2+}, a potent activator of PDH-phosphatase (23). In addition, transformation may have also been mediated by greater muscle pyruvate content, inhibiting PDH-kinase and facilitating dephosphorylation of PDHc. However, a rising acetylCoA-to-CoASH ratio also occurred which should have activated PDH-kinase and lowered the rate of PDHc transformation. At 40% $\dot{V}O_2$max, elevated PDH-phosphatase activity should have overridden the inhibitory effects of PDH-kinase on PDHa during the rest-to-work transition but PDHa was only ~44-47% transformed in the two conditions. Thus, it is possible that the allosteric activating factors which act on PDH-phosphatase are subsaturating and failed to sufficiently activate it and supplant the inhibitory effects the greater acetylCoA-to-CoASH ratio. At 80% $\dot{V}O_2$max PDHc transformation was complete despite the potential inhibitory effects of an even greater acetylCoA-to-CoASH ratio, and therefore PDH-kinase activity must have been overridden by elevated PDH-phosphatase activity mediated by increased Ca^{2+} availability during the more intense muscular contractions (23).

Increasing the power output from 40% to 80% $\dot{V}O_2$max resulted in a decrease in PCr content which indicated indicated a decrease in mitochondrial ATP and in the mitochondrial ATP-to-ADP ratio (36). This should have had an inhibitory effect on PDH-kinase and facilitated greater PDHa. Similarly, increased muscle pyruvate content could have facilitated such changes by inhibiting PDH-kinase and ensuring adequate substrate binding.

3.5.2.3 Rest-to-Work Transition

The rapid transformation of PDHc to PDHa during the rest-to-work transition in the

Na-acetate trial and the resolution of differences in the resting muscle acetylCoA-to-CoASH ratio between trials reveals further information about the regulatory influences that determine PDHa. Using rat models of starvation and diabetes, Sale *et al* (32) demonstrated that the inhibitory effects of altered fuel availability, acting through an increase in the acetylCoA-to-CoASH ratio, initially led to an acute increase in PDH-kinase activity. This resulted in increased phosphorylation of site 1 on the E1 components and lower PDHa. In this (32) and other studies (12,34), lower PDHa was positively correlated with site 1 phosphorylation, while a prolonged increase in PDH-kinase activity resulted in phosphorylation of sites 2 and 3 on the E1 component that did not correlate with PDHa. Phosphorylation of these sites did however reduce the rate of PDHc transformation to PDHa during the rest-to-work transition (32,34). In the present study a lower rate of PDHc transformation was not observed in the Na-acetate trial during the rest-to-work transition, suggesting that only site 1 phosphorylation was responsible for the decrease in PDHa in our subjects after resting infusion.

The present study extends the findings of a previous study completed in our laboratory (26) in which we reported a lower rate of PDHc transformation at rest and during the rest-to-work transition after 3 d of consuming a LCD. The results of that study are consistent with a greater PDH-kinase/PDH-phosphatase activity ratio at rest and during exercise leading to lower PDHa. During rest lower PDHa transformation occurred as a result of the prolonged inhibitory effects of an elevated acetylCoA-to-CoASH ratio, secondary to chronically elevated plasma FFA levels, but during exercise at 75% $\dot{V}O_2$max lower transformation was attributed to lower muscle pyruvate content (26). However when compared to the present study the rate of PDHc transformation during the rest-to-work

transition was lower, suggesting that increased site 2 and site 3 occupancy on the E1 components further restricted PDHc transformation. Taken together the results of these studies suggest that the extent of phosphorylation and the rate of transformation during the rest-to-work transition is influenced by the duration of elevated acetylCoA accumulation preceeding activity. Chronically elevated acetylCoA levels as observed after 3 d of consuming a LCD leads to a prolonged activating stimulus on PDH-kinase, increased occupancy of sites 2 and 3 on the E1 components and a greater more persistent constraint on PDHc transformation. In contrast acute elevations of acetylCoA leads to only site 1 occupancy and minor obstacle to transformation during the rest-to-work transition.

3.5.2.4 PDHa Flux

In vivo PDHa flux is largely a function of the phosphorylation state of PDHc but it is also dependent on the availability of substrates and accumulation of products. Knowing the rate of glycogen utilization, glucose uptake, lactate release and muscle lactate accumulation in the active muscle mass, it was possible to calculate the *in vivo* PDHa flux during cycling exercise at 80% $\dot{V}O_2$max. In order to do this it was assumed that; 1) 10 kg of active muscle was involved in the cycling exercise, 2) lactate released from the 10 kg of exercising muscle accumulated in the total body water (TBW) of the remaining 70 kg of body weight ($\sim$42 L), 3) lactate accumulation in the TBW is reflected in plasma lactate values, 4) glucose uptake in our subjects was similar to other reports (26,30) of cycling for 15-20 min at a similar intensity ($\sim$2.2 mmol/min) and accounts for $\sim$0.4 mmol·min^{-1}·kg wet wt^{-1} of PDHa flux, 5) 3 L of intracellular water is equivalent to 1 kg of dry muscle and 6) most of the glycogen utilization and lactate production occurred during the heavy work load.

During exercise glycogen utilization (2.8 mmol·min^{-1}·kg wet wt^{-1}) lactate release (3.3 mmol·min^{-1}·kg wet wt^{-1}) and muscle lactate accumulation (0.7 mmol·min^{-1}·kg wet wt^{-1}) were similar in the two trials and therefore the mean of both trials can be considered together in the calculation of flux. Addition of the glycogen and glucose contributions, subtraction of lactate release, muscle lactate accumulation and anaplerotic reactions involving pyruvate (0.1 mmol·min^{-1}·kg wet wt^{-1}) (31) gives a resultant *in vivo* PDHa flux of 1.9 mmol·min^{-1}·kg wet wt^{-1} which is similar to measured values for each condition. This confirms that measured PDHa reflected the *in vivo* PDHa catalytic activity in our subjects during cycling exercise. Thus the physiological effect of the acetylCoA-to-CoASH ratio on PDHa in our subjects was limited to rest. The acute increase in the acetylCoA-to-CoASH ratio at rest lowered PDHa but exercise conditions completely transformed PDHc overridding the effects of a rising acetylCoA-to-to-CoASH ratio, allowing maximum flux.

3.5.3 Acetate Metabolism and Acetyl-group Accumulation

Plasma acetate concentration was constant during the last 10 min of resting infusion and at each exercise intensity, indicating steady state utilization of acetate. Previous studies have demonstrated that the primary fate of the metabolized acetate is oxidation by the skeletal muscle mass (17,35). The remaining portion of the metabolized acetate may enter minor pathways of utilization and become involved in exchange of carbons with other substrate pools (35). Given the short duration of the infusion protocol that we employed in this study, it is likely that metabolism in skeletal muscle represents the primary route of acetate utilization in our subjects (33).

When the rate of acetylCoA generation from acetate metabolism or glycolysis exceeds

the rate at which it condenses with oxaloacetate to form citrate, acetylCoA accumulates. Catalyzed by carnitine acetyltransferase (CAT), excess acetylCoA can then react rapidly and reversibly with the mitochondrial carnitine to form acetylcarnitine. Mitochondrial acetylcarnitine is subsequently exchanged for cytosolic carnitine by acylcarnitine translocase. The function of the linked PDHc/CAT reactions has been previously reported in human muscle (6,8,9,15). Its suggested physiological importance is three fold. First, it serves to maintain a viable pool of mitochondrial CoASH presumably to maintain flux through the PDHc and oxoglutarate dehydrogenase complex (OGDHc) reactions (6,15). Second, it serves as a cytosolic store for "active acetate" in the form of acetylcarnitine that is immediately available for use by the TCA cycle (15). Third, a reduction in cytosolic carnitine during acetylcarnitine accumulation would encourage preferential use of carbohydrate sources over fat sources by limiting long chain acylcarnitine transport (15).

During 20 min of rest, 200 mmol of Na-acetate were infused at a constant rate of 10 $mmol \cdot min^{-1}$. Assuming a distribution space of 60% of body weight (29,33) and using the mean plasma acetate concentration, it can be estimated that 127 mmol (63%) accumulated in the distribution space leaving 73 mmol (37%) for metabolism in peripheral tissues at a rate of 101 $\mu mol \cdot min^{-1} \cdot kg$ wet wt^{-1}. Assuming 3 L of intracellular water is equal to 1 kg dry muscle and knowing the rate of acetate metabolism, the rate of acetylCoA accumulation and the rate of acetylcarnitine accumulation during the rest period it is also possible to estimate the proportion of acetate left over for use in other pathways including oxidation. Of the 101 μmol of acetate $metabolized \cdot min^{-1} \cdot kg$ wet wt^{-1} during this time, 0.2 $\mu mol \cdot min^{-1} \cdot kg$ wet wt^{-1} ($\sim$0.2%) accumulated as acetylCoA while 90 $\mu mol \cdot min^{-1} \cdot kg$ wet wt^{-1} (88.8%) accumulated as acetylcarnitine leaving 11.2 $\mu mol \cdot min^{-1} \cdot kg$ wet wt^{-1} (11%) of the metabolized acetate available

for oxidation. During resting acetate infusion acetylCoA and acetylcarnitine rose 2.0 and 2.4 fold, respectively, which is consistent with the transfer of active acetate through the mitochondrial CoASH pool to carnitine and into the cytosol where they were stored.

During exercise at 40% $\dot{V}O_2$max, 50 mmol of acetate was infused at the same rate while 3 mmol (6%) accumulated in the distribution space and 47 mmol (94%) were metabolized at a rate of 261 μmol·min^{-1}·kg wet wt^{-1}. At the same time acetylCoA content did not change but the acetylcarnitine pool was utilized at a rate of 82 μmol·min^{-1}·kg wet wt^{-1}. Thus the combined rate of acetate utilization from infused and stored sources was 344 μmol·min^{-1}·kg wet wt^{-1}. During acetate infusion, acetylcarnitine decreased slightly but acetylCoA did not change. Conversly in the control condition acetylCoA and acetylcarnitine increased 2.0 and 1.8 fold, respectively. During moderate cycling in the acetate trial acetyl-groups were drawn from the acetylcarnitine pool to generate citrate for entry into the TCA cycle. In contrast acetylcarnitine accumulation increased in the control trial as glycolytic flux and PDHa flux exceeded flux through the citric acid cycle.

At 80% $\dot{V}O_2$max 150 mmol of acetate were infused and 58 mmol (39%) accumulated while 92 mmol (61%) were metabolized at a rate of 174 μmol·min^{-1}·kg wet wt^{-1}. AcetylCoA accumulated at a similar rate in the two conditions. Similarly, acetylcarnitine also accumulated at a similar rate in the two trials but absolute accumulation was greater in the acetate trial as a result of the preceeding infusion at rest and during moderate exercise. During heavy exercise the increased transference of acetyl-groups into the cytosol was probably necessary to maintain a viable pool of free mitochondrial CoASH to sustain PDHa and TCA cycle flux.

3.5.4 Summary

The present study examined the role of PDHa in the integration of carbohydrate and fat fuel utilization in human skeletal muscle during rest and incremental exercise. Na-acetate infusion was used to acutely elevate the availability of the short chain fatty acid anion, acetate, as well as increase the rate of acetylCoA and acetylcarnitine generation. The most important finding of this study was that PDHa can be acutely regulated by variations in the acetylCoA-to-CoASH ratio at rest but not during exercise. At rest the data was consistent with the role of PDHa in the operation of the Glc-FA cycle as proposed (27), where PDHc transformation to PDHa was regulated by the acetylCoA-to-CoASH ratio. Conversely, during moderate and intense exercise no evidence was found for the operation of the Glc-FA cycle as classically proposed. Complete transformation of PDHc to PDHa occurred despite a rise in the acetylCoA-to-CoASH ratio. The role of PDHa in fuel selection during exercise may be dependent on the period of fat fuel availability and therefore acetylCoA accumulation in the rest period that precedes exercise.

3.6 References

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CHAPTER 4

SKELETAL MUSCLE PYRUVATE DEHYDROGENASE ACTIVITY DURING MAXIMAL EXERCISE IN HUMANS

(published in Am. J. Physiol. (Endocrinology and Metabolism): In Press, 1995)

4.1 Abstract

The regulation of the active form of pyruvate dehydrogenase (PDHa) and related metabolic events were examined in human skeletal muscle during repeated bouts of maximum exercise. Seven subjects completed 3 consecutive 30 second bouts of maximum isokinetic cycling, separated by 4 min of recovery. Biopsies of the vastus lateralis were taken before and immediately after each bout. PDHa increased from 0.45±0.15 to 2.96±0.38; from 1.10±0.11 to 2.91±0.11 and from 1.28±0.18 to 2.82±0.32 mmol·min^{-1}·kg ww^{-1} during bouts 1, 2 and 3 respectively. Glycolytic flux was 13 fold greater than PDHa in bouts 1 and 2, and 4 fold greater during bout 3. This discrepancy between the rate of pyruvate production and oxidation resulted in substantial lactate accumulation to 89.5±11.6 in bout 1, 130.8±13.8 in bout 2 and 106.6±10.1 mmol·kg dw^{-1} in bout 3. These events coincided with an increase in the mitochondrial oxidation state, as reflected in a fall in the mitochondrial NADH-to-NAD ratio, indicating that muscle lactate production during exercise was not an O_2 dependent process in our subjects. During exercise the primary factor regulating PDHa transformation was probably intracellular Ca^{2+}. In contrast, the primary regulatory factors causing greater PDHa during recovery were lower ATP-to-ADP and NADH-to-NAD ratios, and increased

concentrations of pyruvate and H^+. Greater PDHa during recovery facilitated continued oxidation of the lactate load between exercise bouts.

4.2 Introduction

Since the studies of Hill and Lupton in 1923 (19), muscle lactate production has been attributed to lack of oxygen supply. This theory still receives widespread support (see (23) for review) and is believed to operate as follows. With increasing intensity of contraction, tissue O_2 supply becomes limiting for aerobic metabolism resulting in the eventual cessation of aerobic ATP production. As mitochondrial ATP production decreases below ATP utilization by the muscle, cytosolic ATP levels decrease while ADP, AMP and P_i increase, providing a stimulus to accelerate anaerobic glycolysis. Greater glycolytic rate leads to cytosolic pyruvate and NADH accumulation, which in turn react to form lactate, the end-product of anaerobic glycolysis. Meanwhile in the mitochondria, NADH and ADP also accumulate due a substrate limitation of O_2 at cytochrome oxidase. This theory assumes that the cytosol and mitochondria are not separately regulated compartments and that NADH and ADP levels rapidly achieve equilibrium between compartments. As tissue O_2 decreases the net effect of these events is an accelerated rate of anaerobic ATP production to augment or replace aerobic ATP production.

Considerable controversy still exists regarding the mechanism of lactate production in exercising muscle. Studies that have used direct measurement of whole tissue NADH and NAD report a reduction of the muscle redox state (33) and argue that lower tissue O_2 tension is responsible for both alterations in redox state and lactate accumulation in exercising muscle (3). In contrast, determination of mitochondrial redox state by determining

the concentrations of reactants related to the mitochondrial enzyme, glutamate dehydrogenase, has yielded opposite results. Graham and Saltin (15) have shown that the mitochondrial redox state became more oxidized in human muscle during incremental exercise while lactate accumulation increased. This suggests that tissue O_2 levels are not compromised and that tissue hypoxia is not the cause of muscle lactate production during exercise. Similar studies examining stimulated dog muscle to simulate various exercise intensities also found that muscle lactate production was independent of tissue O_2 levels (8,9,21). Determination of mitochondrial redox state using surface fluorometry (21), has shown that increased mitochondrial oxidation occurs concurrently with lactate accumulation during exercise. Also, studies of myoglobin saturation (8,9) indicate that tissue O_2 levels are maintained constant during work of increasing increments, and are not limiting for optimal function of the electron transport chain.

Muscle lactate production may increase in the presence of adequate O_2 delivery if the rate of pyruvate production by glycolysis becomes greater than the rate of its oxidation by the pyruvate dehydrogenase complex (PDHc). PDHc is a multi-enzyme flux generating complex which controls the rate of entry of pyruvate-derived acetyl-CoA into the TCA cycle (37). During intense exercise when the dependence on carbohydrate oxidation for energy production is maximized, the difference between glycolytic flux and flux through the active form of PDHc (PDHa) may become increasingly large. As the rate of pyruvate generation exceeds the capacity for oxidation by PDHa, pyruvate should accumulate and react with glycolytically generated NADH to form lactate without a decrease in mitochondrial O_2. Conversion of pyruvate to lactate is catalyzed by the near-equilibrium enzyme, lactate dehydrogenase, which maintains lactate concentration an order of magnitude higher than

pyruvate.

The purpose of the present investigation was to test the hypothesis that lactate accumulation in maximally contracting human skeletal muscle results from a greater rate of glycolytic pyruvate production than pyruvate oxidation by PDHa. We also studied the factors regulating PDHa in human muscle during maximal exercise and measured the contribution of PDHa flux to energy production during repeated bouts of maximal cycling exercise. This model was choosen because previous studies have shown a progressive fall in the work achieved during repeated 30 second bouts of maximal cycling, accompanied by a progressive reduction in lactate production (27,34).

4.3 Methods

4.3.1 Subjects

Seven male subjects (mean±SEM, age: 22.4±0.8; height: 178±2 cm; weight: 73.8±4.4) participated in this study. Approval was obtained from the ethics committee of McMaster University. Written informed consent was obtained from each subject after an explanation of the attendant risks associated with the study protocol.

4.3.2 Experimental Protocol and Determination of Muscle Performance

Each subject completed three 30 s bouts of isokinetic cycling at 100 rpm separated by 4 min of rest recovery as previously described (34). Biopsies of the vastus lateralis were taken immediately before and after each 30 s bout. The isokinetic cycle ergometer used in this study has been previously described (26). Briefly, the cycle was fitted with a 3 hp DC motor which set the upper limit of crank velocity at 100 rpm for this study. The forces

exerted by the subjects were recorded by strain gauges attached to the pedal cranks. Electrical signals were transferred to a chart recorder and computer. Work was calculated for each pedal stroke as the product of impulse and angular velocity. Total work was calculated as the sum of 50 pedal strokes for two legs over each 30 second period.

4.3.3 Pre-experimental Protocol

Since it was not possible to make all measurements on the same day, one week before biopsy samples were collected, each subject completed the cycling protocol while expired gases were continuously sampled. Oxygen uptake ($\dot{V}O_2$) and carbon dioxide output ($\dot{V}CO_2$) measurements were made from expired gases using a Quinton metabolic cart (Quinton Q-plex 1, Quinton Instrument, Seattle, WA). Respiratory exchange ratio (RER) was calculated as the quotient of $\dot{V}CO_2$ / $\dot{V}O_2$. Data were averaged over 15 second intervals.

4.3.4 Muscle Sampling

Biopsy sites were prepared on each thigh superficial to the vastus lateralis. Under local anesthesia (2% xylocaine without epinephrine) incisions were made through the skin to the deep fascia. Biopsies of the vastus lateralis was obtained as described by Bergström (2). Biopsies were immediately frozen in liquid N_2 and removed from the needle while still frozen. Under liquid N_2, 10-30 mg of tissue was chipped from each biopsy and dissected free of blood and connective tissue. From this, two clean 5-15 mg portions were stored separately under liquid N_2: one for determination of PDHa and the other for determination of total pyruvate dehydrogenase activity (PDHt). The remaining muscle was freeze dried, dissected free of blood and connective tissue and stored dry at -50 °C, until analyzed for metabolite

concentrations.

4.3.5 PDHc Analysis

PDHa and PDHt were measured as previously described (29,30) using a radiometric method (11) that determines the rate of acetylCoA production as a function of time. Briefly, for the measurement of PDHa the sample was gently homogenized on ice in a solution containing 50 mM tris, 200 mM sucrose, 50 mM KCl, 5 mM EGTA, 5 mM $MgCl_2$, 50 mM NaF, 5 mM dichloroacetic acid (DCA), 1 mM dithiothreitol (DTT) and 0.1% triton X-100 (vol:vol) at pH 7.8. For the measurement of PDHt, the sample was homogenized in a similar buffer with the following differences: exclusion of 50 mM NaF; inclusion of 10 mM $CaCl_2$, 25 mM $MgCl_2$, 4 $U \cdot ml^{-1}$ hexokinase, 10 mM glucose and 10 mM DCA.

The buffer used to assay PDHa and PDHt was modified somewhat from the original (11) as we have previously described (29,30): it contained 100 mM tris, 0.5 mM EDTA, 1.0 mM $MgCl_2$, 3.0 mM NAD^+, 1.0 mM CoASH, 1.0 mM thiamine pyrophosphate and 1 mM DTT, and the pH was adjusted to 7.8. The reaction was initiated by adding pyruvate to a final concentration of 1.0 mM. 200 µl aliquots of the reaction mixture were sampled at 1, 2 and 3 min and the reaction was stopped by adding each aliquot to 40 µl of 0.5 M perchloric acid (PCA). After 5 min each aliquot was neutralized with 1.0 M K_2CO_3 and centrifuged for 3 min at 15,900×G (microcentrifuge E, Beckman Instrument Co., Mississauga, Ont., Canada). The resulting supernatant was stored at -50 °C until analyzed for acetylCoA by the method of Cederblad *et al* (5). Plots of acetylCoA as a function of time were used to determine the reaction rates. For analysis of PDHt, each sample was incubated on ice for 1 h which was found to be optimal for complete transformation to PDHt. PDHt was taken as the mean of

the two highest measurements for each subject.

4.3.6 Metabolite Analysis

Just before metabolite analysis a portion of the dry powdered muscle was extracted in a solution of 0.5 M (PCA) and 1 mM EDTA, and neutralized to pH 7.0 with 2.2 M $KHCO_3$. Three such extractions were completed. The first extraction was used for the determination of ATP, PCr, acetylCoA, total CoA, acetylcarnitine, free carnitine, total carnitine, lactate, glutamate and oxoglutarate. ATP and PCr were measured on the same day as extraction. A second portion of the dry muscle was extracted for analysis of muscle pyruvate and assayed on the same day. A third portion of dry muscle was extracted before ammonia analysis. These samples were stored in liquid N_2 as a PCA extract and were neutralized just before the assay to prevent loss of NH_3 from the samples.

Neutralized PCA extracts were analyzed for ATP, PCr, lactate, oxoglutarate, glutamate, pyruvate and NH_3 as described by Bergmeyer (1). CoASH, carnitine and their acetylated forms were analyzed as described by Cederblad *et al* (5).

4.3.7 Calculations

The mitochondrial redox state (NADH-to-NAD ratio) was calculated from the glutamate dehydrogenase reaction using measurements of glutamate, NH_3 and oxoglutarate as described by Graham and Saltin (15). The mitochondrial redox state was calculated as follows:

$$[NADH] / [NAD] = (k_{eq} \times [glutamate]) / ([oxoglutarate] \times [NH_3]),$$

where the equilibrium constant (k_{eq}) was 3.87×10^{-10} M, as calculated by Williamson and Krebs (38) at a $[H^+]$ of 100 nM (pH 7.0). The cytosolic redox state was calculated as NAD-to-NADH ratio, or the reciprocal of the mitochondrial redox state, as follows:

$$[NAD] / [NADH] = ([pyruvate] \times [H^+]) / (k_{eq} \times [lactate]),$$

where k_{eq} was 1.11×10^{-11} M, also as calculated by Williamson and Krebs (38).

Intracellular pH was calculated from muscle lactate and pyruvate using the empirical relationship of Sahlin *et al* (32) and validated by Spriet (34), in the type of exercise used in the present study. For rest the following equation was used:

$$pH = 7.22 - 0.00521 \times (lactate + pyruvate).$$

For exercise samples the following equation was used:

$$pH = 7.06 - 0.00413 \times (lactate + pyruvate),$$

where the units of expression for lactate and pyruvate are mmol·kg dw^{-1}.

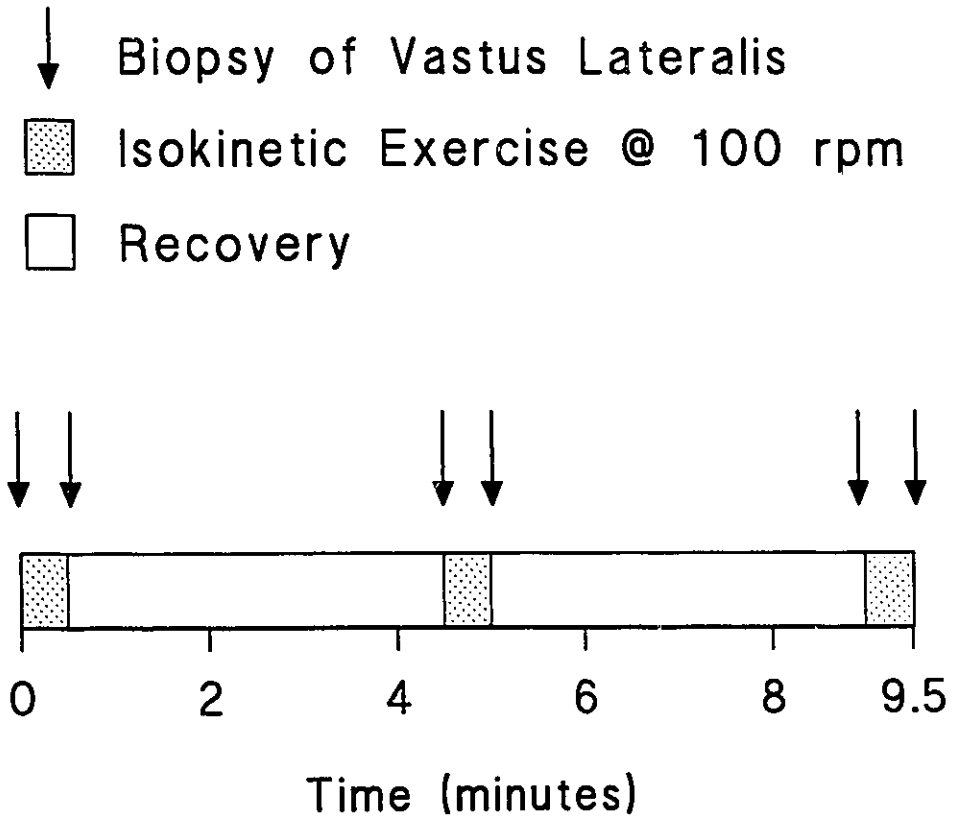
4.3.8 Correlation and Multilinear Regression Analysis

Correlation and multilinear regression analyses were completed to determine which regulatory factors best predicted PDHa at rest and during recovery, and to characterize their relationship to PDHa.

4.3.9 Statistical Analysis

Data were analyzed by a one-way analysis of variance with repeated measures over time. When a significant *F* ratio was found, the Newman-Kuels post hoc test was used to compare means for all data except the NADH-to-NAD ratio. When a significant *F* ratio was found for the NADH-to-NAD ratio, Fisher's pairwise comparisons were used to identify differences. For $\dot{V}O_2$, $\dot{V}CO_2$ and RER data, analysis was completed only on those points demarcated by SE bars. Results were considered significant a $p<0.05$.

FIGURE 1: Experimental Protocol. The shaded boxes on the time axis indicate isokinetic cycling exercise at 100 rpm. The open boxes indicate rest recovery. Biopsies of the vastus lateralis were taken immediately before and after each 30 second bout of maximal isokinetic cycling at 100 rpm.


Biopsy of Vastus Lateralis
Isokinetic Exercise @ 100 rpm
Recovery
0
2
4
6
8
9.5
Time (minutes)

4.4 *Results*

4.4.1 *Total Work*

Total work performed during the first 30 s bout of isokinetic cycling was 18.7±1.2 kJ. It significantly declined to 15.6±1.2 kJ in bout 2 and still further to 14.2±1.3 in bout 3. Total work did not differ between the pre-experimental and experimental trials. During the second trial total work also declined with each successive bout: from 19.3±0.9 to 16.3±1.0 and 14.2±1.2 kJ for bouts one, two and three, respectively.

4.4.2 *$\dot{V}O_2$, $\dot{V}CO_2$ and RER*

Resting $\dot{V}O_2$ (Figure 1) was 0.46±0.04 l·min^{-1} and reached a peak value of 2.84±0.17 after 30 s of cycling. Peak VO_2 increased from 0.83±0.08 to 3.27±0.12 and from 0.84±0.10 to 3.14±0.11 l·min^{-1} in the second and third bouts, respectively. During the rest periods $\dot{V}O_2$ partially recovered but remained elevated over basal resting levels. Before bouts 2 and 3 $\dot{V}O_2$ was 0.83±0.08 and 0.84±0.10 l·min^{-1}, respectively. Four min after bout 3, $\dot{V}O_2$ was 0.86±0.11 l·min^{-1}.

Resting $\dot{V}CO_2$ (Figure 1) was 0.44±0.06 l·min^{-1}, increased to 3.31±0.20 after 30 s of cycling and reached a peak $\dot{V}CO_2$ of 3.80±0.11 15 s later. Peak $\dot{V}CO_2$ was reduced to 3.08±0.08 in bout 2 and 2.45±0.12 in bout 3. During the 4 min rest periods $\dot{V}CO_2$ fell to 1.20±0.11, 0.99±0.11 and 0.86±0.10 l·min^{-1}.

With the onset of cycling in the first bout CO_2 evolution was greater and more rapid than O_2 consumption. This was followed by a slower rate of change in $\dot{V}CO_2$ during the ensuing rest period which resulted in a rise in the RER (Figure 1) that remained elevated throughout the first rest period. During the second and third bouts of cycling the magnitude

of CO_2 evolution declined such that it was lower than O_2 consumption, resulting in a dramatic decline in the RER. The RER (Figure 1) was 0.93±0.05 at rest, rose to 1.17±0.04 after 30 s of cycling and reached a peak value of 2.01±0.07 at 1.25 min into the first 4 min rest period. Thereafter the RER slowly declined to reach 1.45±0.02 prior the second bout where after another 30 s of maximal cycling it declined further to 0.95±0.03. At 1.25 min into the second 4 min rest period the RER again reached a peak value of 1.48±0.07. Before the final bout the RER recovered to 1.19±0.04 and the ensuing 30 s of cycling again resulted in a rapid decrease to 0.78±0.03. RER reached a peak value of 1.26±0.09 at 1.5 min into the last recovery period and was 1.00±0.02 at the end.

4.4.3 PDHc Transformation to PDHa

PDHa (Figure 2) was 0.45±0.15 mmol·min^{-1}·kg ww^{-1} before commencement of exercise which represented 14.4±3.9% transformation of PDHc to PDHa. PDHa rose to 2.96±0.38 during the first bout of maximal cycling exercise which represented complete transformation (98.6±8.3%). The following two bouts of maximal cycling also resulted in complete transformation reaching 2.91±0.11 (100.0±6.9%) after the second bout and 2.82±0.32 (92.7±5.7%) after the third. In two the intervening rest-recovery periods PDHa fell to 1.10±0.11 (34.1±3.3%) in the first and 1.28±0.18 (44.5±8.9%) in the second. During each of the rest recovery periods PDHa remained elevated 2.2 and 2.8 fold over the initial resting value. PDHt for this group of subjects was 2.96±0.17 mmol·min^{-1}·kg ww^{-1}.

4.4.4 AcetylCoA-to-CoASH Ratio

The acetylCoA-to-CoASH ratio (Figure 3) was 0.32±0.04 at rest and rose as a result

of maximal cycling to 0.57±0.04, 0.96±0.14 and 1.73±0.75 after each of the three exercise bouts. In the two intervening rest periods this ratio recovered to resting levels. It was 0.47±0.03 and 0.52±0.03 at the end of the first and second recovery periods, respectively.

4.4.5 ATP-to-ADP Ratio

Muscle ATP (Table 1) remained stable during the first and second bouts but was reduced during the third bout. In contrast, muscle ADP (Table 1) alternately increased with each exercise bout and recovered to basal levels during the ensuing 4 min recovery period. Consequently the resultant ratio of ATP-to-ADP fell during maximal cycling and recovered during the rest periods (Figure 3).

At rest the ATP-to-ADP ratio (Figure 3) was 8.9±0.5 and fell after one bout of maximal cycling to 6.4±0.9. After recovering in the first rest period (7.1±0.9) it fell again after the second bout to 5.2±0.6. During the second recovery period it was partially restored reaching to 6.4±1.1 but did not completely recover to resting levels. After the third bout it decreased even further to 4.5±0.7. Muscle AMP (Table 1) did not vary throughout the experimental protocol.

4.4.6 Mitochondrial and Cytosolic Redox State

Ammonia levels alternately rose and fell with successive bouts of maximal cycling while changes in muscle glutamate were opposite, falling with each successive bout (Table 1). Neither metabolite completely recovered to their pre-exercise resting values: ammonia remained slightly elevated while glutamate levels remained lower than the pre-exercise rest values. In contrast to these two metabolites, oxoglutarate decreased after bout 1 and did not

recover thereafter (Table 1).

The mitochondrial NADH-to-NAD ratio (Figure 3) was 25.7±6.0 at rest and decreased to 9.6±1.7 after the first bout. It partially recovered toward resting levels during the first recovery period, reaching 15.3±2.6. However, the second bout of cycling resulted in a further decline to 4.9±0.6 that remained low throughout the rest period (5.3±0.7) and did not change after bout 3 (4.8±0.9). In contrast, the cytosolic compartment became more reduced (Table 1).

4.4.7 Lactate, Pyruvate and [H⁺]

Intracellular accumulation of lactate, pyruvate and H^+ (Figure 4) occurred as a result of maximal cycling exercise. Muscle lactate (Figure 4) increased as a result of cycling from 6.6±0.8 mmol·kg dw^{-1} at rest to 89.5±11.6, 130.8±13.8 and 106.6±10.1 after each of the three bouts of maximal cycling. Lactate accumulation partially recovered during the first and second rest periods to 51.3±8.6 and 81.7±9.2 mmol·kg dw^{-1}, respectively.

Muscle pyruvate increased from 0.47±0.09 mmol·kg dw^{-1} at rest to 1.95±0.34 and 1.77±0.32 after the first and second bouts, respectively. In the intervening recovery period pyruvate fell to 0.77±0.10 which did not differ from rest. During the second recovery period pyruvate decreased to 0.96±0.12 but did not change after 30 s of maximal cycling in bout 3 (0.93±0.18).

Calculated $[H^+]$ (Figure 4) was 65.6±0.6 nmol·l^{-1} at rest and increased to 216.5±27.6, 277.8±21.2 and 247.1±21.1 after 1, 2 and 3 bouts of maximal cycling. During the first and second recovery periods, $[H^+]$ partially recovered to 116.0±11.2 and 167.1±17.3 nmol·l^{-1}, respectively.

4.4.8 Acetyl-group Accumulation

Total CoA (Figure 5) was relatively constant throughout varying only slightly from 46.7±5.38 μmol·kg dw^{-1} at rest to 54.9±3.93 after the first 30 s bout and 57.6±7.33 after the third. AcetylCoA (Figure 5) alternately rose and fell with successive bouts: it was 11.1±1.6 μmol·kg dw^{-1} at rest and rose to 19.7±1.3, 24.7±3.0 and 31.6±5.2 after each of the three 30 s bouts of maximal cycling. During the two intervening 4 min rest periods acetylCoA partially recovered to 16.2±1.6 and 18.1±1.6 μmol·kg dw^{-1} after the first and second rest period, respectively.

Free CoA (CoASH) (Fig 6) did not change during the first 30 s bout of cycling: it was 35.6±3.9 μmol·kg dw^{-1} at rest and 35.2±2.9 after 30 s of isokinetic cycling. During the second and third exercise bouts, CoASH mirrored changes in acetylCoA, decreasing as the acetylCoA pool grew larger during exercise and rising as excess acetylCoA was metabolized during the rest periods. CoASH fell to 26.9±2.7 μmol·kg dw^{-1} after the second and 26.0±4.4 after the third 30 s bout of cycling and completely recovered to 35.2±3.5 and 36.1±4.3 at the end of the first and second rest periods, respectively.

The pattern of acetylcarnitine accumulation (Figure 6) was similar to acetylCoA in that it rose as a result of 30 s of maximal cycling. However, it differed from the pattern of acetylCoA accumulation observed during the rest-recovery periods by continuing to rise throughout, indicating a transfer of acetyl-groups to the larger carnitine pool. Acetylcarnitine was 4.0±1.0 mmol·kg dw^{-1} at rest and rose to 6.3±0.7, 8.8±0.7 and 8.9±0.6 after bouts one, two and three, respectively. During the first and second recovery periods, acetylcarnitine rose continuously to 7.7±1.4 and 9.1±1.3 mmol·kg dw^{-1}, respectively. Changes in free carnitine (Figure 6) mirrored those of acetylcarnitine, decreasing continuously from 14.1±1.8 mmol·kg

dw^{-1} at rest to 8.4±1.0 after bout 3. Total carnitine (Figure 6) did not change throughout: it was 18.4±1.7 mmol·kg dw^{-1} at rest and remained constant during 3 bouts of maximal exercise and the two rest periods. Total carnitine ranged from 19.3±1.1 after bout 1 to 17.1±1.6 after bout 3.

4.4.9 Correlation and Multilinear Regression Analyses

Correlation analysis of PDHa and the regulatory factors, ATP-to-ADP ratio, NADH-to-NAD ratio, PCr, pyruvate and $[H^+]$ during rest and the recovery periods revealed the following. The ATP-to-ADP ratio (r = -0.115), NADH-to-NAD ratio (r= -0.514) and PCr (-0.733) were negatively correlated with PDHa, while pyruvate (r = 0.536) and H^+ (r = 0.486) were positively correlated. Furthermore, multi-linear regression analysis using these independent variables accounted for 76.5% of the variation in PDHa at rest and during recovery. The resulting regression equation was as follows:

$$PDHa = 1.88 + (0.0295 \times [ATP]/[ADP]) - (0.00473 \times [NADH]/[NAD]) - (0.0253 \times [PCr]) + (0.419 \times [pyruvate]) + (0.00305 \times [H^+]),$$

$$R^2 = 76.5\%, \; p<0.006,$$

where pyruvate and PCr concentration is expressed as mmol·kg dw^{-1} and H^+ concentration was $nmol \cdot l^{-1}$.

FIGURE 2: Oxygen uptake ($\dot{V}O_2$) carbon dioxide output ($\dot{V}CO_2$) and respiratory exchange ratio (RER) during 1 minute of rest before commencement of exercise, rest recovery (open boxes) and during 30 seconds of maximal isokinetic cycling exercise. Data are means ± SEM. *, indicates significantly different from rest. +, indicates post-exercise different from pre-exercise of the same bout. ‡, indicates significantly different from post-bout 1. Only those points demarcated by error bars were analyzed.

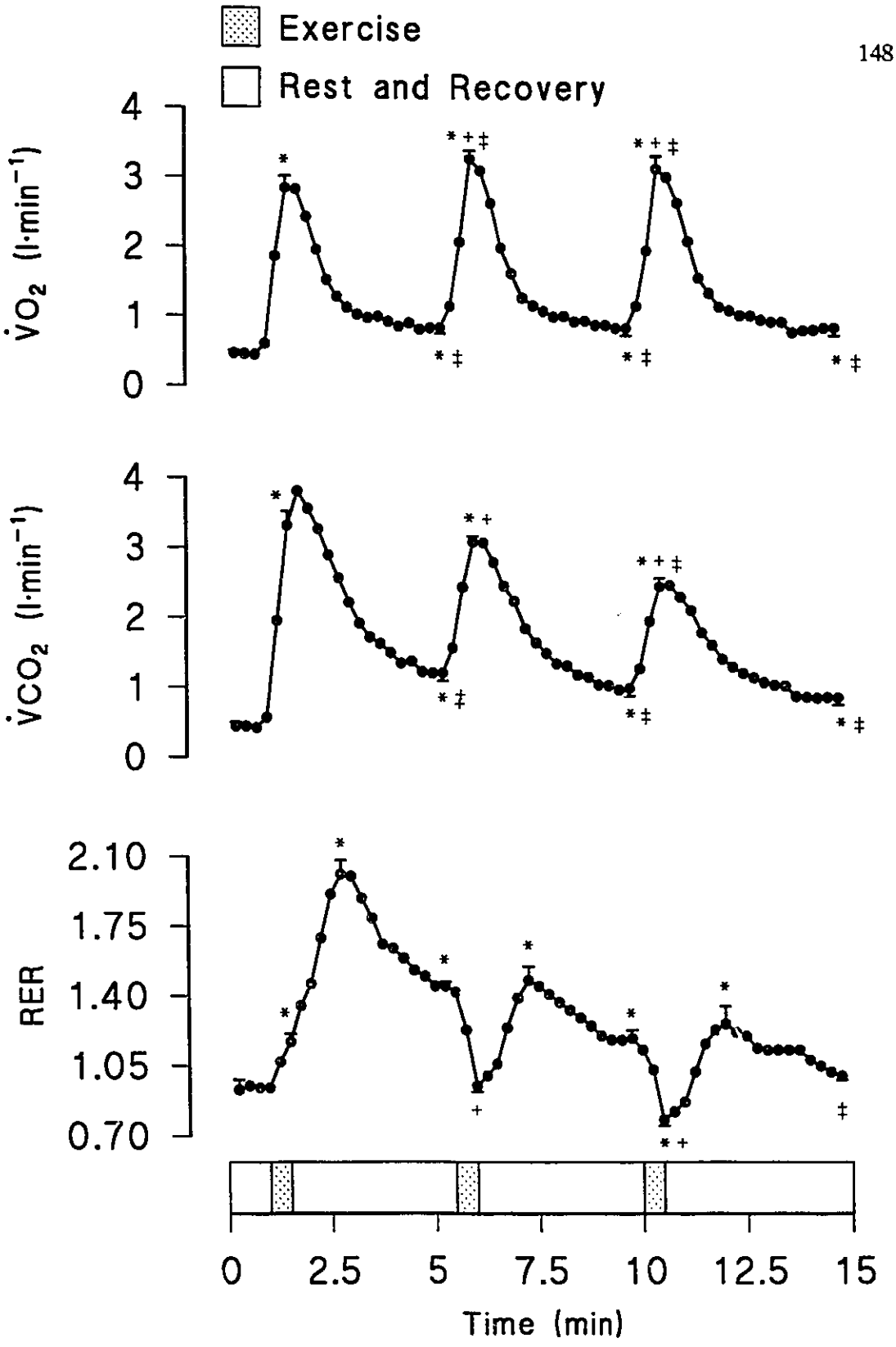

Exercise
Rest and Recovery
$\dot{V}O_2$ (l·min^{-1})
$\dot{V}CO_2$ (l·min^{-1})
RER
4
3
2
1
0
2.10
1.75
1.40
1.05
0.70
0
2.5
5
7.5
10
12.5
15
Time (min)

FIGURE 3: Muscle pyruvate dehydrogenase activity (PDHa) during maximal isokinetic cycling (shaded box) and during rest recovery (open boxes). Data are means ± SEM. *, indicates significantly different from rest. +, indicates post-exercise different from pre-exercise of the same bout. ‡, indicates significantly different from post-bout 1.

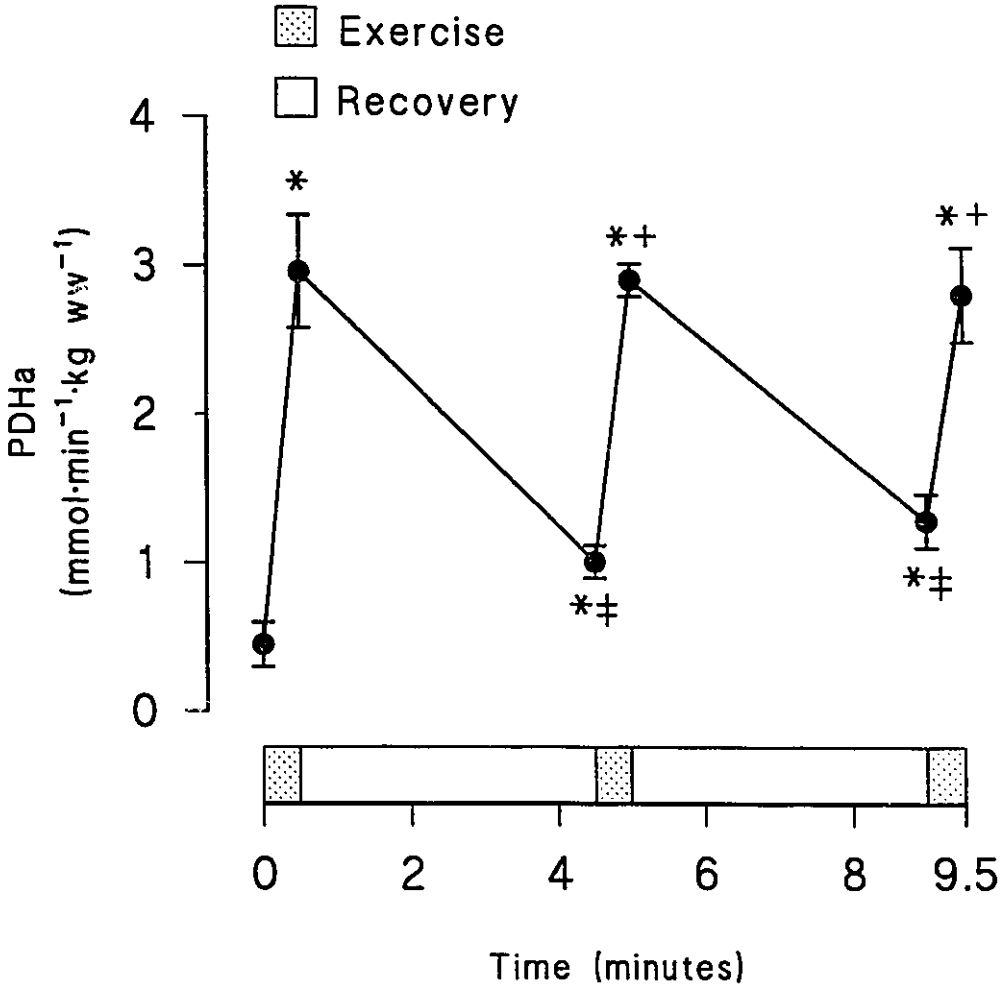

Exercise
Recovery
PDHa
(mmol·min⁻¹·kg ww⁻¹)
4
3
2
1
0
*
*+
*+
*‡
*‡
0
2
4
6
8
9.5
Time (minutes)

FIGURE 4: Mitochondrial acetylCoA-to-CoASH, ATP-to-ADP and mitochondrial NADH-to-NAD ratios during maximal isokinetic cycling (shaded boxes) and during rest recovery (open boxes). Data are means ± SEM. *, indicates significantly different from rest. +, indicates post-exercise different from pre-exercise of the same bout. ‡, indicates significantly different from post-bout 1.

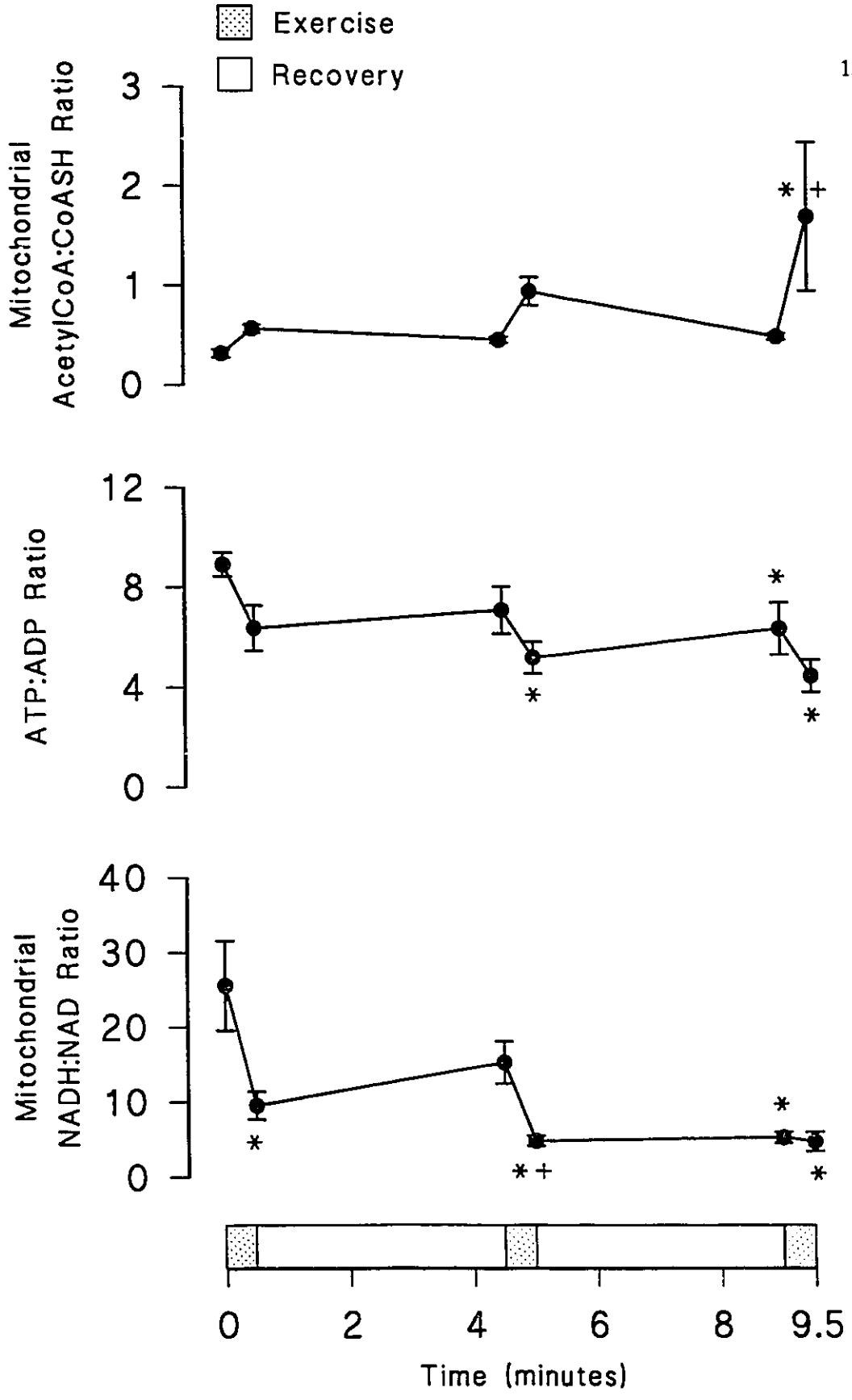

Exercise
Recovery
Mitochondrial AcetylCoA:CoASH Ratio
3
2
1
0
ATP:ADP Ratio
12
8
4
0
Mitochondrial NADH:NAD Ratio
40
30
20
10
0
0
2
4
6
8
9.5
Time (minutes)

FIGURE 5: Muscle lactate, pyruvate and intracellular [H^+] during maximal isokinetic cycling (shaded boxes) and during rest recovery (open boxes). Data are means ± SEM. *, indicates significantly different from rest. +, indicates post-exercise different from pre-exercise of the same bout. ‡, indicates significantly different from post-bout 1.

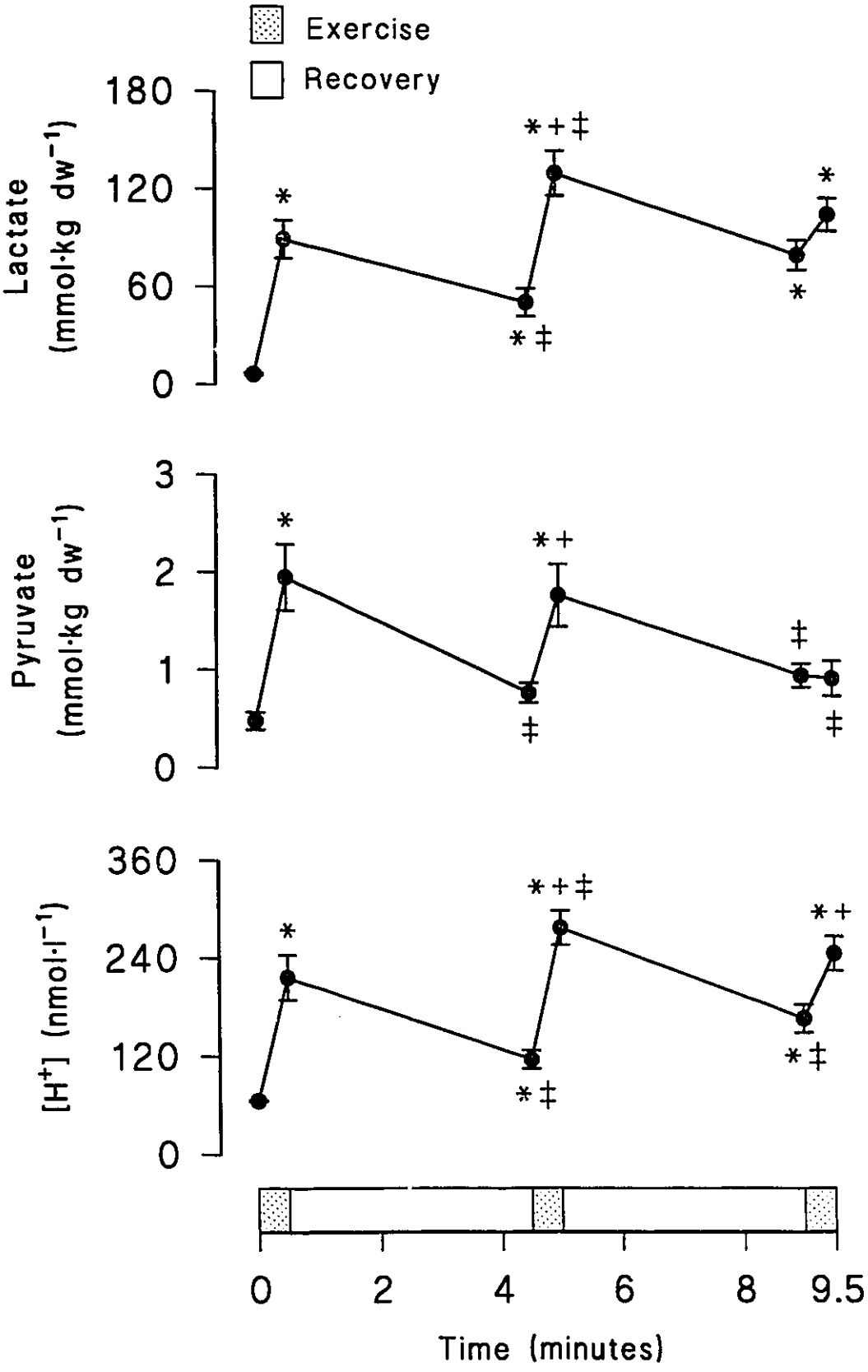

Exercise
Recovery
Lactate (mmol·kg dw⁻¹)
180
120
60
0
Pyruvate (mmol·kg dw⁻¹)
3
2
1
0
[H⁺] (nmol·l⁻¹)
360
240
120
0
0
2
4
6
8
9.5
Time (minutes)

FIGURE 6: Total CoA, acetylCoA and free CoA during maximal isokinetic cycling (shaded boxes) and during rest recovery (open boxes). Data are means ± SEM. *, indicates significantly different from rest. +, indicates post-exercise different from pre-exercise of the same bout. ‡, indicates significantly different from post-bout 1.

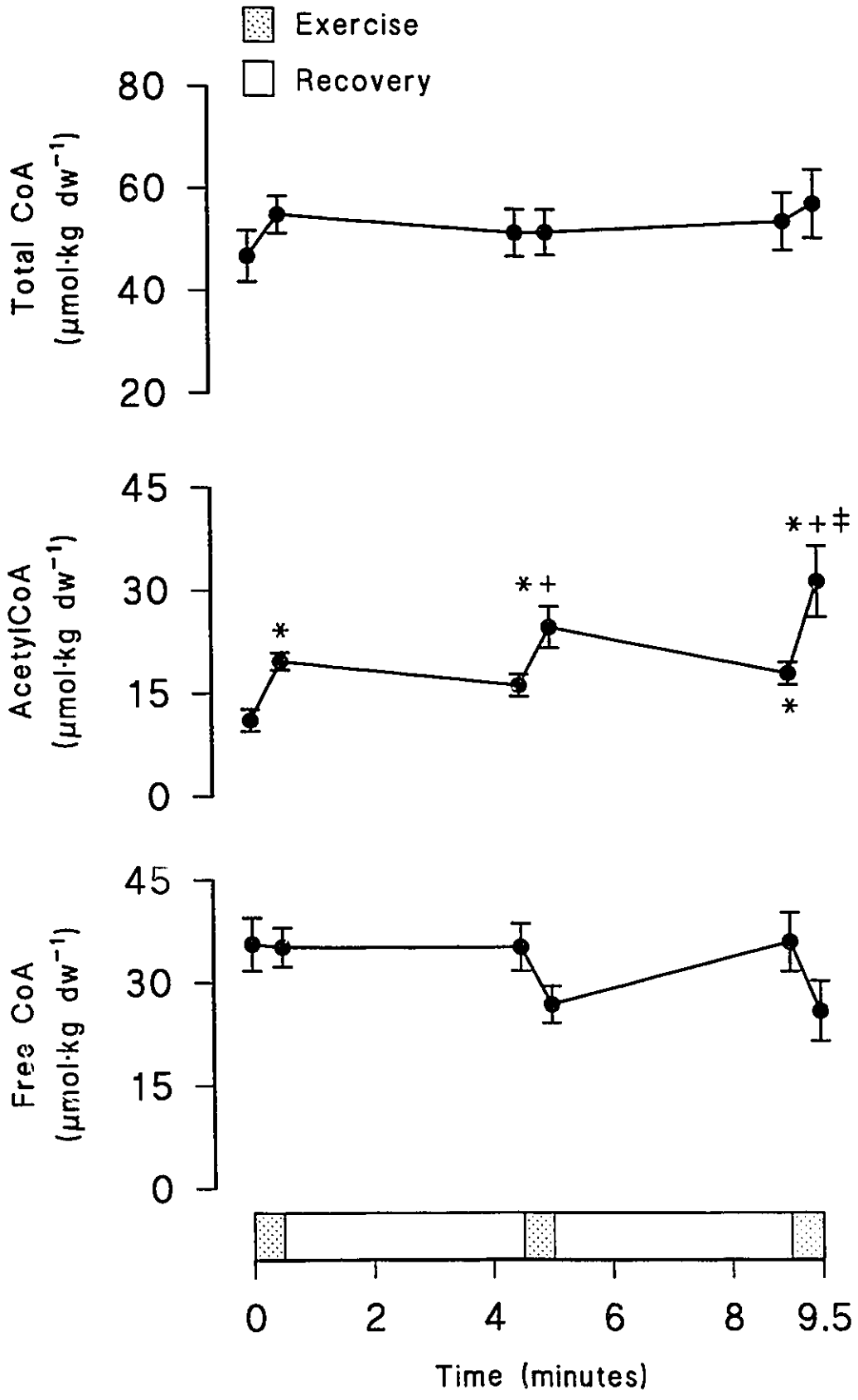

Exercise
Recovery
Total CoA
(μmol·kg dw⁻¹)
80
60
40
20
AcetylCoA
(μmol·kg dw⁻¹)
45
30
15
0
*
*+
*+‡
Free CoA
(μmol·kg dw⁻¹)
45
30
15
0
0
2
4
6
8
9.5
Time (minutes)

FIGURE 7: Total carnitine, acetylcarnitine and free carnitine during maximal isokinetic cycling (shaded boxes) and during rest recovery (open boxes). Data are means ± SEM. *, indicates significantly different from rest. +, indicates post-exercise different from pre-exercise of the same bout. ‡, indicates significantly different from post-bout 1.

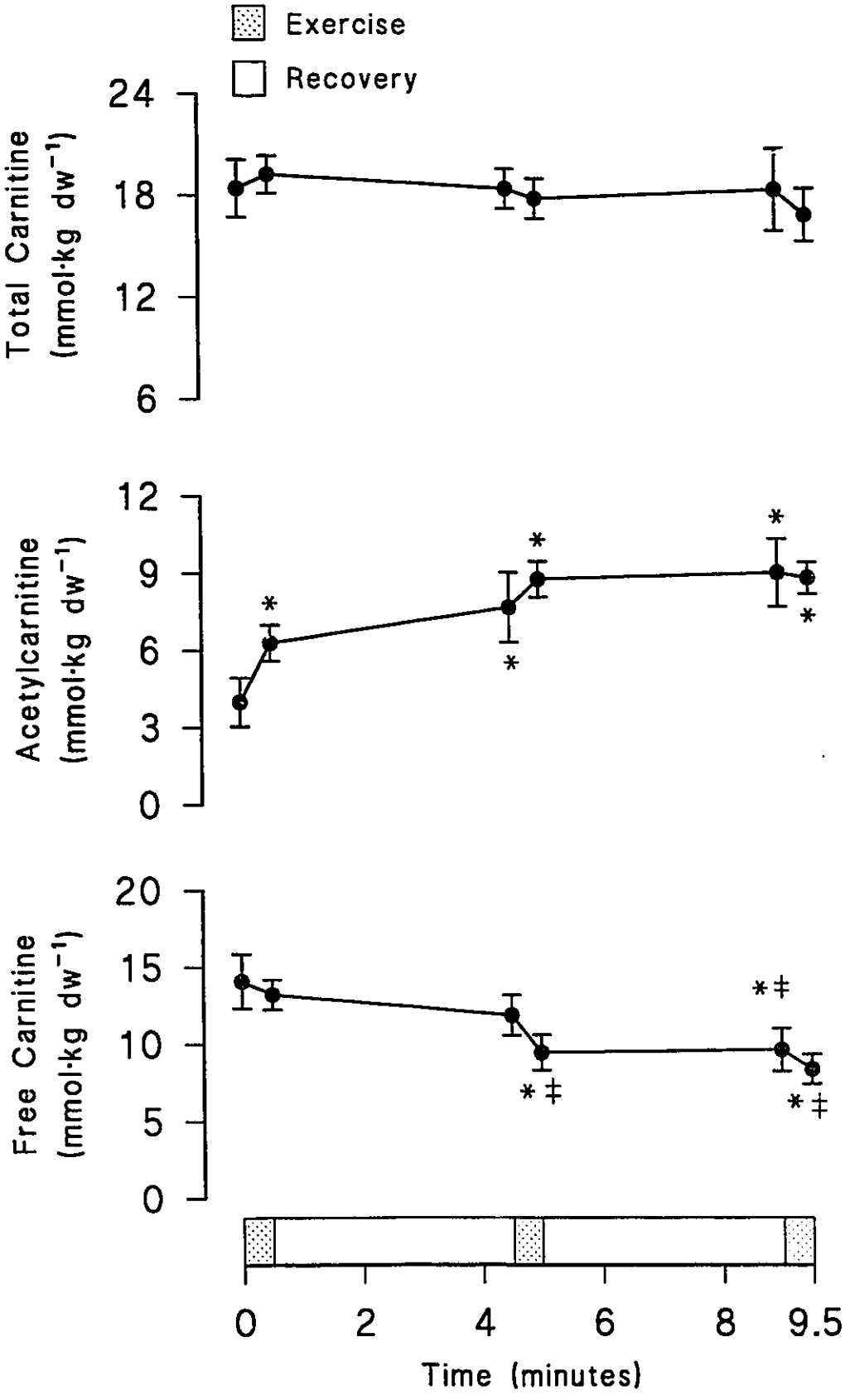

Exercise
Recovery
Total Carnitine (mmol·kg dw⁻¹)
24
18
12
6
Acetylcarnitine (mmol·kg dw⁻¹)
12
9
6
3
0
Free Carnitine (mmol·kg dw⁻¹)
20
15
10
5
0
0
2
4
6
8
9.5
Time (minutes)

Table 1. Muscle metabolite content in the vastus lateralis at rest and during intermittent isokinetic cycling.

Measure	Pre-Bout 1	Post-Bout 1	Pre-Bout 2	Post-Bout 2	Pre-Bout 3	Post-Bout 3
Ammonia	512±88	1376±164 *	773±48 ‡	1311±130 *†	988±57 (6) *	1698±180 (6) *†
Oxoglutarate	13.5±1.0 (4)	7.5±1.0 *	6.9±1.0 *	7.3±1.1 (6) *	8.4±1.2 (6) *	5.3±1.2 (5) *
Glutamate	10.4±1.4	7.0±0.4 *	5.9±0.7 *	4.2±0.6 *†‡	3.6±0.5 (6) *‡	2.6±0.5 (5) *‡
ATP	22.4±1.0	23.1±1.3	21.7±2.2	18.5±1.9	16.1±1.7 (6) *‡	15.9±1.4 (6) *‡
ADP	2.54±0.13	3.53±0.26 *	3.21±0.28	3.67±0.23 *	2.62±0.22 (6) ‡	3.69±0.30 (5) *‡
AMP	0.16±0.02	0.17±0.02	0.20±0.03	0.19±0.01	0.18±0.01 (6)	0.20±0.03 (5)
PCr	75.9±4.1	39.7±3.0 *	66.7±2.3 ‡	27.8±1.7 *†‡	61.1±5.6 (6) *‡	33.2±2.2 (6) *†
Cytosolic NAD/NADH Ratio	158±37	142±23	65±15 *	124±22	60±8 (6) *	66±15 (6) *‡

Data are means ± SEM; n = 7 except where indicated in parentheses. Ammonia and oxoglutarate measures are expressed in μmol·kg^{-1} dw while all other measures are expressed as mmol·kg dw^{-1}. *, indicates different rest. †, indicates different from Pre- of same bout. ‡, indicates different from Post-Bout 1.

4.5 DISCUSSION

In two previous studies which employed the same protocol we found marked lactate production in the first 30 second bout, but in the third bout no change in lactate occurred (27,34). We postulated that the reduction in lactate accumulation in the third bout was related to progressively greater PDHa flux. The purpose of the present study was to examine skeletal muscle PDHa regulation in humans during maximal intermittent exercise and to examine the role of PDHa in regulating muscle lactate production. We also wished to ascertain which regulatory factors were responsible for determining PDHa in humans during exercise and to characterize the contribution of PDHa flux to overall energy production during maximal contractions. The protocol employed was successful at altering muscle lactate content and PDHa transformation during successive periods of exercise, and it provided a good model from which to examine factors regulating PDHa transformation.

4.5.1 Lactate Metabolism

4.5.1.1 Oxygen Availability

The classic theory of muscle lactate accumulation during contraction has centered on the development of tissue hypoxia which results in a substrate limitation of the electron transport chain at cytochrome oxidase and ultimately limits oxidative phosphorylation (39). Consequently, parallel increases in the NADH-to-NAD ratio and P_i and a decrease in the ATP-to-ADP ratio ensue. Accumulation of ADP, AMP and P_i in turn serve as stimuli for continued glycogenolytic and glycolytic flux. AMP activates glycogen phosphorylase and the accumulation of P_i ensures that sufficient substrate is available for phosphorylase activity, while ADP, AMP and P_i provide a stimulus for increased phosphofructokinase activity and

glycolytic flux. Catalyzed by lactate dehydrogenase, accumulated cytosolic NADH is then free to react reversibly with glycolytically generated pyruvate to form lactate.

Support for this theory was largely based on observations that muscle lactate production increases or decreases during exercise in response to breathing hypoxic or hyperoxic gas mixtures, respectively (see (22) for review). This explanation of lactate production is also supported by studies that have examined muscle cell redox state, which is purported to be a sensitive indicator of mitochondrial O_2 availability (22). Sahlin et al (33) reported muscle NADH accumulation coincided with muscle lactate production in humans during incremental cycling exercise under normoxic conditions. However, measurements of NADH and NAD by these investigators were made on whole cell extracts which reflects total NAD and NADH but do not reflect the mitochondrial redox state, except in the case of circulatory occlusion. Furthermore, measurements of muscle PO_2 in exercising humans (3) demonstrated that in muscle that was accumulating lactate, tissue PO_2 was well above 0.1-0.5 mmHg, the critical level for the development of tissue hypoxia (6).

In contrast to whole cell measures of NAD and NADH, indicator measurements of the mitochondrial redox state using the glutamate dehydrogenase equilibrium reaction more accurately reflect tissue O_2 availability (15). In the present study, the mitochondrial NADH-to-NAD ratio decreased during the first bout and recovered during the ensuing rest period (Figure 3). While it also decreased after the second bout, it did not recover during the rest period but remained lower throughout the third bout. Concomitant with these changes during exercise was considerable lactate accumulation (Figure 4). In contrast, muscle lactate decreased during the rest periods when the mitochondria became more reduced. These observations are consistent with muscle lactate accumulation occurring independent of O_2

availability in our subjects and are similar to previous reports of human muscle during cycling exercise (15) and *in situ* stimulated dog muscle (8,9,21,35). In the absence of any apparent oxygen limitation to mitochondrial respiration, the decrease in the ATP-to-ADP ratio observed during each bout (Figure 3) suggests that the controlling factor of mitochondrial respiration was either kinetic regulation by ADP alone or thermodynamic regulation by the cell phosphorylation potential (39).

4.5.1.2 Glycolytic Rate, PDHa Activity and Lactate Production

For the intensity of exercise employed in the present study most or all of the substrate for glycolytic ATP production is derived from muscle glycogen (18,20,27,34). Thus, in our subjects glycolytic flux was initiated by the flux-generating enzyme glycogen phosphorylase and further regulated by the rate limiting enzyme phosphofructokinase (PFK). Normally, during steady state exercise conditions, glycolytic flux will be equal to the PFK activity, which can be calculated as glycogen breakdown minus the sum of accumulated hexose-monophosphates. However, under the non-steady state conditions of the present exercise protocol, the glycolytic intermediates, fructose-1,6-diphosphate (F-1,6-DP) and glycerol-3-phosphate (G-3-P) must also be subtracted, since these intermediates accumulate by a significant amount (27,34).

Assuming most of the substrate utilized during exercise in the present study was from muscle glycogen (18,20) and that 3 kg of intracellular water is equal to 1 kg dry weight of muscle, it is possible to calculate glycolytic flux for each bout as the difference between glycogen breakdown and the accumulation of hexose monophosphates, F-1,6-DP and G-3-P. We have previously reported such values for this specific protocol (27,34), which can serve

as a basis for comparison with measured PDHa. Using this method, glycolytic flux was calculated to be 39 (27), 40 (34) and 11 (34) mmol·min^{-1}·kg ww^{-1} in bouts 1, 2 and 3, respectively, which were similar to the sum of the rates of lactate plus pyruvate accumulation and PDHa flux, in our subjects. The corresponding measures of PDHa for bouts 1, 2 and 3 were 3.0, 2.9 and 2.8 mmol·min^{-1}·kg ww^{-1} (Fig 2). Thus, glycolytic rate was 13 fold greater than PDHa in bouts 1 and 2, and 4 fold greater in bout 3. Since the PDHa values reported here were 100% of PDHt activity, they also represent the upper capacity for glycolytic flux into the TCA cycle. Given that mitochondrial O_2 was not limiting in our subjects, these data argue that muscle lactate production during maximal exercise in normoxia may simply be a function of widely different capacities between the ability to generate and to oxidize pyruvate.

During maximal cycling exercise 265, 254 and 80 mmoles of lactate accumulated in the active muscle mass, in bouts 1, 2 and 3, respectively. In addition, from a previous study (25) it is possible to estimate that during this protocol, 34 (10%), 28 (10%) and 7 (8%) mmoles of lactate are released into the extracellular space during bouts 1, 2 and 3. Thus, total lactate production during bouts 1, 2 and 3 was approximately 299, 282 and 87 mmoles, during 30 seconds of cycling in each of bouts 1, 2 and 3, respectively.

During the first and second recovery periods, the decrease in muscle lactate accumulation (Figure 4) is consistent with greater PDHa flux than glycolytic flux due to both sustained PDHa activity and inhibition of glycolytic rate. Measures of muscle lactate in our subjects (Figure 4) combined with arterial-venous lactate measures from a previous study (25) and using a leg blood flow of 10 l·min^{-1}, allow us to estimate the proportion of accumulated muscle lactate that was oxidized during recovery. Of the 265 mmoles that accumulated in bout 1, 60 mmoles were released into the extracellular space, 62 mmoles

were oxidized and 143 mmoles remained in the active muscle mass. Similarly, of the 254 mmoles that accumulated in the active muscle mass during bout 2, 92 mmoles were released into the extracellular space, 65 mmoles were oxidized in the active muscle mass and 97 mmoles remained in the active muscle mass. Thus during 4 min of recovery from bouts 1 and 2, 23% and 26% of the accumulated lactate was removed by oxidation. Additionally, during 4 min of recovery from bouts 1 and 2, 23% and 36% of the accumulated lactate was released into the extracellular space.

4.5.2 PDHc Regulation

4.5.2.1 Rate of PDHc Transformation

Muscle contraction has previously been shown to result in transformation of PDHc to PDHa in humans (10,12,13,29,30) but the maximum rate of PDHa transformation during muscle contraction has not been characterized. Thus, it was of interest in the present study to also examine the rate of change of PDHa transformation (ΔPDHa/Δt). ΔPDHa/Δt can be calculated from a previous report which employed electrical stimulation at 20 Hz (13). PDHa was transformed from 26% to 64% of PDHt after 16 s and 78% after 32 s. No further transformation occurred after 73 s of stimulation where PDHa was 79% of PDHt. Thus at 20 Hz the majority of transformation occurred in the first 16 s of stimulation and was equal to a ΔPDHa/Δt of 3.1 mmol·min^{-2}kg ww^{-1} during this period. In contrast to that study, complete transformation occurred during each 30 s exercise bout in our subjects (Figure 2). In bout 1 this corresponded to a ΔPDHa/Δt that was $\geq$ 5 mmol·min^{-2}kg ww^{-1} and is to our knowledge the largest observed rate of PDHa transformation reported for human muscle. Given that each contraction was maximal in our subjects, it is possible that most of the

transformation could have occurred early in the exercise bout (13) and the ΔPDHa/Δt may be greater. The difference between the ΔPDHa/Δt in the present study and that calculated from the data of others (13) is probably due to the more intense protocol that we employed.

4.5.2.2 AcetylCoA-to-CoASH, NADH-to-NAD and ATP-to-ADP Ratios

The mitochondrial ratios of acetylCoA-to-CoASH, ATP-to-ADP and NADH-to-NAD are important signals regulating the rate of aerobic metabolism and PDHa transformation. The regulatory influence of these factors has been recently reviewed (37). NADH and acetylCoA inhibit PDH-phosphatase and increase the activity of PDH-kinase, while NAD and CoASH accumulation have the opposite effect, increasing the activity of PDH-phosphatase and inhibiting PDH-kinase activity. Thus, an increase in either of the acetylCoA-to-CoASH or NADH-to-NAD ratios will activate PDH-kinase and inhibit PDH-phosphatase. Conversely, a decrease in the NADH-to-NAD and acetylCoA-to-CoASH ratios will inhibit PDH-kinase and activate PDH-phosphatase. The effects of the acetylCoA-to-CoASH and NADH-to-NAD ratios are thought to be primarily mediated through allosteric effects on the regulatory subunits (37).

Unlike the other regulatory ratios, the ATP-to-ADP ratio acts on PDH-kinase alone and does not exert its effect by allosteric interaction. Since ATP is the substrate for PDH-kinase it is competitive with its product, ADP, which inhibits catalytic activity. Thus, the net balance of acetylCoA-to-CoASH, NADH-to-NAD and ATP-to-ADP ratios are important determinants of the relative activities of PDH-kinase and PDH-phosphatase which in turn determine the extent of PDHc phosphorylation and PDHa activity. When the activity of PDH-kinase is greater than that of PDH-phosphatase, PDHa will decrease. Conversely, when

the activity of PDH-phosphatase is greater, PDHa will increase.

4.5.2.3 AcetylCoA-to-CoASH Ratio

In the present study, each exercise bout resulted in an increase in the mitochondrial acetylCoA-to-CoASH ratio (Figure 3) which should have resulted in lower PDHa. During the ensuing 4 min recovery periods, the changes that occurred in the acetylCoA-to-CoASH ratio during exercise were resolved. Oscillation of the acetylCoA-to-CoASH ratio (Figure 3) and acetylCoA (Figure 5) coincided with a continuous increase in acetylcarnitine and decrease in free carnitine throughout the exercise and rest periods (Figure 6), which is consistent with the reversible transfer of acetyl-groups from mitochondrial CoA to carnitine. The resulting acetylcarnitine is then transported to the cytosol for storage. This system of acetyl-group transfer has previously been characterized in human muscle (10,12,13,17). In our subjects, it acted to buffer against excess acetylCoA when its production through PDHa exceeded its rate of entry into the TCA cycle. The absence of any apparent inhibitory effect of the acetylCoA-to-CoASH ratio on PDHa during cycling exercise indicates that other regulatory factors were sufficient to override this inhibitory stimulus.

During exercise, the continuous transfer of acetyl-groups from CoA to carnitine attenuated the rise in mitochondrial acetylCoA (Figure 5) and the acetylCoA-to-CoASH ratio (Figure 3) and regenerated CoASH (Figure 5) at a rate that was sufficient to sustain maximal PDHa activity. During the recovery periods this mechanism of acetyl-group transfer allowed recovery of the acetylCoA-to-CoASH ratio to pre-exercise levels (Figure 3), thus preventing complete inactivation of PDHa (Figure 2) and allowing continued PDHa flux. During exercise these events would have been vital to ensuring maximal pyruvate oxidation and

minimizing muscle lactate accumulation. In contrast, during the recovery periods continued PDHa flux guaranteed net removal of accumulated lactate.

4.5.2.4 NADH-to-NAD Ratio

Before exercise, the mitochondrial NADH-to-NAD ratio was very large (Figure 3), possibly due to a substrate limitation of ADP at oxidative phosphorylation, resulting in inhibition of the electron transport chain and NADH accumulation (39). Extensive NADH accumulation under rest conditions would have increased PDH-kinase activity and inhibited PDH-phosphatase, thus maintaining PDHc in a phosphorylated state and sustaining low PDHa.

The NADH-to-NAD ratio decreased 2 fold during bout 1 and 4 fold during bout 2, where it remained throughout the remainder of the protocol (Figure 3). Consequently, the stimulatory and inhibitory effects of NADH on PDH-kinase and PDH-phosphatase, respectively, should have been removed, resulting in a shift toward greater PDH-phosphatase activity and greater PDHa transformation (Figure 2). A rise in the NADH-to-NAD ratio during the first rest period would have reversed this transformation and resulted in greater PDH-kinase activity and lower PDHa. In contrast, maintenance of a low NADH-to-NAD ratio during the second rest period would have allowed PDHa transformation to be maintained at a higher level.

Despite the fact that the NADH-to-NAD ratio was altered so as to support greater PDHa during exercise, it is not likely to have exerted a major effect on PDHa transformation since its effects are apparently secondary to the potent stimulatory affects of Ca^{2+} (13). During exercise the role of NADH-to-NAD ratio was probably relegated to one of support,

acting in concert with the other regulatory ratios in PDHa regulation. In contrast, during rest and recovery, when the influence of Ca^{2+} on PDHa transformation is less, the NADH-to-NAD ratio appeared to be an important factor. A continuous decrease in this ratio (Figure 3) occurred from pre-exercise rest to the second recovery period which coincided with an increase in PDHa (Figure 2). This is consistent with the gradual removal of the inhibitory effects of the NADH-to-NAD ratio with each successive recovery period, resulting in progressively greater PDHa.

4.5.2.5 ATP-to-ADP Ratio

Fluctuations in the ATP-to-ADP ratio (Figure 3) were similar to changes in the NADH-to-NAD ratio (Figure 3). At rest the ATP-to-ADP ratio was ~9 and dropped by 33% to ~6 after bout 1. Similar to the effects of the NADH-to-NAD ratio, a high ATP-to-ADP ratio at rest would have stimulated PDH-kinase and served to maintain low PDHa. A fall in this ratio during each exercise bout would have had the opposite effect, reducing PDH-kinase activity and allowing greater PDHa transformation. In our subjects, the fall in the ATP-to-ADP ratio followed a step-wise pattern with each successive bout, never fully recovering during the rest periods. Consequently, there was less of a stimulus for increased PDH-kinase activity with each successive exercise and recovery period. This would have resulted in greater PDHa during the recovery periods and a faster rate of PDHa transformation during bouts 2 and 3.

While the ATP-to-ADP ratios reported here are of the whole cell, it is assumed that similar quantitative changes occur in the mitochondria, since mitochondrial and cytosolic compartments are in equilibrium (36). This is also supported by changes in whole cell PCr

which parallel changes in the mitochondrial ATP-to-ADP ratio (36): a reduction in muscle [PCr] reflects a lack of available mitochondrial ATP and a rise in ADP, while an increase in [PCr] reflects a relative increase in ATP over ADP. In our subjects, alterations in muscle [PCr] also paralleled changes in the ATP-to-ADP ratio.

4.5.2.6 Pyruvate

The primary substrate of PDHc, pyruvate, is also a potent stimulus for PDHa transformation, exerting its effects by inhibiting PDH-kinase, with an estimated K_i of 0.5-2.8 mM (37). During bouts 1 and 2, muscle pyruvate increased 4 fold over pre-exercise rest levels (Figure 4), contributing to PDHa transformation. In the first and second rest periods, pyruvate partially recovered but remained elevated, sustaining a substantial stimulus for greater PDHa transformation. Assuming that the fluid volume of muscle is 75% of the total volume, it is possible to calculate that muscle pyruvate concentration was 0.2 mM at rest and reached a maximum concentration of 0.7 mM after bout 1 which is in the range of the K_i of pyruvate for PDH-kinase. Thus, in our subjects changes in muscle pyruvate concentration would have been in an optimal range to exert a significant inhibitory effect on PDH-kinase, stimulating rapid PDHa transformation during exercise and slowing the conversion back to the inactive form during recovery.

4.5.2.7 [H⁺]

We used the relationship found by Sahlin and coworkers (32) between the sum of pyruvate plus lactate, and muscle homogenate pH to estimate intramuscular $[H^+]$. Although many factors contribute to intracellular $[H^+]$ in heavy exercise (24), they appear to change

in parallel. Spriet et al (34) found a closely similar relationship in maximal cycling exercise of the type used in the present study, supporting its validity, and allowing us to examine the influence of changes in $[H^+]$ on PDHa transformation.

While few reports are available on the effects of $[H^+]$ on PDHa transformation, one study reported that acidosis increased PDHa transformation in perfused rat heart (28). Thus, as muscle $[H^+]$ increased in our subjects (Figure 4) this would inhibit of glycogen phosphorylase (7) and PFK (14), slowing the rate of lactate production. At the same time, increases in $[H^+]$ would have increased PDHa activity and oxidation of lactate. Since PDHa transformation was rapid and complete during each exercise bout and there already exists a multitude of regulators of PDHa transformation, it may seem redundant that this aspect of PDHa regulation should be functionally significant in our subjects during very intense muscular contractions. However, the utility of this mechanism is highlighted during recovery from intense exercise when intramuscular acidosis inhibits lactate production in concert with sustained PDHa activity and lactate oxidation, to reduce muscle lactate concentration and alleviate the acidosis.

4.5.2.8 PDHa Flux

Under normal dietary conditions, measures of PDHa are equal to *in vivo* flux (10,12,29,30). For the present data, calculation of PDHa flux was based on the following axioms: 1) 100 mmol of O_2 occupies 2.24 L; 2) 1 mmol of pyruvate is oxidized by 3 mmol of O_2; 3) the active muscle mass is the primary consumer of the increased O_2 uptake during exercise; and 4) the active muscle mass (13 kg) is equal to the average of three previously reported estimates (4,27,31). Subtracting resting from total O_2 uptake ($mmol \cdot min^{-1}$),

converting the balance to the equivalent pyruvate flux and correcting for the active muscle mass yields a calculated PDHa flux of 3 mmol·min^{-1}·kg ww^{-1} for all three bouts. This is similar to the measured values of PDHa during this time which were 3.0, 2.9 and 2.8 mmol·min^{-1}·kg ww^{-1} (Figure 2) in bouts 1, 2 and 3, respectively. Thus factors that determined PDHa transformation during exercise also determined PDHa flux.

Similar calculations for each of the recovery periods resulted in a calculated PDHa flux of 1 mmol·min^{-1}·kg ww^{-1} which was 2 fold lower than the average measured PDHa for the first and second recovery periods (Figure 2). During recovery from maximal exercise, the average measured PDHa may have been greater than PDHa flux if the enzyme activity decreased in a linear fashion over the 4 min period (Figure 2). If so, this suggests that during recovery PDHa transformation did not present a limitation to oxidation of the lactate load as factors that determined PDHa ensured that the potential for flux was 2 fold greater than actual flux. However, if PDHa actually decreased to ~1 mmol·min^{-1}·kg ww^{-1} upon cessation of exercise, then measured PDHa would have been equal to PDHa flux. It is likely that PDHa actually decreased to 1.1 and 1.2 mmol·min^{-1}·kg ww^{-1} (Figure 2) almost immediately after cessation of exercise in bouts 1 and 2, respectively, since the quantity of lactate oxidized during each of the recovery periods would have required an average PDHa flux of ~1 mmol·min^{-1}·kg ww^{-1}. This suggests that under these conditions, the factors that determined PDHa transformation also determined PDHa flux. Use of lactate as a metabolic fuel during recovery from exercise was probably used to replenish the PCr pool (16), and support intracellular ATP requirements during this period.

4.5.3 Contribution of PDHa Flux to ATP Production

During intense muscular contractions, of the duration employed in the present study, the major sources of ATP production have traditionally been thought to come from PCr breakdown and anaerobic glycolysis (20). However, it is not clear what proportion of ATP production is supported by aerobic metabolism during 30 s of maximal sprint activity. Thus, it was of interest in the present study to determine the proportions of the various fuels utilized to generate ATP and determine what proportion was supplied by flux through PDHa. ATP production from both anaerobic metabolism (PCr and glycolysis) and aerobic glucose oxidation are summarized in table 2.

In the first two bouts, approximately 67-71% of the ATP was generated from anaerobic sources: 16% was from PCr and 51-55% was from anaerobic glycolysis. Meanwhile, aerobic glycolysis contributed 29-33% of the ATP. Since there were no apparent differences between the amount or proportions of fuels utilized to supply ATP, the drop in power output from 19.3 kJ in bout 1 to 16.3kJ in bout 2 was probably related the development of muscle fatigue (24). In contrast, during bout 3 total ATP production decreased, contributing to a further decrease in power output to 14.2 kJ. In bout 3, only 37% of the ATP was generated from anaerobic sources while generation from aerobic glycolysis increased to 63%. The difference in total ATP generation and the proportion of fuels utilized between bout 3 and the first two bouts was primarily attributed to a 3 fold decrease in ATP production from anaerobic glycolysis and a 1.4 fold increase in aerobic glycolysis. Flux through PDHa provided an increasing amount of energy with each bout (220 mmol ATP in bout 1; 255 mmol ATP in bout 2; and 357 mmol ATP in bout 3) and was the result of greater PDHa transformation during each recovery period.

Table 2: Sources of ATP generation during 3 consecutive 30 second bouts of maximal isokinetic cycling exercise, each separated by 4 minutes of rest recovery. Data are expressed as mmol of ATP and are also summarized as % of total ATP generated.

		Anaerobic			Aerobic	Total
		PCr	Glycolysis	*Total*	Glycolysis	
Bout 1	ATP (mmol)	118	410	*528*	220	748
	% of Total	16%	55%	*71%*	29%	100%
Bout 2	ATP (mmol)	126	390	*516*	255	771
	% of Total	16%	51%	*67%*	33%	100%
Bout 3	ATP (mmol)	91	122	*212*	357	569
	% of Total	16%	21%	*37%*	63%	100%

Calculations: ATP production from PCr and anaerobic glycolysis were calculated from the breakdown of PCr and the accumulation of lactate, respectively. Production of ATP from aerobic glycolysis was calculated from total acetylCoA production as the area under the PDHa curves for each bout. 1 mmol of acetylCoA from glycogenolysis was equal to 19.5 mmol of ATP. The contribution of fat fuels was assumed to be negligible (22).

4.5.4 Summary of PDHa Regulation

In summary, during intense cycling exercise PDHc was rapidly transformed to PDHa. The primary stimulus for this transformation was probably Ca^{2+} which activates PDH-phosphatase and inhibits PDH-kinase (37). During exercise, greater PDHa may have been further supported by, falls in the NADH-to-NAD and ATP-to-ADP ratios, increases in pyruvate and H^+ concentrations, and attenuation of the rise in the acetylCoA-to-CoASH ratio. However, the physiological relevance of these changes on PDHa regulation may not be realized until recovery when intracellular Ca^{2+} decreases and the alterations in these regulatory factors persist. During exercise PDHa flux matched measured values of PDHa. It is suggested that the role of PDHa is to supply oxidizable substrate to the TCA cycle for generation of ATP and that this source of energy becomes increasingly important with each successive bout of maximal isokinetic cycling because anaerobic sources become increasingly inhibited.

During recovery, the influence of Ca^{2+} was removed but PDHa remained elevated due to, lower NADH-to-NAD and ATP-to-ADP ratios, greater pyruvate and H^+ concentrations and recovery of the acetylCoA-to-CoASH ratio. Measured PDHa was also similar to PDHa flux, during recovery, which allowed a significant portion of the accumulated lactate to be oxidized. Correlation and multilinear regression analysis were able to explain 76.5% of the variation in PDHa, suggesting that all of the regulatory factors examined may have been important determinants of PDHa transformation and PDHa flux during rest and recovery from exercise.

4.6 Summary

The present study examined PDHa regulation and lactate metabolism in human muscle during repeated bouts of maximal isokinetic cycling. Transformation of PDHa was rapid and complete after each of three 30 s maximal sprints. Lactate accumulation during exercise was attributed to differences in the maximal fluxes of glycolysis and PDHa, while no evidence was found to indicate that lactate production was dependent on O_2 availability. Thus, the results of the present study support our hypothesis, that lactate accumulation in maximally contracting human skeletal muscle results from a greater rate of glycolytic pyruvate production than pyruvate oxidation by PDHa. During exercise, glycolytic flux was much greater than PDHa flux resulting in muscle lactate accumulation. Conversely, during recovery from exercise, maintenance of muscle PDHa flux while glycolytic flux was slowed, appears to be responsible for net lactate oxidation.

The primary determinant of PDHa during exercise appears to have been Ca^{2+}, while the other regulatory factors examined were secondary. Conversely, during rest and recovery from exercise, the important predictors of PDHa were found to be the ratios of ATP-to-ADP and NADH-to-NAD, as well as the concentrations of pyruvate, PCr and H^+. The contribution of PDHa flux to total ATP production during repeated 30 s bouts of maximal sprint activity became increasingly important with successive bouts, accounting for 29%, 33% and 63% of total energy production, respectively. The progressive increase in intramuscular [H^+] during the recovery periods served to simultaneously inhibit glycolysis and maintain greater PDHa transformation, to account for the progressive reduction in muscle lactate accumulation.

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CHAPTER 5

GENERAL DISCUSSION

5.1 Introduction

PDHc regulation has been extensively studied *in vitro* using isolated enzyme preparations and *in situ* using a variety of animal models (see 1,11,32,33,36,37,47,48,49,50,54 for reviews). However, regulation of PDHc transformation by the putative allosteric regulators in human skeletal muscle *in vivo* and the integrated functional role of this enzyme in muscle energy production remains largely unexplored. Excluding the studies of the present thesis, only three previous reports have correctly determined PDHa and PDHt in human skeletal muscle and have examined its regulatory properties *in vivo* (7,8,9). Other reports of PDHc regulation in human skeletal muscle (43,46,51,52,53) have been plagued by methodological problems which preclude any useful conclusions to be drawn from these studies.

In contrast, the present series of studies add new information concerning PDHc regulation in human skeletal muscle *in vivo* and set it in the context of its role in energy metabolism. The first two studies (30,31) examined the role of PDHc in intramuscular fuel utilization and the contribution of the acetylCoA-to-CoASH ratio to the regulation of PDHc transformation. In a third study, the role of PDHc in muscle lactate production was assessed and the putative regulatory factors that control PDHc transformation were examined (28). The present chapter highlights the important findings of these studies and provides suggestions concerning the future directions for research on PDHc regulation in human skeletal muscle.

5.2 PDHc Regulation by the AcetylCoA-to-CoASH, NADH-to-NAD and ATP-to-ADP Ratios

The metabolite ratios of acetylCoA-to-CoASH, NADH-to-NAD and ATP-to-ADP were examined in the present series of studies to assess their importance in regulating PDHc transformation between its inactive (PDHb) and active (PDHa) forms, and PDHa flux. Analysis of these regulatory factors was paramount to gaining a better understanding of the role of PDHc transformation in human skeletal muscle fuel selection during altered states of substrate availability, and during varied states of muscle contractile activity. These ratios are expressed as product/substrate ratios to emphasize their inhibitory effects on PDHc transformation from PDHb to PDHa and their potential inhibitory effect on PDHa flux.

Since the apparent K_m of the substrates (ie. CoASH, NAD and ATP) are very similar to the apparent K_i for their corresponding products (ie. acetylCoA, NADH and ADP), a small change in one or the other of these metabolites will result in a corresponding change in the activities of the regulatory subunits PDH-kinase and PDH-phosphatase. Alterations to any of the three ratios will alter PDH-kinase activity while changes to either of the acetylCoA-to-CoASH or NADH-to-NAD ratios will also alter PDH-phosphatase activity. The cumulative effect of these ratios determines PDHa via their integrated effects on the activities of PDH-kinase and PDH-phosphatase. For example, if one ratio changes in one direction, the second changes in the opposite direction and the third does not change at all, it is conceivable that the sum of these changes would have no net effect on the PDH-kinase/PDH-phosphatase activity ratio or PDHc transformation. Alternatively, an increase in one or more of the ratios, while the remainder are unchanged, will result in a corresponding increase in the PDH-kinase/PDH-phosphatase activity ratio, increased

phosphorylation of E1α and a decrease in measured PDHa. Conversely, a decrease in one or more of these ratios, while the remainder are unchanged, will result in a lower PDH-kinase/PDH-phosphatase activity ratio, dephosphorylation of E1α and greater PDHa.

Previous reports examining the effect of these ratios on PDHc regulation in human skeletal muscle are limited to only a few studies (7,8,9,52,53). Moreover, the data are incomplete and available information on the effect of the regulatory ratios *in vivo* are descriptive in nature and do not demonstrate cause-and-effect relationships. The present studies advance the understanding of the regulatory influences of these key metabolite ratios on PDHc transformation *in vivo*. The effects of these metabolite concentration ratios on PDHc transformation and PDHa flux were assessed in human skeletal muscle during rest and at various exercise intensities. In the following sections each of these ratios is considered individually.

5.2.1 PDHc Regulation by the AcetylCoA-to-CoASH Ratio

The acetylCoA-to-CoASH ratio has been examined in human skeletal muscle, *in vivo*, in only three previous studies (7,8,9). However, these studies were limited to exercise (7,8) and electrical stimulation (9) under normal dietary conditions, when the effect of the elevated acetylCoA-to-CoASH ratio was likely to be overridden by the effect of elevated intramuscular Ca^{2+} (19). In contrast, the present studies (30,31) were the first to independently manipulate this ratio in human skeletal muscle, and to demonstrate corresponding reductions in PDHa and muscle glucose oxidation under resting conditions. In these studies, two approaches were employed to independently manipulate the ratio of acetylCoA-to-CoASH. First, carbohydrate depletion followed by a period of chronic carbohydrate deprivation and fat feeding was used

to increase mobilization and oxidation of fat fuels in skeletal muscle (31). Second, acetate infusion was used to acutely increase the availability and oxidation of an alternative fat fuel by skeletal muscle (30). These approaches resulted in 3.5 (31) and 2.6 fold (30) elevations in the intramuscular acetylCoA-to-CoASH ratios, respectively, which resulted in corresponding 3.2 (31) and 2.3 (30) fold reductions in measured PDHa. During dietary manipulation these changes resulted in decreased glucose utilization and increased oxidation of fat fuels (31). Although similar changes occurred to the acetylCoA-to-CoASH ratio as a result of acetate infusion (30), lack of parallel arterial-venous blood samples prevented direct determination of the corresponding effects on muscle glucose uptake and oxidation at rest.

In all three studies (28,30,31), the acetylCoA-to-CoASH ratio increased in proportion to exercise intensity (28,30,31) but the putative regulatory effects of this ratio on PDHa were absent. During exercise, dietary manipulation (31) and acetate infusion (30) were not successful at increasing the acetylCoA-to-CoASH ratio beyond that which occurred as a result of exercise alone. Under normal dietary conditions, the acetylCoA-to-CoASH ratio increased substantially in parallel with PDHa (28,30,31). In contrast, during prolonged carbohydrate deprivation (31), this ratio decreased during exercise at the same time that PDHa was significantly inhibited. The sum of these observations argue against a direct physiological role for the acetylCoA-to-CoASH ratio in regulating PDHc transformation during muscle contraction in humans. However, it is likely that the direct effect of the acetylCoA-to-CoASH ratio on PDHc transformation occurs during rest and the effect of this ratio on PDHa during muscle contraction in humans is proportional to the extent and duration that it is elevated prior to exercise, causing multi-site phosphorylation of E1α and resistance to transformation.

5.2.2 PDHc Regulation by the NADH-to-NAD Ratio

In the third study (28), the effect of the NADH-to-NAD ratio on PDHa was examined. Although this ratio was not manipulated independently, it was highly correlated with PDHa during rest and recovery from exercise. When the NADH-to-NAD ratio was high during rest, the corresponding PDHa activity was low. With each successive rest period, the mitochondrial NADH-to-NAD ratio decreased and PDHa increased. While these observations are not proof of a direct regulatory effect on PDHc transformation, they are consistent with the gradual removal of the stimulatory effect on PDH-kinase and inhibitory effect on PDH-phosphatase, as has been previously shown *in vitro* (2,27,38,54).

During exercise, the mitochondrial NADH-to-NAD ratio was lower while transformation of PDHc to PDHa was maximal. This observation was also consistent with the NADH-to-NAD ratio causing greater PDHa during muscle contraction. However, it has recently been shown that PDHc transformation to PDHa was maximal during intense electrical stimulation combined with circulatory occlusion (9). This approach resulted in a very large and prolonged elevation of the NADH-to-NAD ratio in the cytosolic and mitochondrial compartments which should have inhibited PDHc transformation and resulted in lower PDHa. The fact that this did not occur suggests that other factors which activated PDH-phosphatase were more important during muscle contraction. The most likely regulatory factor was Ca^{2+}, which is known to reach saturating concentrations for PDH-phosphatase during maximal contractile activity (19).

5.2.2.1 Future Directions for the Study of PDHc Regulation by the AcetylCoA-to-CoASH and NADH-to-NAD Ratios

The existence of multi-site phosphorylation of PDHc has been established in rat skeletal and heart muscles (33,39,40,41,42) but operation of a similar mechanism in human muscle has not been shown. Moreover, the magnitude and importance of changes to the acetylCoA-to-CoASH and NADH-to-NAD ratios to PDHc transformation and intramuscular fuel selection and oxidation remains to be fully characterized in human skeletal muscle. Future studies examining the effect of the acetylCoA-to-CoASH and NADH-to-NAD ratios on PDHc regulation should first attempt to determine the range and extent of PDHc regulation by these ratios. This could best be achieved by infusing sodium-acetate at varying rates and allowing a new steady state to be achieved at each level. This should result in step-wise increases in the intramuscular acetylCoA-to-CoASH and NADH-to-NAD ratios and result in similar step-wise decreases in PDHa. Such an approach would also allow parallel examination of the PDH-kinase/PDH-phosphatase activity ratio and the extent of E1α-phosphorylation, which could both be correlated to changes in these regulatory ratios and to PDHa. To establish the existence of, and delineate the time course for multi-site phosphorylation of E1α by this mechanism, acetate could be infused for a prolonged period. Such an approach would also allow the simultaneous determination of the PDH-kinase/PDH-phosphatase activity ratio over time.

5.2.3 PDHc Regulation by the ATP-to-ADP Ratio

In the present studies, the ATP-to-ADP ratio was not manipulated independently. Thus, the data do not show a cause-and-effect relationship between this ratio and measured

PDHa. However, measurement of ATP, ADP and PCr in whole cell extracts combined with parallel measurements of PDHa and PDHt do permit qualitative statements to be made about the effect of this ratio on transformation of PDHc from PDHb to PDHa. Whole muscle ATP (28,30), ADP (28) and PCr (28,30) concentrations remained unchanged from rest (28,30) to moderate exercise at 40% $\dot{V}O_2$max (30), indicating that the mitochondrial ATP-to-ADP ratio was also unchanged. However, PDHa increased considerably during moderate exercise which suggests that the ratio of ATP-to-ADP did not influence PDHc transformation directly.

In contrast to rest and submaximal exercise, during heavy (30) and maximal exercise (28), and during recovery from maximal exercise (28) muscle PCr was considerably lower (12,28,30) and the ATP-to-ADP ratio from whole cell extracts also decreased (28). The magnitude of changes observed in the ATP-to-ADP ratio during exercise and recovery (ie. 8.9-4.5) were similar to those reported in perfused rat heart (26) and skeletal muscle (18) during electrical stimulation. Similar ATP-to-ADP ratios have also been reported in human skeletal muscle during rest (5.7-9.4), after submaximal exercise (3.1) and after maximal exercise (3.9) (53). However, lack of accurate PDHa and PDHt measurements in that study (53) preclude assessment of the qualitative or quantitative importance attached to PDHc regulation in human skeletal muscle. In contrast, the results of the present studies (12,28,30) argue that, at best, the ATP-to-ADP ratio plays a relatively minor role in the regulation of PDHc transformation in human skeletal muscle during exercise. However, during rest and recovery from exercise, the ATP-to-ADP ratio is an important determinant of PDHc transformation to PDHa.

In spite of the apparent relationship between the ATP-to-ADP ratio and PDHa

measures during rest and recovery in the present studies, the data do not provide a quantitative assessment of the importance of this ratio to PDHc transformation *in vivo*. It is possible that this ratio only becomes physiologically relevant during resting conditions or during recovery from exercise, and acts in co-operation with the ratios of acetylCoA-to-CoASH and NADH-to-NAD to regulate PDHc transformation. However, with the experimental approaches currently available, independent manipulation of the ATP-to-ADP ratio alone without also altering one of the NADH-to-NAD or acetylCoA-to-CoASH ratios is not possible in human skeletal muscle, *in vivo*.

5.2.3.1 Future Directions for the Study of PDHc Regulation by the ATP-to-ADP Ratio

To further examine the physiological importance of the ATP-to-ADP ratio in skeletal muscle, future studies should examine PDHc transformation during rest or recovery from exercise in the presence of uncouplers of oxidative phosphorylation (ie. oligomycin, 2,4-dinitrophenol or cyanide) or the electron transport chain (ie. amytal-rotenone or antimycin), in an *in situ* preparation such as the perfused rat hindlimb (20,44) or perfused dog gracilis (6,45). Gradually increasing the concentration of an uncoupler, in the perfusate, in a step-wise manner would allow controlled reductions in the ratio of ATP-to-ADP and the effects on PDHa could be examined. Unfortunately, such experiments would also be complicated by the simultaneous rise in the NADH-to-NAD ratio in the cytosol and then in the mitochondria, unless inhibitors of the malate-aspartate shuttle or cytosolic malate dehydrogenase could prevent this occurrence. In addition, such an approach would also increase pyruvate production and add to PDH-kinase inhibition, since pyruvate acts synergistically with ADP to inhibit the kinase (54). Therefore, inhibitors of glycolysis should

also be used to prevent pyruvate accumulation.

5.3 PDHc Regulation by Pyruvate

The activating effects of pyruvate on PDHc occur by inhibiting PDH-kinase with a K_i of 0.5-2.8 mM (54). In the present work, muscle pyruvate was determined in two studies (28,31). Knowing that 1 kg dw of muscle is equal to approximately 3 kg of intracellular water, it is possible to convert the measured pyruvate contents to concentrations for comparison with the known kinetic constant for PDH-kinase. At rest, pyruvate concentration was 0.2 mM (28) and increased to 0.3 mM during heavy exercise (31). During maximal exercise pyruvate concentration increased further to 0.7 mM (28). Thus, the range of changes in pyruvate concentration (0.2-0.7 mM) is similar to the range of experimentally determined K_i for pyruvate, and this metabolite is in an optimal position to effect large changes in PDH-kinase activity in response to only small changes in concentration.

Pyruvate regulation of PDHc transformation may be most important during recovery from exercise when sustained PDHa flux provides a route for removal of accumulated intramuscular lactate via oxidation in the TCA cycle (28). In addition, this aspect of PDHc regulation would also be central to the operation of the "lactate shuttle hypothesis" as proposed by Brooks (4,5). According to this hypothesis, lactate produced by actively contracting muscle fibers is oxidized by neighboring inactive fibers of the same muscle or in distally located inactive muscles. Indeed, during recovery from maximal intermittent sprint cycling, PDHa was maintained 2.4-2.8 fold greater than at rest and pyruvate concentration was positively correlated with elevated PDHa (28). In addition, it has recently been shown in humans that uptake of lactate into inactive muscles during recovery from high intensity

exercise resulted in corresponding elevations in intramuscular lactate and pyruvate concentrations, and a 2.3 fold increase in PDHa (29). Thus, the elevation of muscle pyruvate seems to serve as its own feed forward signal, inhibiting PDH-kinase and causing PDHc transformation to PDHa. Consequently, a greater portion of lactate can be oxidized resulting in attenuation of the intracellular acidosis that accompanies lactate accumulation.

5.3.1 Future Directions for the Study of PDHc Regulation by Pyruvate

Future studies examining the role of pyruvate regulation of PDHc transformation should first determine the K_m for E1 of PDHc and the K_i for PDH-kinase under conditions that mimic the intramitochondrial milieu, since it has not been determined if the affinity for pyruvate is altered when this enzyme complex is bound to the inner mitochondrial membrane. It is possible that the K_m of pyruvate for the E1 component and the K_i of pyruvate for PDH-kinase are lower than that reported for isolated enzyme preparations (54). If this proves to be true, the implications for the physiological role of PDHc would likely remain unchanged. However, it would provide a more meaningful set of kinetic constants from which measured tissue concentrations could be compared. Currently available constants were determined using isolated enzyme preparations in solution, which limits their utility for purposes of comparison.

Future studies should examine the transformation of PDHc to PDHa, in inactive muscle during incremental exercise. This approach would increase pyruvate content in a step-wise manner and would confirm the importance of this mechanism of PDHc regulation. In addition, if these measures are obtained in conjunction with measures of total lactate production by the active muscle mass and lactate uptake by the inactive muscle mass, then

the quantitative importance of lactate oxidation by this mechanism of PDHc regulation could be determined.

5.4 The Role of PDHc in the Selection and Oxidation of Metabolic Fuels in Human Skeletal Muscle

In their original article describing the theory of the glucose-fatty acid cycle in skeletal and heart muscle, Randle *et al* (34) suggested that the restriction on glucose utilization was most prominent at the level of pyruvate oxidation, pointing to PDHc as a major control point in the selection of metabolic fuels. Subsequent studies on perfused rat heart and diaphragm confirmed that transformation of PDHc to PDHa was lower at rest and this was due to the inhibitory effects of increased acetylCoA and decreased CoASH accumulation, secondary to elevated fat fuel availability and oxidation (13,14,15,35). This led to the suggestion that changes to the acetylCoA-to-CoASH ratio was the primary signal which reduced PDHa and initiated this inhibitory mechanism of intramuscular glucose oxidation. This was thought to subsequently lead to the accumulation of glycolytic intermediates and key metabolic inhibitors of phosphofructokinase, hexokinase (34) and probably glycogen phosphorylase that also inhibited glucose oxidation at these control points.

The present studies (30,31), and an additional study (12) (Appendix B), examined the existence of this reciprocal cycle of glucose and fat oxidation in human skeletal muscle at rest and during various exercise intensities. At rest, diet manipulation (31) and acetate infusion (30) were successful at elevating the intramuscular acetylCoA-to-CoASH ratio, lowering PDHa and restricting intramuscular glucose oxidation (31). In contrast, infusion of Intralipid (12) did not cause an elevation in this regulatory ratio or inhibition of PDHa. Together, the

results of these three studies indicate that this reciprocal cycle of intramuscular fat and glucose selection is operational in human skeletal muscle during rest. Moreover, acute elevations of alternative fat fuels (ie. acetate and Intralipid) suggest that the source of acetylCoA accumulation is from the oxidation of short-chain fatty acid anions. Thus, under conditions of starvation, high fat feeding or uncontrolled diabetes, the primary fuel source for intramuscular generation of acetylCoA is likely from hepatic generation of the ketone bodies, acetoacetate and β-hydroxybutyrate.

The absence of any acetylCoA accumulation after Intralipid infusion (12) may have been limited by an associated increase in malonyl-CoA (49), which may have inhibited carnitine palmitoyl transferase and therefore provided a feedback mechanism to restrict transport of long chain fatty acid acyl-CoA into the mitochondria. In contrast, acetate (30), acetoacetate and β-hydroxybutyrate rapidly diffuse into the mitochondria where they can be converted to acetylCoA at a rate that is only dependent on the availability of these short chain fatty acid anions. Consequently, acetylCoA can accumulate quickly and to a large extent and inhibit PDHc transformation to PDHa. Alternatively, the capacity to generate acetylCoA via β-oxidation of long chain acyl-CoA was very low in the subjects examined (12).

During moderate (30) and prolonged heavy exercise (12,30,31), reduced intramuscular glucose oxidation and lower PDHa were only observed after a period of prolonged carbohydrate deprivation (31). During acute manipulations of the glucose-fatty acid cycle using acetate infusion, intramuscular glycogen utilization was not impaired and a lower rate of PDHc transformation to PDHa was not observed (30). However, during Intralipid infusion (12), reduced intramuscular glycogen utilization was observed but this occurred independent of changes to the acetylCoA-to-CoASH ratio and PDHa. The apparent site of conservation

of muscle glycogen during Intralipid infusion appeared to have been at the level of glycogen phosphorylase. In each condition, except the LCD condition, the acetylCoA-to-CoASH ratio increased as a direct result of muscle contraction during exercise and transformation of PDHc to PDHa was not inhibited. In contrast, after prolonged consumption of a LCD the acetylCoA-to-CoASH ratio decreased with exercise while PDHc transformation to PDHa was inhibited (31).

Together, these studies suggest that the glucose-fatty acid cycle did not operate in human skeletal muscle during exercise, as originally proposed by Randle *et al* (34). Instead, operation of this reciprocal metabolic cycle during exercise may have occurred via alterations in the phosphorylation state of E1α of PDHc. Prolonged carbohydrate restriction via consumption of a LCD, resulting in increased availability and oxidation of intramuscular fat fuels may have led to a prolonged increase in PDH-kinase activity and increased phosphorylation of the barrier sites (sites 2 and 3) on E1α of PDHc. Consequently, when PDHc was exposed to activating stimuli during muscle contraction, the rate of PDHc transformation to PDHa was inhibited. While this aspect of PDHc regulation has been confirmed in rat heart and skeletal muscle (see section 5.2.2.1 above), it has not been demonstrated in human skeletal muscle.

5.4.1 Future Directions for Studying the Role of PDHc in the Selection and Oxidation of Metabolic Fuels in Human Skeletal Muscle

In the present studies (12,30,31), there is evidence for 2 levels of PDHc regulation in human skeletal muscle as it operates in the glucose-fatty acid. The first is an acute phase that is dependent on the increase in mitochondrial acetylCoA-to-CoASH ratio (and possibly

the NADH-to-NAD ratio) which increases PDH-kinase activity, inhibits PDH-phosphatase activity and increases site 1 occupancy on E1α of PDHc. These events lead to lower PDHa and a short term restriction on pyruvate oxidation. The second level of regulation is also dependent on an increase in the acetylCoA-to-CoASH ratio (and possibly the NADH-to-NAD ratio) resulting in a sustained increase in PDH-kinase activity and a decrease in PDH-phosphatase activity. This may result in additional phosphorylation of the barrier sites of E1α of PDHc (site 2 and 3) and resistance to activation by simulators of PDHc transformation to PDHa.

In the second phase, changes in the PDH-kinase/PDH-phosphatase activity ratio may also be supported by alterations in the affinity of the acetylCoA-to-CoASH and NADH-to-NAD ratios for the regulatory subunits. In addition, it is interesting to speculate that as a further mechanism of restricting pyruvate oxidation and protecting muscle and whole body glucose reserves, alterations in PDH-kinase and PDH-phosphatase content would follow or coincide with these changes during prolonged elevation of fat fuels combined with carbohydrate restriction. Alterations in PDH-kinase and PDH-phosphatase affinity could easily be tested by examining changes in the kinetic constants over time. In addition, the recent availability of a cDNA probe for PDH-kinase (24) makes it possible to examine changes in PDH-kinase expression during long term glucose deprivation and long term elevations in fat fuel availability.

Future studies examining the role of PDHc in the selection and oxidation of intramuscular fuel selection should first establish the time course and extent of E1α phosphorylation in human skeletal muscle, in response to prolonged elevations in fat fuels. Specifically, the time course for multi-site phosphorylation should be established and

characterized. It would also be interesting to characterize parallel changes in the affinity of the regulatory subunits to the key regulatory ratios of acetylCoA-to-CoASH, NADH-to-NAD and ATP-to-ADP. Finally, it would also be possible to examine alterations in the expression of the main catalytic subunits (E1, E2 and E3) (25) and PDH-kinase (24) using cDNA probes. These aspects of PDHc regulation could best be studied in humans using a model of prolonged carbohydrate deprivation combined with high fat feeding. Such an approach would allow subjects to be examined over the course of several weeks and would also allow the prolonged effects of catecholamine and the glucoregulatory hormones on PDHc to be examined.

5.5 The Role of PDHc in Muscle Lactate Production

The role of PDHc regulation in muscle lactate production in humans was examined using a model of repeated 30 seconds bouts of maximal isokinetic sprint cycling separated by 4 minute periods of rest recovery (28). This model allowed simultaneous determination of the rates of muscle lactate production and removal, and allowed determination of the mitochondrial redox state. During maximal exercise, the mechanism of muscle lactate production was found to be due to a rate limitation for pyruvate oxidation at PDHc and it was not due to the acceleration of glycolysis, secondary to the development of tissue hypoxia as suggested by others (10). During exercise, the rate of glycolytic pyruvate production exceeded the rate of pyruvate oxidation by PDHa, by a factor of 13 in the first and second sprint bouts and by 4 fold in the third bout. The remainder of the lactate produced was accounted for by release from the muscles of origin into the extracellular space (21) and accumulation within the muscles of origin (28).

In contrast to exercise, during the recovery periods muscle lactate accumulation decreased concurrently while the mitochondrial redox state became more reduced. During this time PDHc was maintained 34-44% transformed to PDHa and was 2.2 and 2.8 fold greater than at rest. It has previously been shown that during the rest recovery periods there is no change in muscle glycogen content (22,44) and glucose uptake is low (21). Thus, glycolytic flux probably ceased or was at least slowed in our subjects. Together these events suggest that the decrease in muscle lactate accumulation was due, in part, to a greater rate of pyruvate oxidation by PDHa than pyruvate production by glycolysis. The remainder of muscle lactate was probably released from the muscle of origin into the extracellular space (21). During rest recovery, maintenance of PDHa activity provided an important route for the removal of the excess lactate. The mechanism by which PDHa activity was maintained was consistent with a feed forward mechanism mediated by elevated pyruvate and H^+ concentrations causing inhibition of PDH-kinase and activation of PDH-phosphatase, respectively. Additionally, lower NADH-to-NAD and ATP-to-ADP ratios, and attenuation of the acetylCoA-to-CoASH ratio would have also supported a lower PDH-kinase/PDH-phosphatase activity ratio and resulted in greater PDHa.

The findings of the present work (28) show that muscle lactate production in normal healthy individuals exercising maximally under normoxic conditions is not due to the development of tissue hypoxia. This implies that in normoxia, the theory of muscle lactate production termed the "anaerobic threshold" (3,10) may be incorrect. However, this does not preclude the possibility that exposure to a hypoxic environment would lead to tissue hypoxia causing acceleration of anaerobic glycolysis and result in muscle lactate production.

5.5.1 Future Directions for Studying the Role of PDHc in Skeletal Muscle Lactate Production

The present work (28) has shown that muscle lactate production can be explained by a discrepancy between the rates of pyruvate production by glycolysis and pyruvate oxidation by PDHa. However, the present work examines lactate production only at the extremes of muscular activity. Future studies should further characterize and compare glycolytic rate, PDHa transformation and PDHa flux as a function of increasing exercise intensity. This could best be examined in humans during incremental exercise while simultaneous arterial and venous lactate measurements are obtained along with measures of intramuscular glycogen, lactate, PDHa and the mitochondrial redox state. This approach would allow a complete accounting of all of the lactate produced from glycogenolysis, and glycolysis derived from glucose. It would also allow the rate of glycolytically produced pyruvate to be compared to the rate of pyruvate oxidation by PDHa at the same time the redox state could be monitored.

A similar approach could also be used to study muscle lactate production while hypoxia is induced to cause a reduction in the mitochondrial redox state and a corresponding shift in the "threshold for lactate production" to a lower exercise intensity. Alternatively, muscle lactate production could be examined before and after a period of short-term submaximal training which should result in a shift in the "threshold for lactate production" to a higher exercise intensity, independent of changes in $\dot{V}O_2max$ (16,17,23). These approaches would allow independent manipulation of the mechanisms controlling muscle lactate production and assess the importance of PDHc regulation to muscle lactate production. These studies would provide additional insight into the mechanisms of muscle

lactate production and extend the findings of the present study (28).

5.6 Summary and Conclusions

5.6.1 Biochemical Regulatory Properties

Examination of PDHc transformation to PDHa under the conditions of the present studies (12,28,30,31) provide additional insight into the regulation of PDHc transformation *in vivo*. Regulation by the acetylCoA-to-CoASH, NADH-to-NAD and ATP-to-ADP ratios were found to operate as described for isolated enzyme preparations *in vitro*. However, the quantitative effects of these ratios on PDHc transformation to PDHa must be further characterized *in vivo* by independent manipulation through the physiological range. Furthermore, the role of the acetylCoA-to-CoASH and NADH-to-NAD ratios in the short term and long term regulation of PDHc transformation to PDHa during the selection and oxidation of intramuscular fuels must be better defined and the synergistic nature of these ratios remains to be delineated *in vivo*.

Increasing the acetylCoA-to-CoASH ratio at rest resulted in a proportional decrease in PDHa which restricted pyruvate oxidation. In contrast, during exercise, the methods employed were not successful at altering the acetylCoA-to-CoASH ratio over the changes that occurred as a result of exercise alone. The effect of alterations to the NADH-to-NAD and ATP-to-ADP ratios on PDHc transformation also remains to be determined under the conditions of the present studies (30,31). Complete characterization of their regulatory influence on PDHc transformation by these other regulatory ratios and the implications for the selection and oxidation of metabolic fuels remains to be fully determined.

At rest and during recovery from maximal exercise and under normal dietary

conditions, the acetylCoA-to-CoASH, NADH-to-NAD and ATP-to-ADP ratios appeared to act in concert to regulate PDHc transformation to PDHa. At rest, these ratios appeared to maintain lower PDHa and their effects were consistent with a high PDH-kinase/PDH-phosphatase activity ratio. Conversely, during recovery from maximal exercise, alterations in these ratios appeared to have caused greater PDHa and the changes were consistent with a lower PDH-kinase/PDH-phosphatase activity ratio. During exercise, these regulatory ratios had no apparent effects on PDHc transformation to PDHa. The overriding factor controlling PDHc transformation to PDHa was probably Ca^{2+} release from the sarcoplasmic reticulum.

Muscle pyruvate concentration varied within the range of the estimated inhibitory constants of pyruvate for PDH-kinase. In addition, muscle pyruvate content increased in parallel with PDHa. These findings were consistent with pyruvate activation of PDHa during exercise and recovery from maximal exercise. However, the present studies did not assess the quantitative importance of PDHc regulation by pyruvate. Conceivably, pyruvate exerted some regulatory action on PDHc transformation during rest and recovery from exercise and regulation by pyruvate was central to the operation of the "lactate-shuttle" hypothesis. The results of the present studies argue in support of the operation of this mechanism in human skeletal muscle *in vivo*. However, the quantitative importance of this mechanism of PDHc regulation to energy metabolism and acid-base homeostasis remains to be established.

5.6.2 Physiological Relevance

The present series of studies examined the regulation of human skeletal muscle PDHc *in vivo*. These studies sought to determine the physiological importance of this flux-generating enzyme to muscle energy metabolism. Acute and chronic manipulation of fat fuel

availability revealed that PDHc was an important control point for the selection and oxidation of fat and carbohydrate fuels during rest and possibly during exercise. During rest, PDHc transformation to PDHa was inhibited by elevation of the acetylCoA-to-CoASH ratio, restricting pyruvate oxidation at this first control point as predicted by Randle *et al* (34). However, during exercise, lower PDHa could only be induced by chronic dietary manipulation and it occurred seemingly independent of variations in the acetylCoA-to-CoASH ratio. This finding is in conflict with the predictions of Randle *et al* (34) and more study is required to establish the role of PDHc regulation in fuel selection during exercise, as well as the mechanism by which transformation is inhibited.

Examination of PDHc transformation during repeated maximal sprint activity allowed determination of the role of PDHa in muscle lactate production in humans. The results of this study indicated that under normoxic conditions lactate production was simply due to a rate limitation at the level of PDHc. A greater rate of pyruvate production by glycogenolysis than oxidation by PDHa led to accumulation of excess pyruvate which was then converted to lactate via LDH. During recovery from maximal sprint exercise the decrease in muscle lactate accumulation resulted from lower glycolytic rate and maintenance of PDHa by the putative allosteric regulators of PDHc transformation, leading to oxidation of a portion of the lactate load by the TCA cycle.

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APPENDIX A

A COMPARISON OF RADIOMETRIC METHODS FOR DETERMINATION OF PDHa AND PDHt IN SKELETAL MUSCLE

6.1 Abstract

The present study characterized and compared two currently available radiometric assays of pyruvate dehydrogenase activity in skeletal muscle. The limits within which each assay can be reliably used are outlined. Upper limits are suggested for sample volume and reaction time. The $^{14}CO_2$ method of determination was found to be reliable when 50-200 μl of a 19 volume muscle homogenate was assayed over a 2 minute reaction time. Suggestions are also made to further improve the sensitivity of this assay system. The acetylCoA method of determination was found to be reliable when 10-70 μl of a 30 volume homogenate was used over a 3 minute reaction time. In addition, the acetylCoA method of determination can be reliably run for up to 5 minutes at the larger homogenate volume of 70 μl. Direct comparison of these two assays further revealed that each gave similar absolute results. However, the acetylCoA method of determination was found to be much less variable, and much more accurate and precise than the $^{14}CO_2$ method of determination.

6.2 Introduction

Several different methods of determining pyruvate dehydrogenase activity in tissue extracts are currently available (see Stansbie et al (34) and Weiland (43) for reviews). However, most have inherent problems which limit their practical application for analysis of small muscle samples such as those obtained from a biopsy of human skeletal muscle. Spectrophotometric assays that are designed to measure the rate of acetylCoA and NADH formation lack the sensitivity required to determine the active fraction (PDHa) or the total activity (PDHt) of PDHc in very small biopsy samples. In addition, several other problems limit their use.

Spectrophotometric determination of PDHa and PDHt from the rate of NADH formation is limited by simultaneous oxidation of NADH by other metabolic processes (34). Similarly, spectrophotometric determination from the rate of acetylCoA formation is made cumbersome by the need to isolate acetylCoA:arylamine N-acetyltransferase (EC 2.3.1.5.) from pigeon liver which is not commercially available. Depending on the particular assay used, this enzyme is required to transfer the acetyl moiety from acetylCoA to either p-(p-aminophenylazobenzene sulphonic acid) or cresyl violet acetate, producing their corresponding acetylated forms and causing increased absorbance and fluorescence, respectively.

The only other assay system for PDHa and PDHt determination that existed until just recently was a radiometric method that determines the rate of $^{14}CO_2$ formation from 1-^{14}C-Na-pyruvate (35,36,39,40). The $^{14}CO_2$ liberated then reacts with a strong base (hyamine hydroxide), is "captured" and the amount of CO_2 produced is calculated as a function of time. This system has been extensively used for determination of PDHa and PDHt in the following

human tissues: fibroblasts (3,6,15,16,31), platelets (4,13,15) and skeletal muscle (14,27,29,37,39,40). It has also been used to analyze PDHa and PDHt in adipose tissue (35,36) and various other tissues in animal models (21,22). This method is ideal for PDHa and PDHt determination in small skeletal muscle biopsy samples, since it is many fold more sensitive than spectrophotometric assays. However, when used to make measurements in skeletal muscle, as outlined in the literature (3,4,6,14,15,16,29,31,35,36,37,39,40), serious limitations arise rendering it inaccurate. The main problems are very low and highly variable results. Therefore, the first purpose of the present work was to improve this assay system and to characterize its utility for analytical use.

Recently, another radiometric method for PDHc determination in muscle was reported (9). This assay was adapted from one previously reported by Dohm et al (5), that was designed for PDHa and PDHt determination in kidney. Constantin *et al* (9) improved this system by using a second radiometric assay to determine the concentration of acetylCoA (7) in aliquots obtained at 1, 2 and 3 minutes into the reaction. Like the former radiometric method, this method was also much more sensitive than any of the spectrophotometric methods and was found to be ideal for PDHa and PDHt determination on small tissue samples (5-15 mg wet wgt.). The original report of this assay demonstrated very good reproducability and precision of measurement. Therefore, given the problems encountered with the $^{14}CO_2$ method, the second purpose of the present work was to examine this new radiometric assay and to compare it to the $^{14}CO_2$ method, to determine which of the two was better for analytical purposes in skeletal muscle.

6.3 Methods

6.3.1 Muscle Sampling

The red gastrocnemius, plantaris and soleus muscles were excised from male Sprague-Dawley rats (300-500 grams), while under a general anaesthetic (phenobarbital injected intraperitoneally). The rats were then euthanized with an overdose of the anaesthetic followed by intracardial injection of a saturated solution of KCl. After excision, muscles were immediately freeze clamped and stored in liquid N_2. Prior to use in the PDHa and PDHt assays muscle samples were pulverized under liquid nitrogen. The resulting frozen muscle powder was then dissected free of blood and connective tissue, also under liquid nitrogen, using a stereomicroscope with paired 10X wide-field eyepieces. The resulting clean muscle powder was then stored frozen in liquid N_2 and portions were used on a daily basis for PDHa and PDHt analysis.

Human muscle biopsies were obtained from healthy male volunteers between the ages of 19 and 29 years. Biopsies were obtained by the method of Bergström (1). One thigh was prepared for needle biopsy of the vastus lateralis with incisions of the skin through to the deep fascia, under local anesthesia (2% xylocaine without epinephrine). Biopsies were immediately frozen in liquid N_2, removed from the needle while frozen and stored in liquid N_2 until analyzed. Between 10 and 35 mg was chipped from each biopsy and dissected free of blood and connective tissue, under liquid N_2 using a stereomicroscope with paired 10X wide-field eyepieces. From this, two clean 5-20 mg portions were stored separately in liquid N_2. Rat and human skeletal muscle samples were collected as part of larger ongoing studies and had the approval of McMaster University Ethics Committee.

6.3.2 $^{14}CO_2$ Method of PDHa and PDHt Determination

6.3.2.1 Reagents

6.3.2.2 Preparation of 1-^{14}C-Na-pyruvate

The labelled substrate (1-^{14}C-Na-pyruvate) was obtained from Amersham (1-^{14}C-Pyruvic Acid, Sodium Salt, Code CFA.85) and had a specific activity of 32 mCi·mmol^{-1}. 6.0 ml of double distilled, millipore purified H_20 was placed into a 250 μCi ampule and mixed. 200 μl of this mixture was placed into 30 separate 1 ml glass vials with screw on caps. These were then frozen in liquid nitrogen and freeze dried. The resulting vials each contained 260 nmol of 1-^{14}C-Na-pyruvate (8.3 μCi) and were stored frozen at -50 °C, under desiccant until used.

On the day of the assay 804 μl of 0.004 N HCl was added to 1 vial, resulting in a stock solution that was 10.02 μCi·ml^{-1}. 230 μl of stock solution was then added per 10 ml of assay buffer just before commencing the assay each day. Suspension of 1-^{14}C-Na-pyruvate in an acidic medium was necessary to minimize nonspecific spontaneous decarboxylation of the ^{14}C-label (33) and to prevent the formation of aldol condensation products that can form when pyruvate is suspended in solution (38).

6.3.2.3 PDHa Homogenizing Buffer (HB_{active})

For PDHa the sample was homogenized in a solution containing 10 mM K_2HPO_4, 0.1% bovine serum albumin, 1 mM DTT, 1 mM EDTA and 0.1% triton X-100 (vol:vol) at pH 7.4. Throughout this chapter the homogenizing buffer for PDHa will be referred to as HB_{active}.

6.3.2.4 PDHt Homogenizing Buffer (HB_{total})

For PDHt determination, the homogenizing buffer used was the same as outlined for PDHa, with the following differences: 1) 100 μl of a solution containing 1.0 M glucose and 10 mM K_2HPO_4 (pH 7.4) was added to 10 ml of the homogenizing buffer before homogenization of the sample, 2) hexokinase was added to a final concentration of 2 $U \cdot ml^{-1}$ before homogenization and 3) after homogenization, 8 μl of a preincubation reagent containing 385 mM $CaCl_2$ and 385 mM $MgCl_2$ was added for every 300 μl of muscle homogenate. These changes resulted in a final concentration of 10 mM for each of $CaCl_2$, $MgCl_2$ and glucose.

Ca^{2+} and Mg^{2+} were used to activate PDH-phosphatase and inhibit PDH-kinase, while glucose and hexokinase were used to deplete the homogenate of ATP, thus removing any available substrate for the kinase and further inhibiting PDH-kinase activity. Examination of the time course for transformation of PDHa to PDHt revealed that conversion by these reagents was complete in 4-6 min. PDHt measures are stable for up to 30 min under these conditions, as previously reported (39,40).

6.3.2.5 Assay Buffer

The assay buffer utilized for PDHa and PDHt determination contained the following reagents: 25 mM K_2HPO_4, 0.1% bovine serum albumin, 2.5 mM DTT, 2.5 mM EDTA, 2.0 mM $MgCl_2$, 10 mM Na-pyruvate, 1-^{14}C-Na-pyruvate (0.13 $\mu Ci \cdot ml^{-1}$ or 230 μl of stock solution per 10 ml of assay buffer), 3.0 mM CoASH, 30.0 mM NAD^+ and 1.5 mM thiamine pyrophosphate (TPP), at pH 7.6. The fraction of pyruvate labelled was varied at times simply by increasing the amount of stock 1-^{14}C-Na-pyruvate added to the assay buffer.

6.3.2.6 Acid Reagent

An acid reagent containing 80 mM citric acid and 40 mM Na_2HPO_4 (pH 2.9) was used to stop the reaction.

6.3.2.7 Preparation of Tissue Homogenate

For PDHa determination muscle samples were gently homogenized in a 2 ml glass homogenizer in 19 volumes of HB_{active} (1 volume sample plus 19 volumes of buffer) while on ice (0-5 °C). For PDHt determination, samples were homogenized in HB_{total} in a similar manner. In addition, 8 μl of the preincubation reagent was added and the sample was incubated for 6-10 min at 37 °C. Samples were homogenized untii all tissue was in suspension which usually required 1-2 min of slow gentle twisting of the glass pestle. Homogenization by this procedure resulted in a homogenate with a muscle tissue content of 50 $mg \cdot ml^{-1}$.

6.3.2.8 PDHa and PDHt Assays

Before the assay, 250 μl of the *assay buffer* was transferred into all of the 10 ml reaction flasks (Knootes Glass Co, New Jersey, USA) in a cold room (4 °C), while on ice. 200 μl of 1.0 M hyamine hydroxide was then placed into a plastic center well (Knootes Glass Co., New Jersey, USA) for each flask. Center wells were then fastened to the top rubber stopper (Knootes Glass Co. New Jersey, USA) and the top of the flask was sealed. A side arm rubber stopper (Knootes Glass Co, New Jersey, USA) was then placed on each flask, creating an air tight environment in which the reactions were run.

Before starting the reaction, each flask and sample was prewarmed for 2-3 minutes in a shaking water bath at 37 °C. The reaction was initiated by adding 50-200 μl of muscle

homogenate and mixing. The reaction was stopped after 2 minutes by adding 800 μl of the acid reagent. Each flask was then incubated for 60 min at 37 °C to allow complete recovery of the CO_2 generated.

After 60 min, the plastic center well was cut from each of the top rubber stoppers, placed in 10 ml of scintillation fluid and counted. The efficiency of counting was determined using a standard quench curve. PDHa and PDHt were calculated as the rate of CO_2 generated as a function of time and tissue content in each flask. Since only the labelled CO_2 was detected by scintillation counting, the results were corrected for the total amount of pyruvate (labelled + unlabelled) present in the assay buffer. Since nonspecific spontaneous decarboxylation of 1-^{14}C-Na-pyruvate is variable with temperature and time, at least 2 blanks were run with each set of sample replicates. Blank tubes were prepared exactly as the samples, except that the appropriate homogenizing buffer was used in place of the actual muscle homogenate.

6.3.2.9 Pyruvate Requirement of PDHt

The pyruvate requirement for the PDHt assay was examined using 200 μl samples of a 19 volume muscle homogenate and a 2 min reaction time. The following pyruvate concentrations were used for this analysis: 0, 0.004, 0.014, 0.05, 0.104, 0.504, 1.004, 3.004, 5.004 and 10.004 mM. Several blanks were run at each of the 10 different concentrations. The data were then fit to a Michaelis-Menton model and the K_m and V_{max} were determined.

6.3.2.10 CO_2 Recovery

The recovery of the CO_2 was examined in the following manner. 3.0 ml of 36 mM $NaHCO_3$, 2.98 ml of 25 mM K_2HPO_4 buffer (pH 8.9) and 0.02 ml of 540 mM $NaH^{14}CO_3$ (100 μCi·mmol^{-1}) were mixed resulting in a solution with the following composition: 18 mM $NaHCO_3$, 12.4 mM K_2HPO_4 and 1.8 mM $NaH^{14}CO_3$ (100 μCi·mmol^{-1}), at pH 8.9. This resulted in total $NaHCO_3$ and $NaH^{14}CO_3$ (100 μCi·mmol^{-1}) contents of 101 and 11 μmoles, respectively, and a solution with 9.1 % of the $NaHCO_3$ labelled.

A center well containing 200 μl of hyamine hydroxide was placed in each flask and 500 μl of the $NaHCO_3$ solution was then added. This resulted in 0.9 μmole of $NaH^{14}CO_3$ and 10.1 μmole of $NaHCO_3$ in each flask. Flasks were then placed at 37 °C for 2 min and then acidified with 800 μl of the acid reagent. Each flask was then allowed to equilibrate for 60 min at 37 °C after which the center wells were removed, placed in 10 ml of scintillation fluid and counted. Given that the specific activity of the label was 100 μCi·mmol^{-1}, the expected DPM per flask was 20.0×10^4. Standards were run in parallel by adding 500 μl of the original 9.1% mixture of labelled-to-unlabelled $NaHCO_3$ directly into 10 ml of scintillation fluid for counting. Recovery of the CO_2 was determined as follows:

$$(\text{sample DPM} / \text{standard DPM}) \times 100.$$

200 μl of acidified assay mixture from each reaction flask was also counted after the 60 min incubation period and compared to 200 μl of a solution containing only 18 mM $NaHCO_3$ and 12.4 mM K_2HPO_4 and a series of scintillation vials with no additions. This was done to determine if any of the label remained in solution after the 60 min incubation period.

6.3.2.11 *Linearity of the PDHa and PDHt Assays As a Function of Homogenate Volume*

CO_2 production was examined as a function of homogenate volume. The volume of added homogenate was varied from 0 (blank) to 200 μl, in 50 μl increments. Three such experiments were repeated for both PDHa and PDHt on the same muscle sample and the data were pooled. A 19 volume muscle homogenate preparation was used for these experiments. A 2 min reaction time was used for all replicates.

6.3.2.12 *Effects of Freezing and Thawing, Triton X-100 and NaF*

The effects of freezing and thawing, and adding a mild detergent (Triton X-100) to the homogenization procedure were examined as a means to improve PDHa and PDHt measures. The effect of freezing and thawing the homogenate was determined by measuring PDHa and PDHt on one day, then freezing and storing the homogenate overnight in liquid nitrogen and measuring PDHa and PDHt on the same homogenate the following day.

The effect of adding a mild detergent was also assessed by first homogenizing a tissue sample in HB_{active} or HB_{total} and splitting this into two portions. To one portion of muscle homogenate Triton X-100 was added to a final concentration of 0.1% (vol:vol), while gently mixing. Nothing further was added to the remaining portion. The samples were then assayed according to the method for PDHa or PDHt determination described above.

The effect of NaF on PDHa was examined by adding NaF, to a final concentration of 50 mM, to HB_{active} and homogenizing one portion of a muscle sample. Another portion of the same muscle sample was homogenized in HB_{active} that did not contain NaF. The resulting homogenates were then assayed using the method for PDHa determination described above.

6.3.3 The AcetylCoA Method of PDHa and PDHt Determination

6.3.3.1 PDHc Analysis

PDHa and PDHt were determined using a method that relies on the measurement of acetylCoA as a function of time (5) and has recently been improved by using a radiometric determination of acetylCoA (9). Briefly, for the measurement of PDHa the sample was gently homogenized on ice in a solution containing 50 mM tris, 200 mM sucrose, 50 mM KCl, 5 mM EGTA, 5 mM $MgCl_2$, 50 mM NaF, 5 mM dichloroacetic acid (DCA) and 0.1% triton X-100 (vol:vol), at pH 7.8.

For the measurement of PDHt, the sample was homogenized in a similar buffer but NaF and triton X-100 were omitted, and 4 $U \cdot ml^{-1}$ of hexokinase and 10 mM glucose were included. Before the assay, 40 μl of the sample was mixed with 10 μl of a preincubation reagent containing 6 mM $CaCl_2$ and 150 mM $MgCl_2$, and was incubated for 15-30 min at 37 °C. Where specified the preincubation step was removed. In such cases the sample was simply homogenized in a buffer containing 50 mM tris, 200 mM sucrose, 50 mM KCl, 5 mM EGTA, 10 mM $CaCl_2$, 25 mM $MgCl_2$, 4 $U \cdot ml^{-1}$ hexokinase, 10 mM glucose, 10 mM DCA and 0.1% triton X-100 (vol:vol), at pH 7.8.

The assay buffer used for the PDHa and PDHt assays contained 100 mM tris, 0.5 mM EDTA, 1.0 mM $MgCl_2$, 0.5 mM NAD^+, 0.5 mM CoASH and 1.0 mM thiamine pyrophosphate (TPP), at pH 7.8. The reaction was initiated by adding pyruvate to a final concentration of 0.5 mM. When the pre-incubation step was used for PDHt determination, triton X-100 was dissolved to 0.1% (vol:vol) in the pyruvate solution and added with the pyruvate. 200 µl aliquots of the reaction mixture were sampled at 1, 2 and 3 min and the reaction was stopped by adding each aliquot to 40 µl of 0.5 M PCA contained in 1.5 ml

eppendorf tubes. After 5 min, each aliquot was neutralized with 1.0 M K_2CO_3 and centrifuged for 3 min at 15,900×G (microcentrifuge E, Beckman Instrument Co., Mississauga, Ont., Canada). The resulting supernatant was stored at -50 °C until analyzed for acetylCoA by the radiometric method of Cederblad *et al* (7). Plots of acetylCoA as a function of time were used to determine the reaction rates.

6.3.3.2 Linearity of PDHa and PDHt Assays With Time and Concentration

Using human muscle biopsy samples, 30 volume muscle homogenates were prepared for PDHa and PDHt determination as outlined above. Using a 70 µl sample from each preparation, acetylCoA production was determined as a function of time. Both the PDHa and PDHt reactions were run as outlined above except that 100 µl aliquots of the reaction mixture were obtained at 1, 2, 3, 4, 5 and 6 minutes. Also using a 30 volume homogenate, the rate of acetylCoA production was determined as a function of homogenate volume as the volume of added homogenate was varied from 10 to 70 µl (ie. 10, 20, 30, 50 and 70 µl). Assays were completed as described above for both the PDHa and PDHt methods of determination.

6.3.4 Calculations

The Coefficient of variation (CV), accuracy and precision were calculated for repeated duplicate measures, as summarized below.

CV: $(SD / Mean) \times 100$

Accuracy: $\Sigma (x_i - m_{population})^2 / N$

Precision: $\Sigma (x_i - m_{sample})^2 / N-1$,

where "m" is the mean and x_i refers to individual data values.

6.3.5 Statistical Analysis

Most data were analyzed by a two-tailed t-test. A one-tailed test was used to assess the effects of adding Triton X-100, freezing and thawing, and NaF to the homogenization procedure. The CO_2 recovery data were analyzed by a one-way analysis of variance with repeated measures over time. When a significant *F* ratio was found, Fisher's pairwise comparisons was used to identify differences. Data are means ± SD. Results were considered significant at $p < 0.05$.

6.4 *Results*

6.4.1 *Pyruvate Requirement for the $^{14}CO_2$ Method of PDHt Determination*

PDHt activity increased as a function of pyruvate concentration from 0.006 mmol·min^{-1}·kg ww^{-1} when the pyruvate concentration was 4 μM, to 2.03 mmol·min^{-1}·kg ww^{-1} at a pyruvate concentration of 10 mM (Figure 1). The data fit a Michaelis-Menton model and accounted for 99% (R^2) of the variation in the data. The resulting regression equation was as follows:

$$PDHt = 2.27 \times [pyruvate] / (1.07 + [pyruvate]),\ R = 0.996,$$

where [pyruvate] is mM and PDHt activity is mmol·min^{-1}·kg ww^{-1}. The asymptote of this curve, which is equivalent to the V_{max}, was 2.27±0.09 mmol·min^{-1}·kg ww^{-1}. Under the present conditions and using 200 μl samples of a 19 volume muscle homogenate the apparent K_m was 1.07±0.15 mM.

6.4.2 *Capacity for CO_2 Recovery for the $^{14}CO_2$ Method of PDHa and PDHt Determination*

The quantity of $^{14}CO_2$ recovered in the hyamine hydroxide of each flask was similar to the quantity of $^{14}CO_2$ present in the same volume of the standard solution (Figure 2). 500 μl of the standard (ST) solution resulted in 21.2 × 10^4 DPM (SD 0.19 × 10^4), while 21.4 × 10^4 DPM (SD 0.08 × 10^4) was recovered in the sample (SA) tubes. The standards and samples were not statistically different and the corresponding efficiency of recovery was 100.8±0.30% which was not different from 100%. Similarly, Figure 3 illustrates that none of the $^{14}CO_2$ or $H^{14}CO_3^-$ remained in solution after the 60 minute incubation period. 200 μl of a blank assay mixture (ABL), standard assay mixture (AST) and the sample assay mixture

(ASA) resulted in 38.7± 2.4, 36.7±0.12 and 40.8±3.19 DPM, respectively, which were not different from one another. Thus the present experiments illustrate that at least 11,000 nmol of CO_2 can be absorbed by the hyamine hydroxide in 60 min, which is in excess of the amount of CO_2 that could be liberated from homogenate samples during 2 minutes of incubation.

6.4.3 Linearity of the PDHa and PDHt Assays As a Function of Homogenate Volume for the $^{14}CO_2$ Method of Determination

Using a saturating concentration of pyruvate alleviated the limitation in this assay system. Using a 2 min reaction time, CO_2 production was observed to increase linearly as a function of homogenate volume (Figure 4). For PDHt, total CO_2 production during the 2 min reaction time for 50, 100, 150 and 200 μl samples of muscle homogenate was 11±3.2 , 23±5.8, 32±8.0 and 44±10.5 nmol, respectively. For PDHa, total CO_2 production during the 2 min reaction time for 50, 100, 150 and 200 μl samples of muscle homogenate was 5±1.4, 9±2.7, 14±3.0 and 19±4.2 nmol, respectively. Since the reaction time was 2 min, the corresponding rate of CO_2 production was half of the total CO_2 produced. In each case the fraction of PDHa/PDHt was 41-42% in the absence of NaF. The resulting regression equations were as follows:

$$PDHt,\ CO_2 = 4.453 \times (mg\ tissue) + 0.306\ ,\ R = 0.999, p<0.0001,$$

$$PDHa,\ CO_2 = 1.877 \times (mg\ tissue) - 0.011\ ,\ R = 0.999, p<0.0001,$$

where CO_2 production is expressed as nmol and the homogenate volume is expressed as

milligrams of wet muscle tissue in each flask.

6.4.4 *Effects of Freezing and Thawing, Triton X-100 and NaF*

The addition of freezing and thawing to the extraction procedure increased PDHa and PDHt measures 2 and 3 fold, respectively (Table 1). Similarly, addition of Triton X-100 to the homogenizing buffer increased PDHa and PDHt activities (Table 1). Addition of 50 mM NaF to HB_{active} resulted in a 2.8 fold decrease in PDHa (Table 1).

6.4.5 *Linearity of PDHa and PDHt Assays With Time and Concentration for the AcetylCoA Method Of Determination*

Examination of PDHa and PDHt as a function of time (Figures 5 & 6) did not reveal any limitation to this method of PDHa or PDHt determination. Using a 30 volume homogenate of human muscle sample, acetylCoA production increased as a linear function of reaction time. The PDHa reaction was linear for the entire 6 min duration of the test (Figure 5). However, the PDHt assay was only linear over 5 minutes (Figure 6). The resulting linear regression equations for PDHa, PDHt and their respective blanks were as follows:

$$PDHa\ AcetylCoA = 25.038 \times (min) - 1.210,\ R = 0.999,\ p<0.0001$$

$$PDHa_{Blank}\ AcetylCoA = 3.632 \times (min) + 3.035,\ R = 0.958,\ p<0.003,$$

$$PDHt\ AcetylCoA = 34.080 \times (min) - 7.632,\ R = 0.996,\ p<0.0001$$

$$PDHt_{Blank}\ AcetylCoA = 4.105 \times (min) + 4.289,\ R = 0.920,\ p<0.004$$

where acetylCoA is expressed as pmol.

A similar analysis of acetylCoA production as a function of homogenate volume from 10 to 70 μl did not reveal any limitations to the measurement of PDHt by this method (Figure 7). Using a 3 min reaction time, acetylCoA production also increased as a linear function of homogenate volume. For the particular sample of human muscle used, the rate of acetylCoA production in the sample and blank were described by the following equations:

$$PDHt, \quad AcetylCoA = 0.322 \times (\mu l\ homogenate) + 0.244\ ,$$
$$R = 0.998, p<0.0001,$$

$$PDHt_{Blank}, \quad AcetylCoA = 0.063 \times (\mu l\ homogenate) - 0.039\ ,$$
$$R = 0.963, p<0.002,$$

where the units of acetylCoA production are pmol·min^{-1}. Blank values were not subtracted from PDHt data. Since PDHt represented the maximum catalytic activity and any limitation due to sample volume would have been identified more easily under these conditions, acetylCoA production was examined as a function of homogenate volume only for the PDHt method of determination.

6.4.6 Comparison of Two Radiometric Methods of PDHc Determination

The sample and day-to-day coefficients of variation (CV) for PDHa and PDHt measures were lowest for the acetylCoA method of determination. The CV_{sample} (same day) was 2.9 and 3.6 fold greater for the $^{14}CO_2$ method (Table 2) compared to the acetylCoA methods (Table 3) of PDHa and PDHt determination, respectively. Similarly, day-to-day variation of PDHa and PDHt measures (same sample) were greater using the $^{14}CO_2$ method. The CV_{day} was 3.0 and 2.0 fold greater for the $^{14}CO_2$ method compared to the acetylCoA method of PDHa and PDHt determination, respectively. The accuracy and precision terms were also much greater for PDHt determination, using the CO_2 method. Similarly, the accuracy term was significantly greater for PDHa determination using the CO_2 method.

FIGURE 1: Pyruvate requirement for the $^{14}CO_2$ method of PDHt determination. PDHt activity is plotted as a function of pyruvate concentration and fitted to a Michaelis-Menton model. Using 200 μl samples of a 19 volume rat muscle homogenate the V_{max} was 2.27±0.09 mmol·min^{-1}·kg ww^{-1} and the apparent K_m was 1.07±0.15 mM. The data were described by the following equation;

$$PDHt = 2.27 \times [pyruvate] / (1.07 + [pyruvate]),\ R = 0.996,$$

where [pyruvate] is mM and PDHt activity is mmol·min^{-1}·kg ww^{-1}.

PDHt = 2.27 * [pyruvate] / (1.07 + [pyruvate])

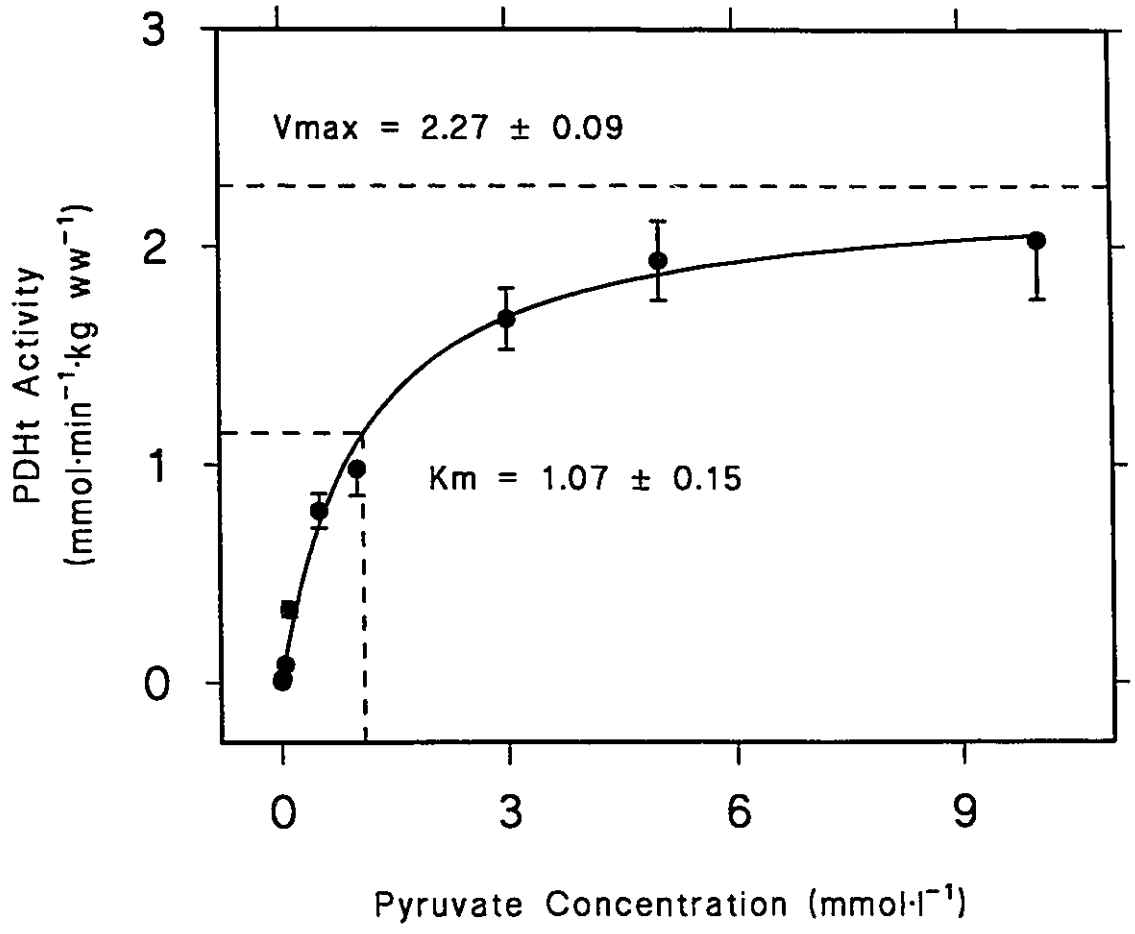

FIGURE 2: Capacity for CO_2 recovery for the $^{14}CO_2$ method of PDHa and PDHt determination I. DPM resulting from 500 μl of the stock solution (ST) and from the center wells of the samples (SA) containing 200 μl of hyamine hydroxide after 60 min of incubation at 37 °C. ST contained 18 mM $NaHCO_3$, 12.4 mM K_2HPO_4 and 1.8 mM $NaH^{14}CO_3$ (100 μCi·mmol^{-1}).

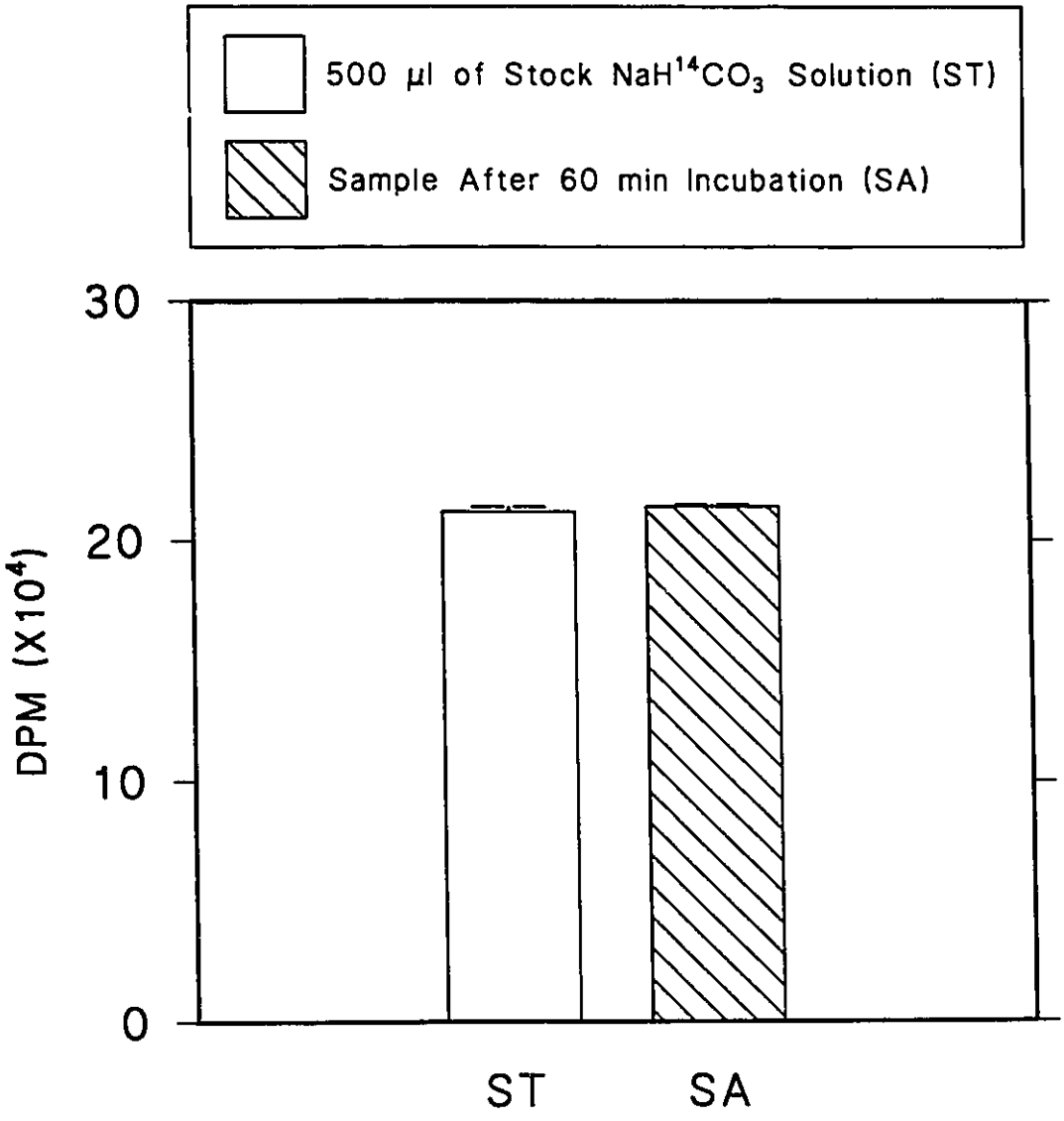
500 µl of Stock NaH$^{14}CO_3$ Solution (ST)
Sample After 60 min Incubation (SA)
30
20
10
0
DPM (X10^4)
ST
SA

FIGURE 3: Capacity for CO_2 recovery for the $^{14}CO_2$ method of PDHa and PDHt determination II. DPM resulting from 200 μl of a blank assay mixture (ABL), standard assay mixture (AST) and the sample assay mixture (ASA). ABL contained only scintillation fluid and AST was composed of 18 mM $NaHCO_3$ and 12.4 mM K_2HPO_4. 200 μl of ASA was obtained after 60 min of incubation at 37°C from those tubes that originally contained the $NaH^{14}CO_3$.

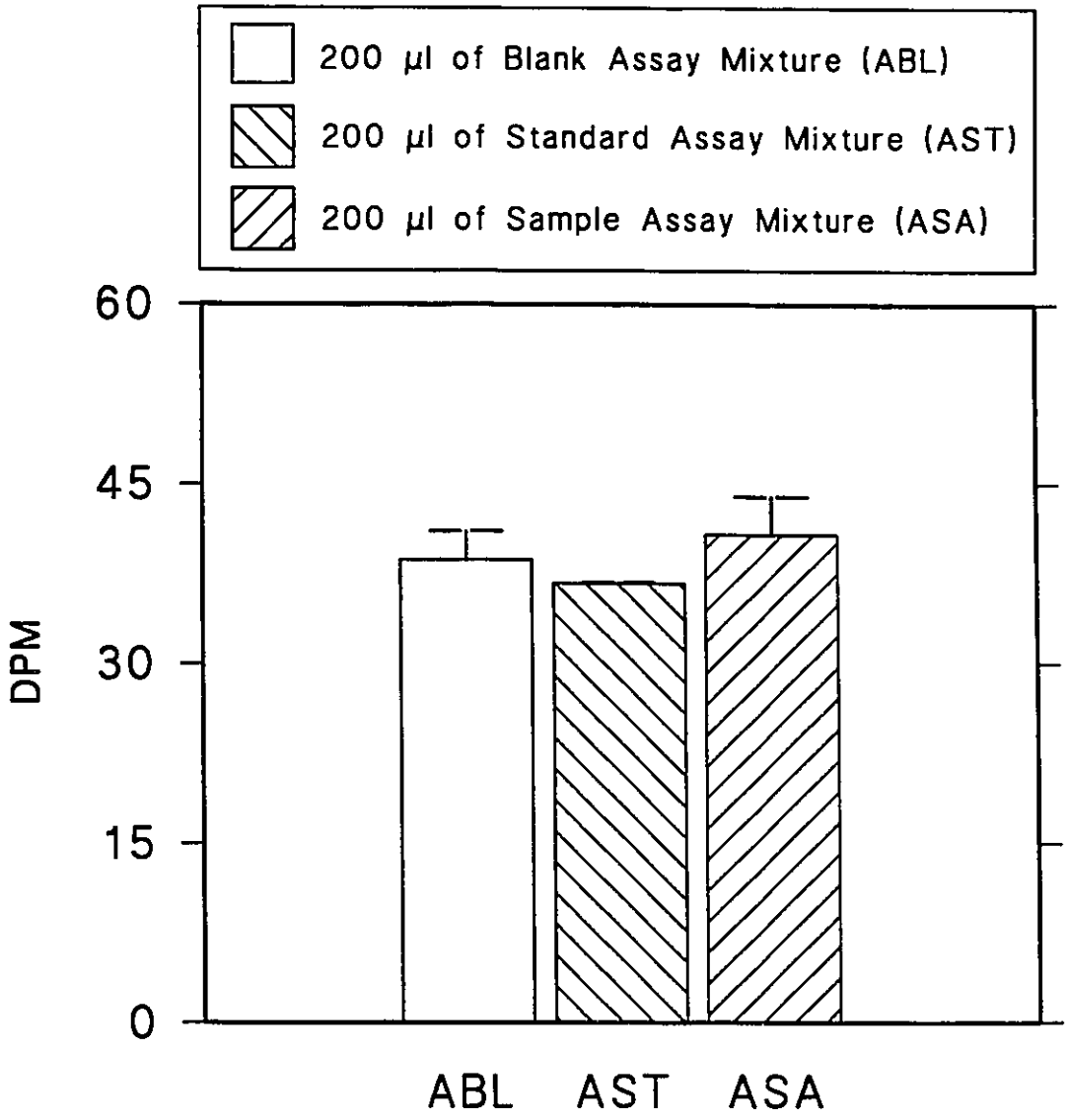
200 µl of Blank Assay Mixture (ABL)
200 µl of Standard Assay Mixture (AST)
200 µl of Sample Assay Mixture (ASA)
60
45
30
15
0
DPM
ABL
AST
ASA

FIGURE 4: Linearity of the PDHa and PDHt assays with homogenate volume, for the $^{14}CO_2$ method of determination. CO_2 production and the corresponding rate of CO_2 production are plotted as a function of homogenate volume. Homogenate volume is expressed as milligrams of wet muscle tissue per flask. For PDHt determination, 50, 100, 150 and 200 μl of homogenate were equivalent to 2.4, 4.9, 7.3 and 9.7 mg of wet muscle tissue per flask, respectively. For PDHa determination, 50, 100, 150 and 200 μl of homogenate were equivalent to 2.5, 5.0, 7.5 and 10.0 mg of wet muscle tissue per flask, respectively.

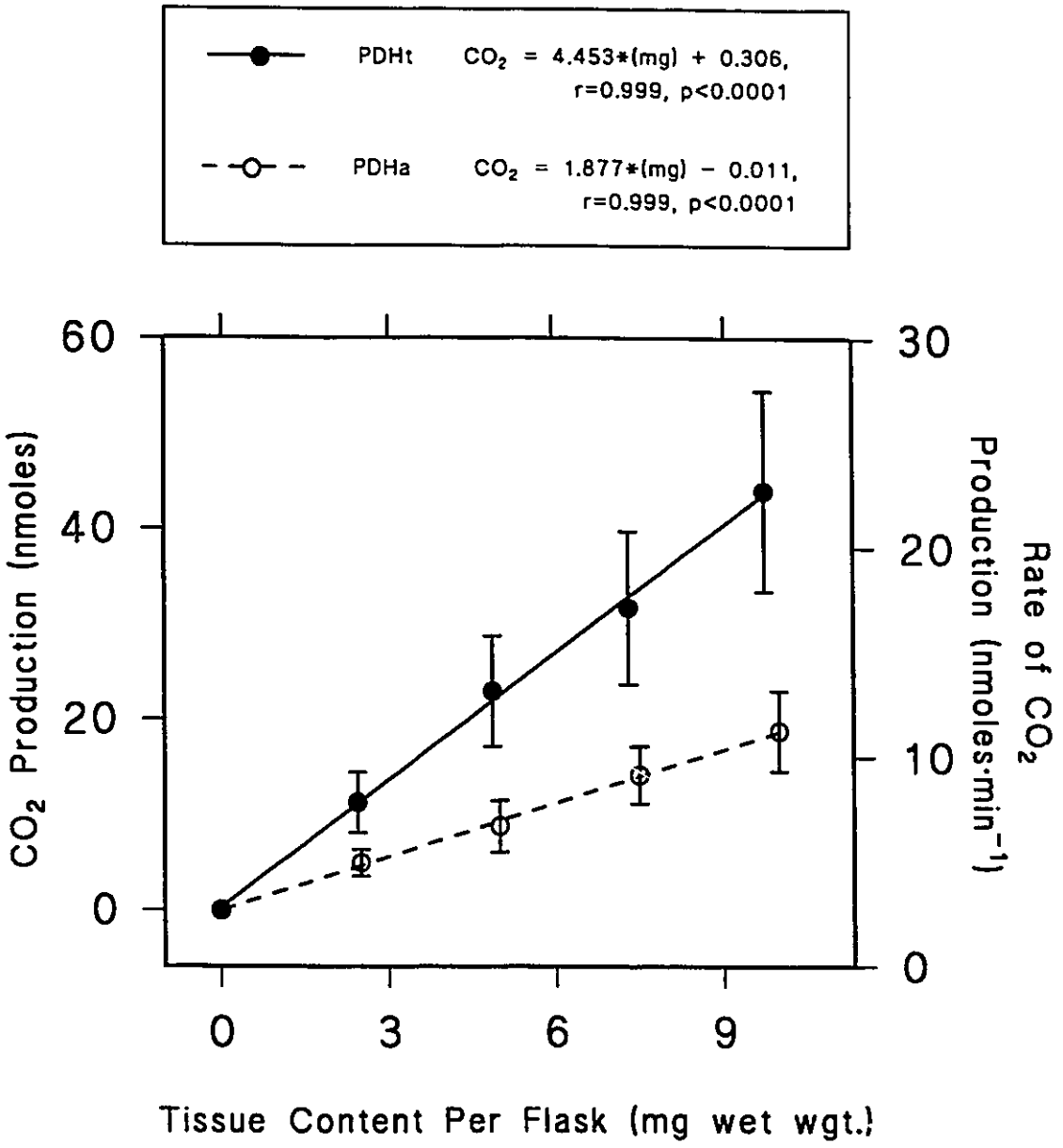
PDHt CO_2 = 4.453*(mg) + 0.306, r=0.999, p<0.0001
PDHa CO_2 = 1.877*(mg) - 0.011, r=0.999, p<0.0001
60
40
20
0
30
20
10
0
0
3
6
9
CO_2 Production (nmoles)
Rate of CO_2 Production (nmoles·min^{-1})
Tissue Content Per Flask (mg wet wgt.)

Table 1: The effects of freezing and thawing, triton X-100 on PDHa and PDHt, and the effect of NaF on PDHa, for the $^{14}CO_2$ method of determination.

Additions	PDHa			PDHt		
	Con	Exp	*n*	Con	Exp	*n*
Freezing & Thawing	0.10 ±0.007	0.22 † ±0.021	3	0.72 ±0.422	2.23 ‡ ±0.110	3
Triton X-100	0.10 ±0.007	0.16 † ±0.012	3	0.91 ±0.342	1.26 ‡ ±0.070	6
NaF (% of PDHt)	41.0 ±11.0%	14.8 γ ±2.3%	20	NA	NA	NA

Data are mean ± SD. "Con" indicates control condition and "Exp" indicates the experimental condition. γ indicates that Exp is different from Con at $p<0.0002$. † indicates that Exp is different from Con at $p<0.007$. ‡ indicates that Exp is different from Con at $p<0.03$. NA indicates not applicable.

FIGURE 5: Linearity of PDHa assay with time for the acetylCoA method of determination. AcetylCoA production is plotted as a function of time for the PDHa assay. A 70 μl sample of a 30 volume human muscle homogenate was used for this analysis (2.3 mg of muscle tissue per tube). Assay reagents and conditions for PDHa determination were carried out as outlined in the methods section.

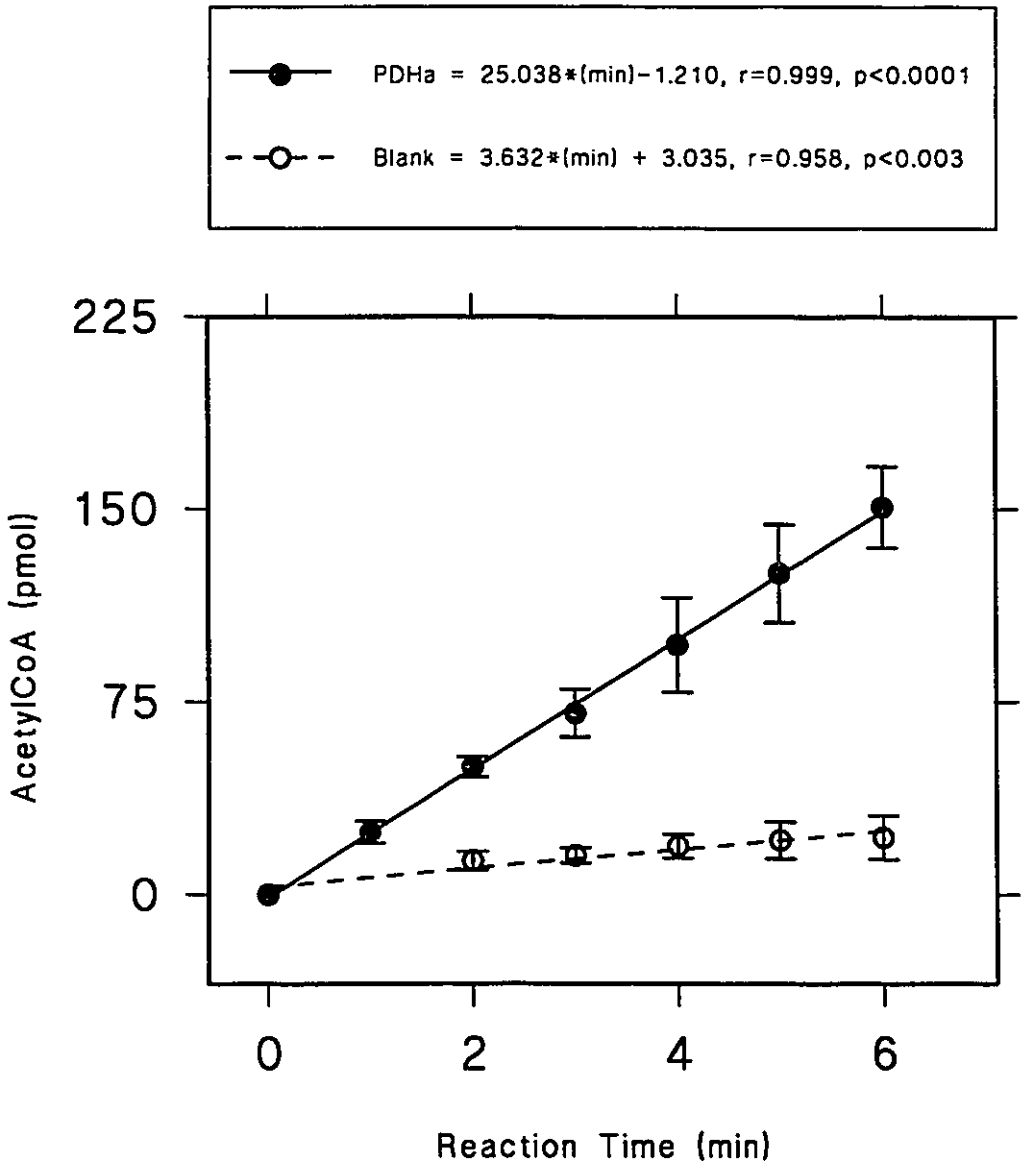
PDHa = 25.038*(min)-1.210, r=0.999, p<0.0001
Blank = 3.632*(min) + 3.035, r=0.958, p<0.003
225
150
75
0
AcetylCoA (pmol)
0
2
4
6
Reaction Time (min)

FIGURE 6: Linearity of PDHt assay with time for the acetylCoA method of determination. AcetylCoA production is plotted as a function of time for the PDHt assay. A 70 μl sample of a 30 volume human muscle homogenate was used for this analysis (1.7 mg of muscle tissue per tube). Assay reagents and conditions for PDHt determination were carried out as outlined in the methods section, with the incubation step.

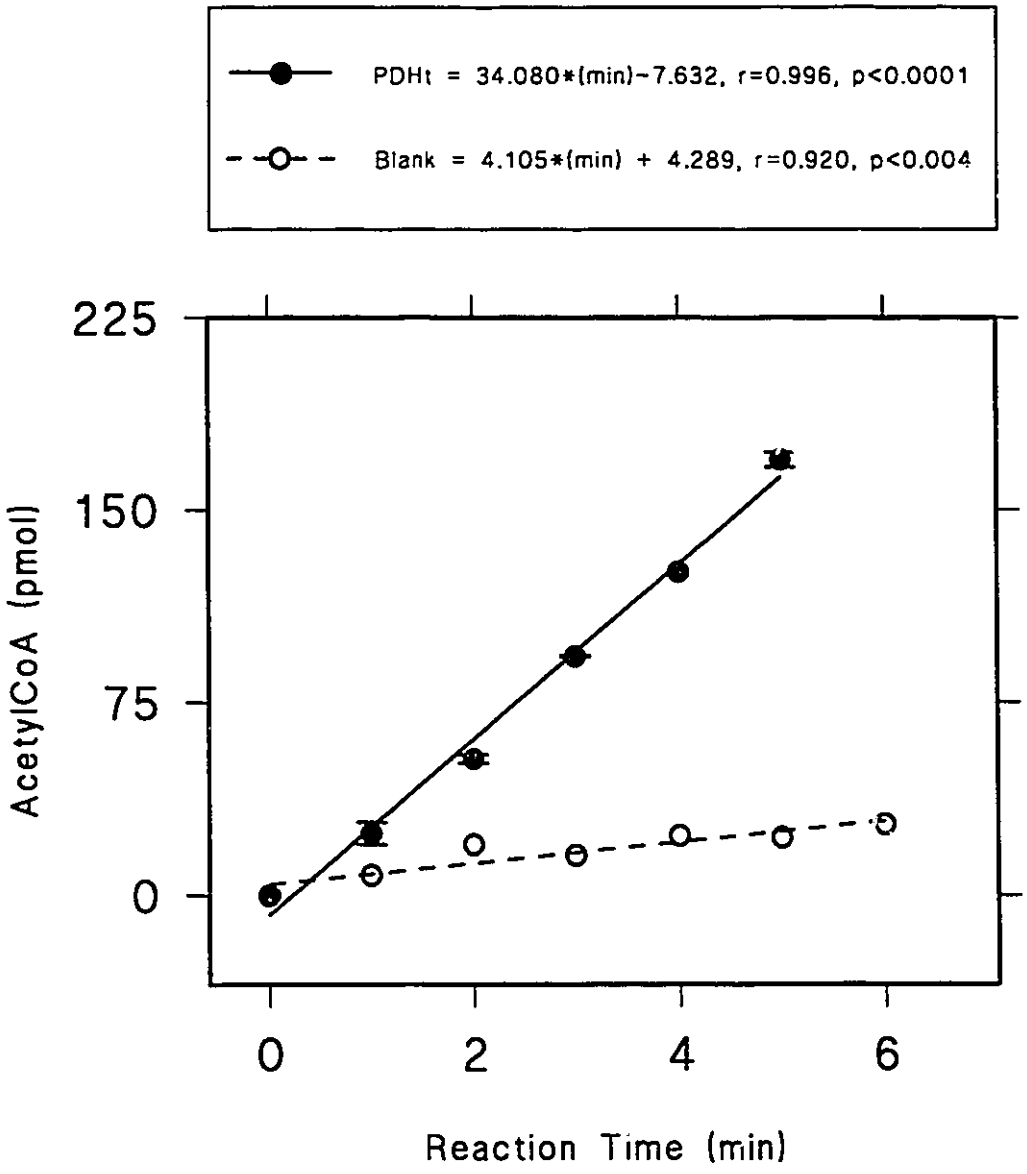
PDHt = 34.080*(min)-7.632, r=0.996, p<0.0001
Blank = 4.105*(min) + 4.289, r=0.920, p<0.004
225
150
75
0
AcetylCoA (pmol)
0
2
4
6
Reaction Time (min)

FIGURE 7: Linearity of PDHt assay with homogenate volume for the acetylCoA method of determination. AcetylCoA production is plotted as a function of muscle homogenate volume, for the PDHt assay. Tissue homogenate volume is expressed as μl of a 30 volume homogenate. 10, 20, 30, 50 and 70 μl samples were analyzed which were equivalent to 0.3, 0.6, 1.0, 1.6 and 2.3 mg of wet muscle tissue per tube, respectively.

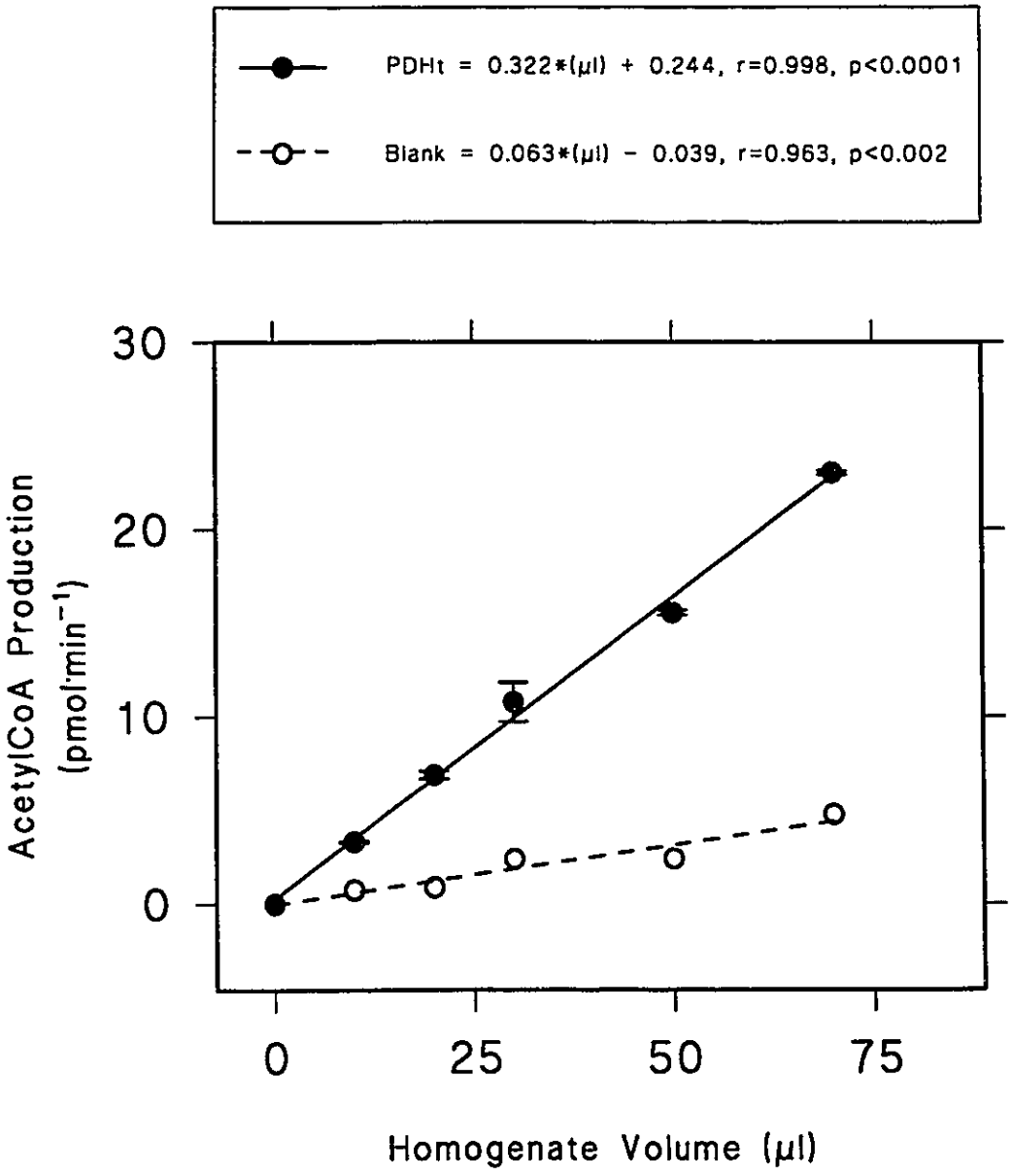
PDHt = 0.322*(µl) + 0.244, r=0.998, p<0.0001
Blank = 0.063*(µl) - 0.039, r=0.963, p<0.002
30
20
10
0
0
25
50
75
AcetylCoA Production
(pmol*min⁻¹)
Homogenate Volume (µl)

Table 2: Characterization of variation, accuracy and precision for the $^{14}CO_2$ method of PDHa and PDHt determination. 19 volume rat muscle homogenates were used for these analyses.

Measures	PDHa			PDHt		
	Mean	SD	*n*	Mean	SD	*n*
CV_{Sample}	19.3% †	13.94%	20	16.6% ‡	11.77%	20
CV_{Day}	21.9% ‡	12.30%	18	21.0% ‡	11.01%	32
Accuracy	0.0578 ‡	0.0387	20	0.3097 ‡	0.2497	19
Precision	0.0768	0.0680	21	0.5890 ‡	0.4890	20

Data are mean ± SD. CV_{Sample} indicates coefficient of variation for repeated duplicate measures on the same day. CV_{Day} indicates day-to-day variation of measures for the same muscle sample. † indicates that the $^{14}CO_2$ method is different from the acetylCoA method of PDHa and PDHt determination at $p<0.002$. ‡ indicates that the $^{14}CO_2$ method is different from the acetylCoA method of PDHa and PDHt determination at $p<0.0005$.

Table 3: Characterization of variation, accuracy and precision for the acetylCoA method of PDHa and PDHt determination. 30 volume human muscle homogenates were utilized for these analyses.

Measures	PDHa			PDHt		
	Mean	SD	*n*	Mean	SD	*n*
CV_{Sample}	6.7%†	3.98%	7	4.6%‡	2.91%	12
CV_{Day}	7.3%‡	5.13%	11	10.3%‡	7.87%	16
Accuracy	0.0033‡	0.0014	3	0.0024‡	0.0014	11
Precision	0.0493	0.0776	7	0.0669‡	0.0994	23

Data are mean ± SD. CV_{Sample} indicates coefficient of variation for repeated duplicate measures on the same day. CV_{Day} indicates day-to-day variation of measures for the same muscle sample. † indicates that the $^{14}CO_2$ method is different from the acetylCoA method of PDHa and PDHt determination at $p<0.002$. ‡ indicates that the $^{14}CO_2$ method is different from the acetylCoA method of PDHa and PDHt determination at $p<0.0005$.

6.5 *DISCUSSION*

The purpose of the present work was to improve a radiometric method of PDHa and PDHt determination that was previously used for measurement in tissue extracts (3,4,6,14,15,16,29,31,35,36,37,39,40) and to characterize its utility for measurement in skeletal muscle. This method relies on detection of the rate of $^{14}CO_2$ liberated from Na-1-^{14}C-pyruvate. Unfortunately, Na-1-^{14}C-pyruvate is very labile in solution and this method gives highly variable results. In addition, other reports of PDHa and PDHt measurements made in human skeletal muscle using this assay (14,27,29,37,39,40) have reported low values when compared to another radiometric method (9) which measures acetylCoA production. PDHt measures obtained using the $^{14}CO_2$ method were typically 3-10 fold lower than measures obtained using the acetylCoA method.

Therefore, in pursuit of a more reliable method of PDHa and PDHt determination the $^{14}CO_2$ method of PDHc determination was compared to an alternative method that has recently been developed for measurement in skeletal muscle extracts. Characterization of these two methods individually, delineated the parameters within which each can be reliably used. In addition, a direct comparison of the variability, accuracy and precision of the two methods, indicates that the newer method which employs radiometric measurement of acetylCoA is a more reliable method of both PDHa and PDHt determination.

6.5.1 *$^{14}CO_2$ Method of PDHa and PDHt Determination*

6.5.1.1 *Pyruvate Requirement for the $^{14}CO_2$ Method of PDHt Determination*

Previous reports of PDHa and particularly PDHt measurements in human (14,27,29,37,39,40) and rat (19) skeletal muscle, using the $^{14}CO_2$ collection method, have

typically reported very low values. Similarly, when initial measurements were made by the author, using this method, comparably low values were also obtained. This suggested that in its present form, one or more of the substrates were limiting. Since the K_m's of CoASH, NAD^+ and TPP for isolated PDHc preparations are approximately 36 fold lower than the K_m of pyruvate for PDHa, the pyruvate requirements were examined. It was hypothesized that insufficient pyruvate availability was the source of the limitation for measurement of PDHa and PDHt activities. Moreover, since PDHt represents the maximal activity of this enzyme, it was only necessary to examine pyruvate requirements under conditions of maximal transformation.

The apparent K_m for pyruvate using 200 μl of a 19 volume muscle homogenate was approximately 1 mM (Figure 1). Thus, to ensure that pyruvate is saturating and does not limit the maximal rate of catalytic activity, the pyruvate concentration for this large volume of homogenate should be 10 times the value of the K_m (ie. 10 mM). This finding suggests that the low values previously reported by others (14,19,27,29,37,39,40) may be the result of insufficient pyruvate. Previous reports have used pyruvate concentrations between 0.3 and 1.0 mM (14,19,27,29,37,39,40), with a fraction of this existing as 1-^{14}C-Na-pyruvate. However, the present data indicate that under similar conditions the appropriate concentration of pyruvate is 10 fold higher (ie. 10 mM). Still, others have used smaller volumes to determine PDHa and PDHt but have experienced the same problem as they allowed the reaction to proceed for 20-30 minutes (37). This also results in a limitation to PDHc determination as the concentration of pyruvate is not sufficient to support maximal activity for this period of time. The longer reaction time may also lead to inaccurate measurements due to insufficient concentrations of CoASH and NAD.

When the $^{14}CO_2$ method was previously employed to measure PDHa and PDHt in skeletal muscle (14,37,39,40), it was applied as previously described for measurement in human fibroblasts (3,6,15,16,31), platelets (4,13,15) and adipose tissue (35,36). However, the PDHc content in skeletal muscle is at least ten fold greater which could account for the limitation if applied directly to skeletal muscle without modification. Instead, if the sample volume had been limited to 20 μl of muscle homogenate and the reaction time had been limited to 2-3 minutes, previous measures probably would not have been in error.

It is recommended that the method of PDHc determination described here should be used with a pyruvate concentration of 10 mM. In addition, it would be advisable to limit the amount of tissue in each flask to 5.0 mg (wet wgt.) or less. This could best be achieved by preparing a 30-40 volume homogenate and limiting the sample volume to less than 150-200 μl, respectively. While this will not eliminate the variability encountered with this particular assay, it will at least remove any further limitation to the accuracy of PDHa and PDHt measurements.

Alternatively, when sample size is limited to 5-15 mg, it is advisable to prepare a 30-40 volume homogenate and limit the sample volume to 20-30 μl. Under these conditions, it is also possible to decrease the pyruvate concentration to ~1 mM. In addition, to reduce counting time, the proportion of labelled-to-unlabelled pyruvate can also be increased.

6.5.1.2 Capacity for CO_2 Recovery for the $^{14}CO_2$ Method of PDHa and PDHt Determination

Another possible limitation to this assay system was the collection of CO_2 after the reaction was stopped by addition of the acid reagent. The maximum capacity for CO_2 entrapment in 200 μl of 1.0 M hyamine hydroxide contained in the center well was 200

μmoles. Since the amount of CO_2 produced is not likely to exceed 100 nmol in 2 min, then it is not likely that the capacity for CO_2 absorption by hyamine hydroxide would be approached. However, the recovery of the CO_2 produced is also dependent on the rate of its reaction with the hyamine hydroxide and its solubility in the assay solution. Therefore, it was necessary to examine CO_2 recovery in the current assay system to determine if all of the CO_2 could be recovered in the 1 hour incubation period following acidification (Figures 2 & 3). While the present data do not characterize the kinetics of the reaction of CO_2 with the hyamine hydroxide, they do at least confirm that all of the CO_2 can be recovered in the hyamine hydroxide within the 1 hour period (Figure 2) and that no radioactivity remains in the assay mixture (Figure 3). Thus, the present data illustrate that recovery of a volume of CO_2, which far exceeds the volume that can be generated in the same period by a sample of muscle homogenate, can be recovered by this method in only 1 hour.

6.5.1.3 Linearity of the Assay With Homogenate Volume

CO_2 production increased as a linear function of homogenate volume, as the sample volume was varied from 50 to 200 μl in 50 μl increments (Figure 4). The linear nature of CO_2 production for the PDHa and PDHt assays suggest that over the 2 min reaction time employed, there was no limitation to measurement of PDHa or PDHt. A limitation to measurement of either of these two assays would have resulted in a lower relative rate of CO_2 production at the larger homogenate volumes. However the catalytic activity was exactly the same for each volume of muscle homogenate examined. For PDHa, the measured catalytic activity was ~0.9 mmol·min^{-1}·kg ww^{-1} at all four homogenate volumes. Similarly, for PDHt, the measured catalytic activity was ~2.3 mmol·min^{-1}·kg ww^{-1} at all four homogenate

volumes, which is similar to the calculated V_{max} for this tissue as illustrated in Figure 1.

The present data demonstrate that this reaction was not limiting over a 2 min duration. However, with the current data it is not possible to estimate at what time point the substrates become limiting. If this particular assay system were to be utilized beyond 2 min, PDHt should first be characterized as a function of time, using a 200 μl sample of a 19 volume muscle homogenate. Thereafter, it is only necessary to ensure that the proportion of homogenate-to-pyruvate present in the assay is not increased, although a much smaller homogenate volume could be used.

Given that biopsy samples from human skeletal muscle are very small and often must be used for multiple analyses, this method could be further down-scaled to use one-tenth of the homogenate volume. As described above, a similar result could be obtained by increasing the dilution of the sample by preparing a 30-40 volume homogenate and reducing the sample volume to as low as 20 μl. At the lower sample volume it would also be possible to decrease the unlabelled pyruvate concentration to ~1 mM. This would increase the specific activity of the pyruvate in the assay and allow measurement of PDHa and PDHt on samples that vary in weight from 5-15 mg, and place it in the range of sensitivity that currently exists for the acetylCoA method (9).

6.5.1.4 Effects of Freezing and Thawing, Triton X-100 and NaF

To further improve the extraction of PDHc and thus improve PDHa and PDHt measures, the effects of freezing and thawing, and adding a detergent (Triton X-100) to the homogenization procedure were examined. It was hypothesized that freezing and thawing the homogenate would rupture the mitochondrial membrane and the presence of Triton X-100

in HB_{active} and HB_{total} would prevent the re-formation of membrane vesicles in solution (10). Collectively, these additions to the homogenizing procedure should increase the solubility of the PDHc complex, allowing unrestricted access to all reagents and result in greater activities of PDHa and PDHt. As the data in Table 1 illustrate, both PDHa and PDHt increased as expected. Thus, freezing and thawing and inclusion of Triton X-100 to the homogenizing buffer appeared to have improved the extraction of PDHc during homogenization.

Homogenization of a muscle sample is associated with increased release of endogenous Ca^{2+} from the sarcoplasmic reticulum which could potentially result in activation of PDH-phosphatase, inhibition of PDH-kinase and artificial elevation of PDHa *in vitro*. Therefore, to control for this, 50 mM NaF was added to the homogenizing buffer. The presence of NaF in the homogenizing procedure resulted in a 3 fold decrease in PDHa measures (Table 1) and is consistent with previous reports which have shown that a concentration of 50 mM is sufficient to inhibit PDHc transformation in vitro (9,17,31). The apparent K_i of NaF for PDH-phosphatase is 0.8 mM, in the presence of 10 mM Mg^{2+} (17). Thus, the high concentration of NaF employed for PDHa determination in both assay methods should have saturated PDH-phosphatase and inhibited Ca^{2+} induced transformation of PDHb to PDHa. The lower PDHa observed after NaF treatment indicates that the release of endogenous Ca^{2+} during homogenization is significant and must be controlled. Moreover, the concentration of NaF employed in the present work was successful at preventing increased PDH-phosphatase activity due to Ca^{2+} release during the homogenization procedure.

6.5.1.5 Determination of PDHt Using the $^{14}CO_2$ Method

In the present study, 10 mM Ca^{2+} and 10 mM Mg^{2+} were used to activate PDH-phosphatase and inhibit PDH-kinase. In addition, glucose and hexokinase were used to deplete the homogenate of ATP, thus removing any available substrate for the kinase and further ensuring inhibition of PDH-kinase activity. These additional reagents appear to have worked as previously described (5,9,39,40), since PDHt measures were approximately 2.5 fold greater than PDHa in the absence of NaF and 6.7 fold greater than PDHa measured in the presence of NaF.

6.5.1.6 Source of Variability in the $^{14}CO_2$ Method of PDHa and PDHt Determination

The results obtained from the $^{14}CO_2$ method of PDHa and PDHt determination were highly variable. It was not uncommon for the SD of replicates to be as large as 25-50% of the mean sample value and for blank values to be as a high as 50% of the sample values. Most of the variation in the CO_2 method was probably the result of nonspecific spontaneous decarboxylation of the labelled pyruvate which has previously been described (33,34,38), where reported blank values have been shown to be up to 50% of the sample values (34).

In the present study high blank values occurred despite attempts to circumvent much of the nonspecific spontaneous decarboxylation of the labelled pyruvate. The labelled pyruvate was frozen and stored *in vacuo* over desiccant and dissolved in a solution of 0.004 N HCl only on the day of the assay. In the present study this step was used to lessen the degree of decarboxylation since pyruvate is most stable in its acid form (33,38). This step also should have prevented the formation of aldol condensation products that can form in solution and contribute to disappearance of pyruvate (38).

Other means of reducing nonspecific spontaneous decarboxylation of the pyruvate label have also been suggested but were not examined in the present study. Schofield (30) showed that lactate dehydrogenase (LDH) could be added to the assay mixture with 1-^{14}C-Na-lactate, allowing labelled pyruvate to be generated through LDH. Using this approach, much of the nonspecific spontaneous decarboxylation was eliminated since lactate is stabile in solution. In addition, others have reported limited success in reducing the degree of nonspecific spontaneous decarboxylation by: 1) storing the 1-^{14}C-Na-pyruvate *in vacuo* at -20 °C under phosphorous pentoxide (4), 2) pre-incubating the label under hyamine hydroxide before use (31,30) and 3) inclusion of 5% rat serum in the incubation mixture (18).

6.5.2 AcetylCoA Method of PDHa and PDHt Determination

6.5.2.1 Linearity of PDHa and PDHt Assays With Time and Concentration

The linear increase in the acetylCoA production as a function of time (Figure 5) and homogenate volume (Figure 6) indicates that there were no limitations to the measurements of either PDHa or PDHt. In addition, these data delineate the limits within which these assays must be used. As indicated in the original publication describing this method (9), the PDHa and PDHt assays can be reliably used within in a range of homogenate volumes from 10-70 μl over a 3 min duration. However, for a 70 μl sample volume the PDHt assay reaches its limit of reliability after 5 minutes. If PDHa is maximally transformed, such as during maximal muscular contractions, the assay for this fraction of PDHc could also result in a limitation after 5 minutes.

Therefore to circumvent the possibility of a limitation arising, when using this assay, it is important to use a 30 μl sample volume and to sample aliquots at 1, 2 and 3 minutes but

not after 3 minutes. In addition, since mitochondrial content of skeletal muscle is variable between species and between fiber types (12), the concentrations of critical substrates can also be further increased to reduce the possibility that one or more of the reagents could become limiting. The concentrations of substrates should be increased as follows: pyruvate to 1.0 mM, NAD^+ to 3.0 mM, CoASH to 1.0 mM and TPP to 1.0 mM. These alterations to the acetylCoA method of PDHa and PDHt determination will ensure that a limitation does not arise due to differences in mitochondrial content between subjects and fiber types. These changes should also allow the direct application of this method of PDHa and PDHt analysis to skeletal muscle of different species.

6.5.2.2 Determination of PDHt Using the AcetylCoA Method

The measurement of PDHt using the acetylCoA method of determination, as originally described (9), resulted in low values in samples obtained from a resting skeletal muscle biopsy (Chapter 2). The reason for the incomplete transformation was not obvious, since several possibilities existed. First, it was possible that the concentrations of Ca^{2+} or Mg^{2+} were not saturating to ensure maximal PDH-phosphatase activity and inhibition of PDH-kinase activity. However, increasing the concentrations to 10 mM and 25 mM, respectively, did not completely resolve the problem.

A second possibility was that since Triton X-100 was not present during the initial homogenization and incubation step, access of the reagents to PDHc were limited due to the reformation of smaller membrane vesicles after freezing and thawing, resulting in low PDHt values. Therefore, the preincubation step was omitted and the reagents responsible for activating PDH-phosphatase (ie. Ca^{2+} (11,20), Mg^{2+} (11,20)) and inhibiting PDH-kinase (ie.

DCA (42,41), glucose and hexokinase (9,39,40)) were added directly to the homogenizing buffer with Triton X-100. These alterations to the homogenization and treatment of the muscle sample gave similar results to the method that employed the preincubation step. However, under these conditions, incubation at 37 °C resulted in a loss of PDHt activity with time. The reason for this is unclear but it may be related to the very vigorous effects of Triton X-100 on PDHc, causing it to be destroyed at high temperatures. When PDHt was determined shortly after homogenization of the sample and kept on ice, the activity was not lost and was similar to values obtained when the incubation step is included.

Since PDHt decreased when the sample was incubated at 37 °C in the presence of Triton X-100, PDHt was examined as a function of incubation time on ice. Only one such experiment was completed but it revealed that PDHt did increase over time and was stabile between 30 min and 2 hours.

Lastly, in other experiments to increase PDHt measures from resting muscle biopsies, a white potato phosphatase (2) was added to the assay and the preincubation step was employed as originally described (?). This phosphatase was chosen since it is commercially available and, like PDH-phosphatase, is specific for mono-phosphate esters located on serine residues. Initial experiments did not show an increase in PDHt but in fact there was a decrease in PDHt measures. The most likely explanation was that the high concentration of ammonium sulphate, in which the potato phosphatase was suspended, caused an increase in PDH-kinase activity and lower PDHt, since ammonium is a potent activator of PDH-kinase (23,26,28). Unfortunately, dialysis of the phosphatase preparation before use did not result in greater PDHt measures when the sample was preincubated with the phosphatase. This appears to be related to the extremely labile nature of this particular phosphatase, which

requires the presence of high concentrations of ammonium sulphate as a stabilizing reagent (2). Even if the these problems could be overcome, the specific activity of this preparation is low compared to PDH-phosphatase (32). Therefore, large quantities of this particular preparation would be required to obtain the necessary activity to completely transform PDHb to PDHa. Since the phosphatase is currently expensive, the cost of such large quantities would make this approach prohibitive.

A more viable alternative would be to isolate PDH-phosphatase from bovine or porcine heart as previously described (31,32). This preparation is also labile but could be reliably used with two modifications. First, this particular phosphatase preparation is contaminated with PDHc (31,32), which would artificially elevate the measures of PDHt. This could easily be corrected by subtracting the PDHc activity in blanks, containing only the phosphatase preparation. If this particular method is employed, it is also imperative that the phosphatase not be separated from the PDHc until just before it is required in the assay. This is because the phosphatase is highly labile and rapidly looses activity when stored separately from PDHc (31). This can be completed by following the isolation procedure previously described (32) up to but excluding the last step, whereupon it should be stored frozen at -20 °C. Isolation of PDH-phosphatase should be completed just before use in the PDHt assay.

An alternative that requires much less work and is more practical for PDHt determination in human muscle biopsies, is to simply use the average of PDHt measures from exercise samples above 60% $\dot{V}O_2max$. This is known to result in maximal transformation of PDHc (8,24,25).

6.5.3 Comparison of Two Radiometric Methods of PDHc Determination

Characterization of the variability of PDHa and PDHt determination by these two radiometric assay systems illustrates that the acetylCoA method was far better than the CO_2 method of PDHa and PDHt determination. The CV_{sample} for PDHa and PDHt were 2.9 and 3.6 fold greater for the CO_2 method as compared to the acetylCoA method of PDHc determination. Similarly, the CV for day-to-day variation of PDHa and PDHt measures of the same sample were 3 and 2 fold greater for the CO_2 method as compared to the acetylCoA method.

The variation observed for both assay methods were also expressed as accuracy and precision terms. The acetylCoA method was 20-100 fold more accurate and 2-10 fold more precise than the $^{14}CO_2$ method of PDHa and PDHt determination.

The variation observed in the acetylCoA method of PDHa and PDHt determination is similar to that originally reported by Constantin-Teodosiu *et al* (9) and is adequate for analytical purposes. The only draw back of this method of determination is that it requires two days to complete analyses of 8-10 biopsy samples, in duplicate. By comparison, the $^{14}CO_2$ method of determination has similar sensitivity but the same number of samples can be analyzed in only one day. However, since it is also necessary to run more blanks and more replicates for the $^{14}CO_2$ method, it may ultimately take a similar amount of time to get sufficiently accurate measures.

6.5.4 Summary and Conclusions

The present study examined and compared two radiometric methods of PDHa and PDHt determination in skeletal muscle. The $^{14}CO_2$ method of determination has been used extensively to study PDHa activity in skeletal muscle but has some inherent problems which have limited its accuracy and precision. As a result of the present analysis, several modifications were made to improve the accuracy of measurement and suggestions were made to further improve the sensitivity of this assay system. In addition, the limits within which this assay system can be reliably used were defined and should facilitate its correct application for PDHa and PDHt analysis in skeletal muscle. While precautions were also taken to reduce the major source of variability in this assay system, it remains highly variable.

In contrast, the acetylCoA method of determination has just recently been adapted for use in small muscle samples. The present study has also examined the limitations of this assay system. The boundaries within which it can be reliably used were found to be as originally reported (9). The present analysis also illustrates that the PDHa and PDHt assays can be reliably run for 5 minutes. It is also suggested that increasing the concentration of substrates will facilitate use of this method for analysis of skeletal muscle samples from other species that have a higher mitochondrial content.

A comparison of the acetylCoA method of PDHa and PDHt determination with the $^{14}CO_2$ method indicates that the former method varied less within the same muscle sample on a given day and varied less from day-to-day. Calculation of accuracy and precision for the two methods indicate that the acetylCoA method is a far more accurate and precise method of PDHa and PDHt determination.

6.6 References

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APPENDIX B

Regulation of fat-carbohydrate interaction in skeletal muscle during intense aerobic cycling

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Dyck, D. J., C. T. Putman, G. J. F. Heigenhauser, E. Hultman, and L. L. Spriet. Regulation of fat-carbohydrate interaction in skeletal muscle during intense aerobic cycling. *Am. J. Physiol.* 265 (*Endocrinol. Metab.* 28): E852-E859, 1993. —Six male subjects received either a saline (control) or Intralipid infusion during 30 min rest and 15 min cycling at 85% maximal O_2 uptake ($\dot{V}O_{2\,max}$) to examine the regulation of fat-carbohydrate interaction (glucose-fatty acid cycle) in skeletal muscle. Muscle biopsies were sampled immediately before and at 3 and 15 min of exercise in both trials. A muscle biopsy was also taken at −30 min rest in the Intralipid trial. Intralipid infusion significantly elevated plasma free fatty acids above control during rest (0.21 ± 0.04 to 0.94 ± 0.09 mM) and exercise (5 min: 1.27 ± 0.15 mM; 15 min: 1.42 ± 0.13 mM). Muscle glycogen degradation was significantly lower in the Intralipid trial (109.7 ± 29.3 vs. 194.7 ± 32.1 mmol/kg dry muscle). Muscle lactate accumulation after 15 min was similar in both trials (control, 60.7 ± 12.2 and Intralipid, 60.9 ± 12.4 mmol/kg dry muscle). Muscle citrate increased at rest during Intralipid (0.32 ± 0.06 to 0.58 ± 0.06 mmol/kg dry muscle) but was not different between trials at 3 min (control, 0.73 ± 0.07 and Intralipid, 0.68 ± 0.06 mmol/kg dry muscle) and 15 min of cycling. Resting acetyl-CoA was unaffected by Intralipid and increased similarly in both trials at 3 min of cycling (control, 59.0 ± 10.3 and Intralipid, 50.7 ± 13.6 μmol/kg dry muscle) and remained unchanged at 15 min. Pyruvate dehydrogenase activity increased five- to sevenfold during exercise and was similar in both trials (15 min: control, 2.42 ± 0.30 and Intralipid, 2.79 ± 0.41 mmol·min^{-1}·kg wet wt^{-1}). The results suggest that, during 15 min of cycling at 85% $\dot{V}O_{2\,max}$, muscle citrate and acetyl-CoA are not responsible for reduced glycogenolysis in the Intralipid trial as proposed in the classic glucose-fatty acid cycle. It is suggested that regulation exists at the level of muscle glycogen phosphorylase.

Intralipid; muscle glycogenolysis; pyruvate dehydrogenase; phosphofructokinase; citrate; acetyl-coenzyme A; glucose-fatty acid cycle

THE CONCEPT OF the glucose-fatty acid cycle was proposed in the 1960s by Randle and co-workers (16, 17, 31) while studying rat heart and diaphragm muscles. It was postulated that enhanced fat oxidation elevates muscle citrate and acetyl-CoA contents, resulting in the down-regulation of carbohydrate metabolism due to inhibition of the enzymes phosphofructokinase (PFK) and pyruvate dehydrogenase (PDH). This concept has been supported by in vitro studies (1, 13, 15) demonstrating regulation of these enzymes as classically proposed. However, since the studies by Randle and co-workers (16, 17, 31), there has been controversy regarding the existence of the glucose-fatty acid cycle in human skeletal muscle during exercise. The majority of studies have been concerned with the regulation of glucose uptake in the face of enhanced fat oxidation, whereas fewer studies have examined the aspect of glycogen utilization. Furthermore, there is a lack of information regarding the mechanisms by which fat-carbohydrate interaction occurs in skeletal muscle and whether the regulation is consistent with that proposed in the classic glucose-fatty acid cycle.

Several studies have investigated the effects of elevated plasma free fatty acids (FFA) on carbohydrate utilization in humans during fasting, fat feeding, and infusion of triglyceride emulsion at rest (2, 14, 27, 38, 40) and exercise (12, 19, 25, 27, 32). Studies using fasting or high-fat meals to elevate plasma FFA (12, 25, 27) have the disadvantage of altering substrate and hormone levels and possibly confounding the results. Infusion of a triglyceride emulsion (Intralipid) has the advantage of acutely elevating arterial FFA levels without additional substrate or hormonal changes. Intralipid infusion studies in resting humans (2, 14, 38, 40) have clearly demonstrated a reduced glucose utilization subsequent to elevated plasma FFA. However, few studies have investigated the effects of Intralipid infusion during exercise, and these studies have yielded inconsistent results. Ravussin et al. (32) were unable to demonstrate a lower respiratory exchange ratio with Intralipid during prolonged low-intensity exercise. Hargreaves et al. (19) were unable to demonstrate reduced muscle glycogen utilization during dynamic knee extensions with Intralipid infusion but demonstrated a significant reduction in thigh glucose uptake during rest, exercise, and recovery.

Although several studies have examined various aspects of the glucose-fatty acid cycle during rest and exercise, there has not been a detailed examination in exercising humans. A previous experiment in our laboratory demonstrated significant sparing of muscle glycogen during the initial 15 min of intense aerobic exercise subsequent to caffeine ingestion (37). Caffeine may enhance FFA mobilization via increases in plasma epinephrine concentration. The measurements of citrate, acetyl-CoA, and acetyl-CoA-to-CoA ratio in muscle were not consistent with the classic regulation of the cycle. However, the muscle measurements were not made before 15 min, which was the period during which the glycogen sparing occurred. PDH activity was also not measured. Therefore, the initial purpose of the present study was to determine whether enhanced exogenous FFA availability (Intralipid and heparin infusion) would spare muscle glycogen during 15 min of intense aerobic cycling, independent of hormonal and substrate changes. Second, if muscle glycogen was spared in the presence of elevated FFA during exercise, we measured muscle citrate, acetyl-CoA, and PDH activity to determine if the regulation of fat-carbohydrate interaction was consistent with the classically proposed theory.

METHODS

Subjects. Six healthy males of varied training status, mean age 27.0 (range, 21-31) yr, and weight 74.9 (range, 60.2-84.2) kg volunteered for the experiment. Two subjects were classified as well trained, two as moderately trained, and two as untrained (see Table 4). Subjects were informed of the experimental procedure and potential risks both verbally and in writing and gave consent. The experiment was approved by the University Ethics Committee.

Preexperimental protocol. All participants reported to the laboratory before the onset of experiments to perform an incremental maximal O_2 uptake ($\dot{V}O_{2\,max}$) test on a cycle ergometer. The mean $\dot{V}O_{2\,max}$ for the group was 3.97 ± 0.31 (SE) l/min. Subjects reported to the laboratory on a second occasion before the experiment for a practice trial at a power output designed to elicit ~85% $\dot{V}O_{2\,max}$.

For the actual experiment, each subject reported at the same time of day on two occasions, separated by 7-10 days. Subjects cycled for 15 min at ~85% $\dot{V}O_{2\,max}$, while receiving, in randomized order, an infusion of either saline (control) or a triglyceride emulsion of soybean oil (20% Intralipid). Identical pan balance weights, cadence, and seat height were used to ensure that the power outputs were identical between trials.

Subjects were instructed to maintain normal training and dietary patterns during the study. Analysis of the diets indicated a moderately high percentage of carbohydrate intake (57% of total caloric consumption), ensuring adequate liver, muscle, and blood carbohydrate stores before each trial. Subjects refrained from caffeine consumption and intense physical activity 48 h before each trial and reported to the laboratory having eaten a meal high in carbohydrates 2-4 h before the experiment.

Experimental protocol. Subjects rested in the supine position, and a catheter was placed percutaneously in an antecubital vein. Catheter patency was maintained with a saline drip (~100-175 ml/h), and a resting blood sample was obtained at −30 min (Fig. 1). In the control trial, subjects remained resting for 30 min while receiving a saline infusion. At the end of the rest period, a final blood sample and a muscle biopsy from vastus lateralis were taken. In the Intralipid trial, a second catheter was placed in the contralateral antecubital vein for infusion of Intralipid at a rate of 102 ml/h. An initial resting muscle biopsy and blood sample were taken at −30 min just before the initiation of Intralipid infusion. Intralipid infusion was started, and 1,000 U heparin (1-ml bolus) were immediately administered. Blood samples were taken at −20, −10, and 0 min at rest. Additional heparin (500 U) was administered at −15 and 0 min during rest, and a second resting muscle biopsy was taken at 0 min (Fig. 1).

Subjects then cycled at ~85% $\dot{V}O_{2\,max}$ for 15 min (Fig. 1). The Intralipid infusion rate was increased to 180 ml/h during cycling. Muscle biopsies were taken at 3 and 15 min of cycling in both trials. Blood samples were taken while the subject was exercising at 1.5, 5, 10, and 14 min of exercise. Expired gas samples were collected for 1-min periods in Douglas bags starting at 6 and 11 min of exercise. A total of 2,000 U heparin was administered during the Intralipid trial. Although this is less than the dose given to promote anticoagulation, we guarded against the possibility of excessive bleeding during biopsy sampling in several ways; subjects refrained from using aspirin during the week preceding the trial, pressure was maintained on the resting biopsy site during the 30-min resting infusion, the exercise component of the experiment was limited to 15 min, and the leg was bandaged for several hours after the trial.

Analyses. Expired gas samples were analyzed for O_2 and CO_2 content with an Applied Electrochemical S-3A O_2 analyzer and a Sensor Medics LB-2 CO_2 detector. Expired volumes were measured with a Parkinson-Cowan volumeter calibrated with a Tissot spirometer.

Muscle biopsies were immediately frozen (5-7 s from the insertion of the needle) in liquid N_2. A small piece of each biopsy (15-20 mg) was removed under liquid N_2 and used for the determination of PDH activity (9). The remainder of the sample was freeze-dried, dissected free of nonmuscular elements, and powdered. Two aliquots (2-3 mg each) were used for the enzymatic determination of muscle glycogen (21) on the resting (0 min) and 15-min exercise biopsies. Approximately 10 mg of each sample were extracted in 0.5 M $HClO_4$ (1.0 mM EDTA) and neutralized with 2.2 M $KHCO_3$ for the determination of ATP, phosphocreatine (PCr), creatine, lactate, and citrate (3). The remaining muscle was extracted in identical fashion and analyzed for acetyl-CoA and acetylcarnitine, as outlined by Cederblad et al. (6).

The mean muscle wet-to-dry ratio (±SE) at rest was 4.04 ±

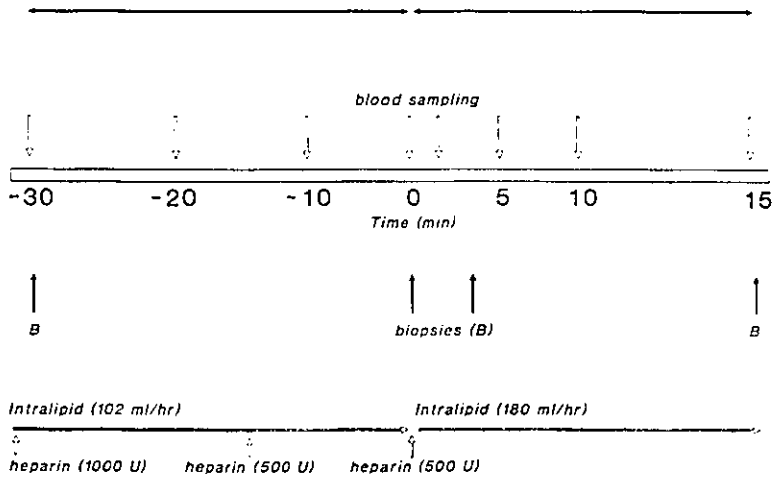


Fig. 1. Experimental protocol. $\dot{V}O_{2\,max}$, maximal O_2 uptake.

0.05 and increased to 4.50 ± 0.05 after 15 min of cycling. All muscle metabolites were expressed per kilogram dry muscle and were corrected to the highest total creatine content for an individual's biopsies. The mean total creatine was 110.2 ± 3.5 mmol/kg dry muscle.

PDH activity in the transformed or active form (PDHa) was measured in muscle homogenates, as outlined by Constantin-Teodosiu et al. (9), with the modification of increasing the concentration of NAD, coenzyme A (CoASH), and thiamine pyrophosphate to 3.0, 1.0, and 1.0 mM, respectively. Briefly, the reaction was initiated by the addition of pyruvate, and 200 μl homogenate aliquots were sampled at 1, 2, and 3 min. The reaction was terminated in each sample by adding 40 μl of 0.5 M $HClO_4$ and subsequently neutralized with 1.0 M K_2CO_3. Acetyl-CoA production was measured in each aliquot, as outlined by Cederblad et al. (6), and regressed against time to determine the reaction rate. PDHa was expressed as millimoles acetyl-CoA produced per minute per kilogram wet muscle. Total creatine was measured on the muscle homogenate to correct for variations in nonmuscular elements of the biopsies.

Blood samples were divided into two aliquots (5 ml into a sodium-heparinized tube for metabolite and hemoglobin measurements and 7 ml into a second sodium-heparinized tube for catecholamine determination). A 200-μl aliquot of heparinized blood was added to 1.0 ml of 0.6 N $HClO_4$, and the supernatant was used for fluorometric determination of whole blood glycerol, lactate, and glucose (3). A 1-ml aliquot of heparinized blood was analyzed for hemoglobin content and O_2 saturation with a hemoximeter IL 482 (Instrument Laboratory, Lexington, MA). Heparinized blood collected for catecholamine determination was mixed with 120 μl 0.24 M ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA)-reduced glutathione and analyzed by high-performance liquid chromatography (Waters), as described by Weiker et al. (41).

For valid plasma FFA determination after heparin administration, in vitro lipolysis due to the presence of lipoprotein lipase (LPL) must be prevented. A 1.5-ml aliquot of heparinized blood was added to an Eppendorf tube containing 30 μl 0.2 M EGTA to prevent in vitro lipolysis and was centrifuged. Plasma was stored at −20°C and analyzed with an enzymatic colorimetric technique (Wako NEFA C kit; Wako Chemicals, Dallas, TX). EGTA inactivation of LPL was reported to be as effective as an alternative method of LPL inactivation involving the addition of 200 μl 5 M NaCl to 800 μl plasma and heating at 56°C for 30 min (19). Our own comparison of the two techniques demonstrated that initial FFA concentrations were similar but that repeated bouts of freezing and thawing resulted in elevation of FFA in the EGTA-treated samples only. Therefore, all FFA concentrations reported in the present study were determined after the initial thaw.

Statistics. All blood and muscle metabolites were compared between trials at a given time point using a paired *t* test. Statistical significance was accepted at $P \leq 0.05$. All data are expressed as means ± SE.

RESULTS

Oxygen uptake and heart rates were not significantly different between the control and Intralipid trials at 6 and 11 min of exercise (Table 1).

Intralipid infusion during 30 min of rest increased plasma FFA concentration from 0.21 ± 0.04 to 0.94 ± 0.09 mM (Fig. 2*A*). No change was observed at rest in the control trial. During exercise, FFA levels increased in the Intralipid trial to 1.27 ± 0.15 and 1.42 ± 0.13 mM at 5 and 15 min, respectively, and were unchanged in the control trial (Fig. 2*A*). Plasma FFA were significantly higher at all time points beyond −30 min during the Intralipid trial compared with control.

Table 1. *Oxygen uptake and heart rate during cycling*

	Time, min	
	6	11
Percentage of $Vo_{2\,max}$		
Control	86.9±2.5	90.8±2.6
Intralipid	85.9±2.0	89.0±3.7
Heart rate, beats/min		
Control	168±2	174±3
Intralipid	167±4	176±4

Values are means ± SE. $Vo_{2\,max}$, maximal O_2 uptake.

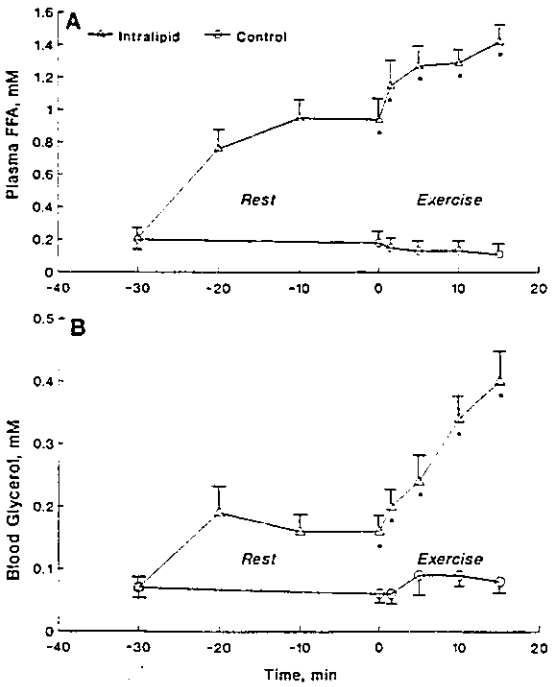


Fig. 2. Plasma free fatty acid (FFA) and blood glycerol concentrations at rest and during intense aerobic cycling with saline (control) or Intralipid infusion. *Significantly different from control.

Changes in blood glycerol were similar to the observed changes in plasma FFA (Fig. 2*B*). Intralipid infusion resulted in a significant elevation of resting blood glycerol in comparison with control from 0.07 ± 0.01 to 0.16 ± 0.02 mM and nearly a sixfold increase by 15 min of exercise (Fig. 2*B*).

There were no significant differences in blood lactate at rest or during exercise as a result of Intralipid infusion (Fig. 3). Blood glucose concentration was similar at −30 min in both trials but was significantly lower in the Intralipid trial just before exercise (3.5 ± 0.1 vs. 4.2 ± 0.3 mM; Fig. 3*B*).

Intralipid infusion had no effect on blood catecholamine levels during rest or exercise (Table 2). Epineph-

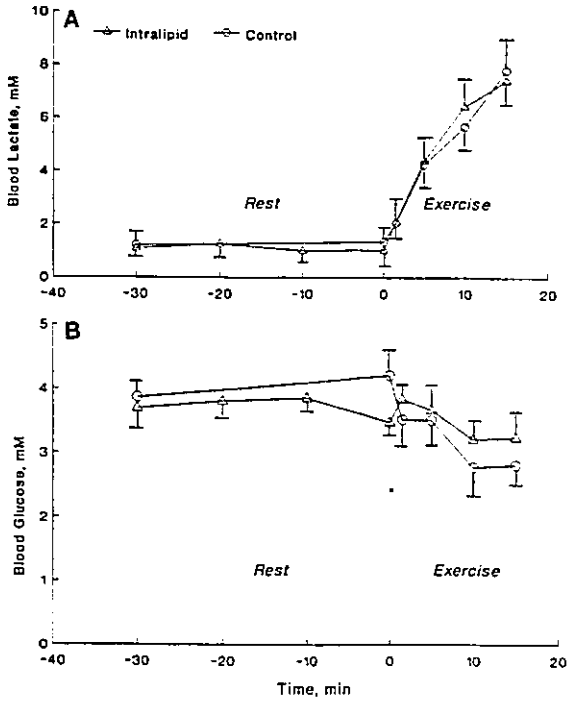


Fig. 3. Blood lactate and glucose concentrations at rest and during intense aerobic cycling with saline (control) or Intralipid infusion. *Significantly different from control.

Table 2. *Plasma epinephrine and norepinephrine concentrations at rest and during intense cycling*

Time, min	Epinephrine		Norepinephrine	
	Intralipid	Control	Intralipid	Control
−30	0.39±0.05	0.44±0.09	1.72±0.15	1.55±0.09
0	0.36±0.02	0.41±0.09	1.50±0.12	1.58±0.11
5	0.85±0.07	1.09±0.20	8.23±0.85	11.11±2.54
10	1.76±0.34	2.09±0.45	20.99±2.94	24.87±2.57

Values are means ± SE in nmol/l.

rine and norepinephrine concentrations progressively increased throughout exercise in both trials.

Muscle ATP content was unaffected by exercise and Intralipid infusion (Table 3). Muscle PCr decreased by 50–60% at 3 min of exercise in both trials. After 15 min

Table 3. *Muscle ATP and PCr contents at rest and during intense cycling*

Time, min	ATP		PCr	
	Intralipid	Control	Intralipid	Control
−30	26.5±0.6		79.0±2.0	
0	27.0±1.2	26.1±1.0	79.4±2.0	79.4±3.7
3	26.4±1.2	25.6±0.9	37.2±6.1	31.5±4.0
15	26.4±0.6	24.9±1.1	37.5±4.7*	24.9±3.6

Values are means ± SE in mmol/kg dry muscle. PCr, phosphocreatine. * Significantly different from control.

Table 4. *Individual muscle glycogen data before and after 15 min of intense cycling*

Subject	Intralipid			Control		
	0 min	15 min	Δ	0 min	15 min	Δ
1	793.3	669.7	123.6	840.8	548.9	291.9
2	429.1	348.7	80.4	425.4	301.7	123.7
3	340.9	255.5	85.4	260.4	129.9	130.5
4	829.6	582.3	247.3	635.4	341.5	293.9
5	250.3	175.0	75.3	287.4	107.0	180.4
6	367.5	321.5	46.0	326.5	178.6	147.9
Means	501.8	392.1*	109.7*	462.7	267.9	194.7
±SE	100.8	78.7	29.3	93.9	67.9	32.1

Values are expressed as mmol glucosyl units/kg dry muscle. Training status as follows: *subjects 1* and *4*, well trained; *subjects 2* and *5*, moderately trained; *subjects 3* and *6*, untrained. * Significantly different from control.

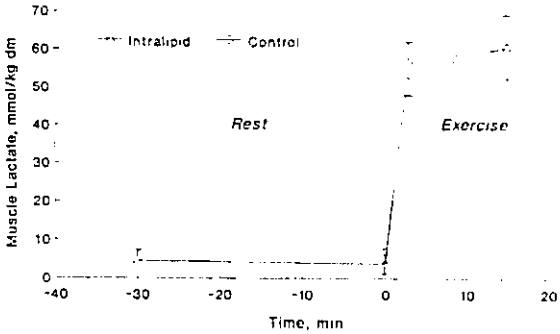


Fig. 4. Muscle lactate content at rest and accumulation during intense aerobic cycling with saline (control) and Intralipid infusion. dm, dry muscle.

of cycling, PCr content was significantly higher in the Intralipid trial vs. control.

Resting muscle glycogen contents were not significantly different between trials (Table 4). Muscle glycogen degradation during the Intralipid trial (109.7 ± 29.3 mmol/kg dry muscle) was significantly less than during the control trial (194.7 ± 32.1 mmol/kg dry muscle) and represented a 44% sparing of muscle glycogen. Muscle glycogen was also measured in three subjects in the −30-min resting biopsy to establish that there was no change in resting glycogen as a result of Intralipid infusion. The mean glycogen contents of the −30-min and 0-min resting biopsies were 545.9 ± 127.2 and 552.0 ± 141.2 mmol/kg dry muscle, respectively.

Intralipid infusion had no effect on resting muscle lactate or accumulation during exercise, despite the magnitude of glycogen sparing (Fig. 4). Muscle citrate content increased from 0.32 ± 0.06 to 0.58 mmol/kg dry muscle at rest during Intralipid infusion, a value significantly greater than the control resting citrate content (0.37 ± 0.08 mmol/kg dry muscle; Fig. 5). Muscle citrate continued to increase during exercise, but at 3 and 15 min there was no difference between trials.

Both muscle acetyl-CoA and acetylcarnitine were unaffected by Intralipid infusion at all time points (Fig. 6). Acetyl-CoA increased during the first 3 min of cycling (control, 59.0 ± 13.5 mmol/kg dry muscle; Intralipid, 50.7

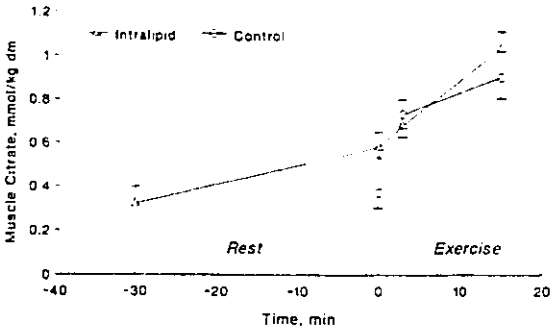
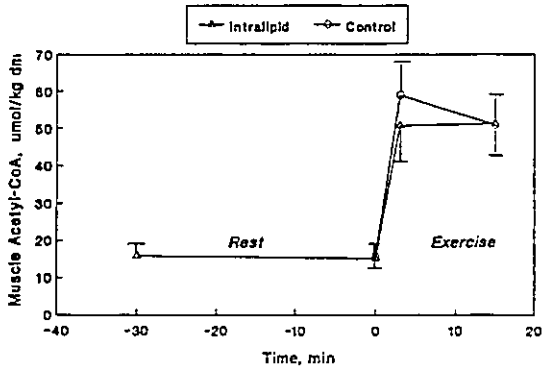
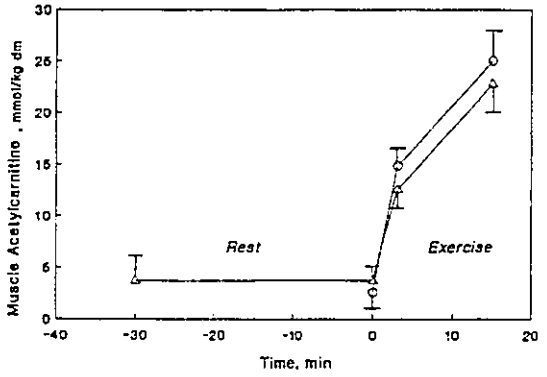


Fig. 5. Muscle citrate content at rest and accumulation during intense aerobic cycling with saline (control) and Intralipid infusion. *Significantly different from control.





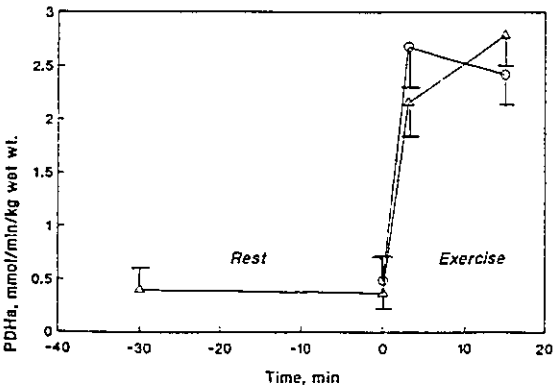


Fig. 6. Muscle acetyl-CoA and acetylcarnitine contents and transformation of pyruvate dehydrogenase (PDHa) at rest and during intense aerobic cycling with saline (control) and Intralipid infusion.

± 13.6 mmol/kg dry muscle) and was unchanged thereafter. After 3 and 15 min of exercise, acetylcarnitine increased to 14.9 ± 0.8 and 12.6 ± 0.7 mmol/kg dry muscle and 25.0 ± 3.5 and 22.8 ± 2.5 mmol/kg dry muscle in the control and Intralipid trials, respectively. PDHa was similar at 0 min rest in the Intralipid trial compared with control (0.37 ± 0.05 vs. 0.49 ± 0.08 mmol acetyl-CoA units·min^{-1}·kg wet wt^{-1}; Fig. 6). There were dramatic increases in PDHa by 3 min of exercise and no further changes in activity at 15 min. PDHa values were not statistically different between trials at 3 and 15 min.

DISCUSSION

The present study demonstrated that Intralipid and heparin infusion, resulting in elevated plasma FFA, produced a significant sparing of muscle glycogen during 15 min of intense aerobic cycling. The glycogen sparing that occurred with Intralipid infusion was unrelated to changes in muscle citrate and acetyl-CoA contents. These results demonstrate that the downregulation of glycogenolysis at 85% $\dot{V}O_{2\,max}$ in the presence of high FFA was not mediated as proposed in the classic glucose-fatty acid cycle. These findings were observed in all subjects, regardless of their training status and resting glycogen contents.

Regulation of fat-carbohydrate interaction. Several studies have used Intralipid infusion both at rest (2, 14, 38, 40) and exercise (19, 32) to investigate the effect of elevated plasma FFA on carbohydrate metabolism. In addition, fat feeding (12, 25) and fasting (27) have been used to elevate plasma FFA before exercise. Studies at rest have unequivocally demonstrated a significant reduction in glucose utilization subsequent to elevated plasma FFA. Relatively few studies have measured glucose flux in exercising humans, although Hargreaves et al. (19) and Knapik et al. (27) demonstrated a reduced glucose uptake during exercise subsequent to Intralipid infusion and fasting, respectively.

There is also little direct information regarding muscle metabolism during exercise when FFA availability is enhanced, and the information is controversial. Costill et al. (12) and Knapik et al. (27) demonstrated a reduced glycogen utilization during cycling at 70 and 45% $\dot{V}O_{2\,max}$, respectively, but Hargreaves et al. (19) reported no sparing during knee extensor exercise. Jansson and Kaijser (25) reported significantly lower muscle lactate during exercise after a fat meal but did not measure glycogen. However, in a related series of experiments, Jansson and

Kaijser reported less glycogen utilization after 6 min (24) and 25 min (23) of submaximal cycling (65% $\dot{V}O_{2\,max}$) after a high-fat diet. Ravussin et al. (32) were unable to demonstrate a reduction in carbohydrate utilization based on respiratory exchange measurements as a result of Intralipid infusion. From the previous studies, only Jansson and Kaijser (25) measured muscle citrate and reported higher citrate values at 5 min of exercise in the high-fat trial. No studies measured acetyl-CoA or PDH activity. Therefore, controversy exists whether carbohydrate is spared when fat availability is increased during exercise in humans, and there is a paucity of information regarding the mechanisms that regulate fat-carbohydrate interaction.

Increases in muscle citrate and acetyl-CoA content as a result of enhanced lipid oxidation may inhibit carbohydrate metabolism at the nonequilibrium reactions catalyzed by PFK and PDH, respectively, as proposed in the classic glucose-fatty acid cycle (16, 17, 31). This is supported by in vitro studies demonstrating inhibition of PFK and PDH by citrate and acetyl-CoA, respectively (1, 13, 15). In addition, several animal studies have demonstrated a relationship between reduced glycogen utilization in contracting oxidative fibers and elevated citrate, subsequent to enhanced fat availability (22, 33, 34). However, the present findings do not support this argument. Infusion of Intralipid at rest resulted in a significant elevation of muscle citrate above control, but this difference was abolished within 3 min of exercise. This extends the findings of Spriet et al. (37) in which muscle citrate was elevated at rest after caffeine ingestion but was similar to control at 15 min of cycling. Acetyl-CoA content was unaffected by Intralipid at all time points. In agreement with the similarity of acetyl-CoA content in the control and Intralipid trials, there was also no effect of Intralipid on PDHa during rest or exercise. Under the present experimental conditions, it is unlikely that either muscle citrate or acetyl-CoA are responsible for the downregulation of muscle glycogenolysis. However, there were no muscle measurements during the initial 3 min at this intensity, and it is possible that the glucose-fatty acid cycle may be regulated as originally proposed under conditions of rest or lower-intensity exercise.

The measurements of PDHa are made under optimal in vitro conditions and represent maximal flux through the transformed (active) form of the PDH complex. Therefore, it is possible that the actual flux through PDHa in the cell is less than what is measured in vitro. In the present study, the maximal activity through the transformed PDH was similar in the two trials, and, therefore, the total possible acetyl-CoA production from pyruvate was also similar. However, if glycogen utilization is corrected for muscle intermediate and lactate accumulation and estimated lactate release, it can be calculated that a lower than expected acetyl-CoA production must have occurred in the Intralipid trial. In the control trial, the calculated acetyl-CoA production was similar to that predicted from the in vitro measurements. This suggests that, depsite similar degrees of PDH transformation in the two trials, there existed a lower flux through this enzyme in the Intralipid trial. This reduced flux must have been due to the presence of intracellular variables. However, acetyl-CoA cannot be the regulator responsible for the reduced flux, as it was similar throughout exercise in the two trials. Recent evidence also suggests that elevated acetyl-CoA does not alter the activity of transformed (active) PDH during muscular contraction (10, 11). It is possible that muscle free CoASH content was lower during the Intralipid trial due to the formation of thioesters with coenzyme A, resulting in a reduced PDH flux (acetyl-CoA formation from pyruvate).

Acetyl-CoA/acetylcarnitine relationship. Formation of acetylcarnitine during exercise provides a sink or temporary store for excess acetyl groups, maintaining a viable pool of free CoA (CoASH) during situations where the production of acetyl-CoA is greater than its condensation with oxaloacetate (5, 20). We are unaware of any previous reports of acetylcarnitine after 15 min of exercise at an intensity similar to that used in the present study. However, the acetylcarnitine value obtained at 3 min of exercise in the present study is similar to that reported after 4 min of intense electrical stimulation (20). Previous reports have demonstrated a linear relationship between muscle acetyl-CoA and acetylcarnitine formation in humans (8, 11), horses (5), and rats (36). However, the present study demonstrates a continuous increase in acetylcarnitine throughout exercise despite a lack of increase in acetyl-CoA beyond 3 min. It can be calculated that the Δacetylcarnitine-to-Δacetyl-CoA ratio from rest to 15 min is ~530 but from rest to 3 min is only ~250-280. This indicates that the buffering of acetyl units is not facilitated to its full extent during the initial stages of exercise. This is likely due to the rapid increase in turnover of the tricarboxylic acid (TCA) cycle at the onset of exercise and the resultant consumption of acetyl-CoA units to establish a new equilibrium with the TCA cycle. This is also supported in a study of prolonged moderate intensity (75% $\dot{V}O_{2\,max}$) where it can be calculated that the Δacetylcarnitine-to-Δacetyl-CoA ratio in the first 3 min was ~360 and thereafter increased to ~700 during the subsequent 37 min of exercise (10).

Elevation of muscle citrate. The present results have clearly indicated that there is no relationship between glycogen sparing in the Intralipid trial and the changes in muscle citrate and acetyl-CoA content. It has been proposed that elevation of citrate subsequent to oxidation of fatty acids or ketones may be the result of increases in oxaloacetate or acetyl-CoA-to-CoA ratio (17). It has also been suggested that oxaloacetate may be substrate limiting for the citrate synthase reaction (18, 29, 42). This is consistent with the findings of Sahlin et al. (35), who demonstrated a reduction in TCA cycle intermediates in exercising humans at fatigue despite adequate acetylcarnitine levels (reflecting availability of acetyl units). In the present study, muscle citrate increased significantly at rest during Intralipid infusion despite the absence of change in acetyl-CoA, suggesting that oxaloacetate may have been the limiting factor. It has been demonstrated in isolated liver mitochondria that oxidation of palmitoylcarnitine increases the carboxylation of pyruvate to oxaloacetate (30). Acetyl-CoA has been demonstrated to be a stimulator of liver pyruvate carboxylase (26, 30, 39), as

well as other acyl-CoA compounds such as propionyl-CoA (26). It is possible that an elevation of fatty acyl-CoA during the Intralipid trial may have stimulated pyruvate carboxylase, resulting in elevation of the oxaloacetate pool and subsequently citrate.

Glycogen sparing. A previous study in this laboratory demonstrated glycogen sparing (37) after caffeine ingestion during intense exercise (80% $\dot{V}O_{2\,max}$). It was postulated that enhanced lipid utilization may have been the cause of sparing, although this could not be proven. The present study was undertaken to determine whether elevation of plasma FFA would spare muscle glycogen during the initial 15 min of intense exercise when the rate of glycogen degradation would be the greatest (4). The degree of glycogen sparing in the present study (44%) is in close agreement with our previous finding (37). It is interesting to note that there was a strong correlation between resting glycogen content and the magnitude of glycogen degradation (control, $r = 0.81$ and Intralipid, $r = 0.87$), although this does not necessarily imply cause and effect. For example, well-trained subjects had the highest resting glycogen contents, and the large glycogen utilization in vastus lateralis in these subjects may have been due to preferential recruitment of this muscle in comparison with lesser trained individuals who were not experienced in cycling. There was no relationship between resting glycogen content or training status and the magnitude of glycogen sparing.

Regulation of glycogenolysis. It is not possible to discuss with any certainty the mechanism of glycogen sparing in the present study. However, regardless of the mechanism, there must ultimately be regulation at the level of glycogen phosphorylase. It is interesting to note that there was less PCr degradation during exercise in the Intralipid trial, which reached significance at 15 min. This suggests a closer match between ATP hydrolysis and phosphorylation of ADP to ATP in the Intralipid trial, resulting in a lower free ADP concentration. This should translate into a decreased free AMP concentration, which is a stimulator of glycogen phosphorylase *a* (7) and *b* (28). In addition, it may be possible that the formation of fatty acyl-CoA subsequent to entry of FFA into the cytoplasm may have a direct effect on glycolytic enzymes, including phosphorylase, resulting in the downregulation of glycogenolysis. Further study will be required to investigate these possibilities.

Summary. The present study has demonstrated a reduction in muscle glycogenolysis during intense aerobic cycling subsequent to an Intralipid-induced elevation of plasma FFA. The glycogen sparing could not be explained by citrate-induced inhibition of PFK or acetyl-CoA-induced inhibition of PDHa. Therefore, during 15 min of cycling at 85% $\dot{V}O_{2\,max}$, the interaction of fat-carbohydrate metabolism does not appear to be regulated as classically proposed in the glucose-fatty acid cycle. It is suggested that regulation exists at the level of glycogen phosphorylase.

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APPENDIX C

Plasma volume and ion regulation during exercise after low- and high-carbohydrate diets

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Lindinger, Michael I., Lawrence L. Spriet, Eric Hultman, Ted Putman, Robert S. McKelvie, Larry C. Lands, Norman L. Jones, and George J. F. Heigenhauser. Plasma volume and ion regulation during exercise after low- and high-carbohydrate diets. *Am. J. Physiol.* 266 (*Regulatory Integrative Comp. Physiol.* 35): R1896–R1906, 1994.—This study compared plasma volume (PV) and ion regulation during prolonged exercise in control vs. glycogen-depleted (GD) conditions, with emphasis on the initial minutes of exercise. In two trials separated by 1–2 wk, four adult males cycled at 75% of peak oxygen consumption (V_{O_2}) until exhaustion (50 ± 7 min for GD) or until the GD exhaustion time in the control trial. Blood was sampled from catheters placed in the brachial artery and retrograde in the femoral vein (fv). Arterial PV decreased rapidly and by 15 min PV was 83% (control) and 88% (GD) of initial. The decrease in PV was accompanied by a net osmotic flux of water from plasma and inactive tissues to contracting muscles. The significantly greater decrease in PV in control compared with GD was associated with a higher muscle lactate content (Lac^-; 36 vs. 17 μmol/g dry wt, respectively). Increases in plasma $[Cl^-]$ and $[Na^+]$ were less than predicted from decreased PV, indicating net loss of these ions from the plasma compartment. Increases in arterial and fv $[K^+]$ were 50% greater than could be accounted for by decreased PV, corresponding with increased arterial and fv plasma K^+ contents. The rapid net release of K^+ and Lac^- from contracting muscle during the first few minutes of exercise in both trials was abolished (control) or reversed (GD) within 15 min of beginning exercise. In summary, *1*) the greatest rate of water and ion flux occurred during the initial 2 min of exercise; *2*) the reduced magnitude of PV and ion disturbances during exercise in the GD trial, compared with control, was associated with a reduced accumulation of lactate in contracting muscle; and *3*) a steady state was achieved in 10–15 min, at which time there was no evidence of net lactate or K^+ release from contracting muscle to the circulation. It can be concluded that water flux between vascular and tissue compartments during exercise occurred independent of ion flux.

blood volume; erythrocyte volume; potassium; chloride; sodium; lactate; hemoglobin; hematocrit; human; skeletal muscle; glycogen depletion

EXERCISE IS ASSOCIATED with ionic disturbances in blood and muscle, which are due in part to a redistribution of water and ions between body fluid compartments. Moderate-to-intense exercise results in a decrease in blood and plasma volume (7, 9, 21, 29, 30) as water moves from the plasma compartment into both the interstitial and intracellular fluid compartments of contracting skeletal muscle (17, 29, 30). This is partially counteracted by a simultaneous movement of water to the circulation from inactive tissues, resulting in a reduced volume in these tissues (21). During maximal exercise the water does not originate from the red blood cells (RBC), since RBC volume (RCV) remains unchanged (22) or changes very little (27). This, however, may not be the case during submaximal exercise where 1 to 7% decreases in RCV have been reported (4, 7, 9, 30).

The net movement of water from the vascular compartment at the onset of exercise is, at least in part, independent of net solute movement and results in increased plasma concentrations of protein, Na^+, Cl^-, K^+ and other constituents (9, 20, 29, 30). Typically, concentrations of Na^+ and Cl^- do not increase in proportion to the decrease in plasma volume, indicating some solvent drag of Na^+ and Cl^- as water flows out of the vascular compartment. Simultaneous and independent ion movements between plasma and contracting muscle, inactive tissues, and RBCs further modulate the ion concentrations of these tissues (12, 16, 17, 19, 20, 23).

The main driving forces for the net flux of water from the plasma compartment into contracting muscle during moderate-to-intense exercise are the increases in intracellular and interstitial osmolality of contracting muscle (6, 21, 32) and the increases in capillary pressure and surface area (15, 21). The former is thought to be due to the rapid hydrolysis of phosphocreatine at the onset of muscle contraction yielding the osmolytes creatine and inorganic phosphate, as well as muscle lactate (Lac^-) accumulation resulting from increased rates of glycolysis (6, 10).

Exercise at ~75% of peak oxygen consumption (V_{O_2}) in the glycogen-depleted (GD) state results in reduced Lac^- production and lower muscle and blood $[Lac^-]$ compared with exercise under conditions of normal or high carbohydrate diet and high liver and muscle glycogen stores (24). The present study examined the hypothesis that exercise at 75% of peak V_{O_2} by subjects who were GD and consumed a low-carbohydrate diet would result in an attenuated decrease in plasma volume, which would correspondingly affect the exercise-induced changes in plasma ion concentrations. This follows from the prediction that a reduced intracellular-interstitial osmolality in contracting muscle, consequent to the reduced muscle Lac^- production and accumulation, would result in a smaller transcapillary flux of water into contracting muscles.

Because the rapid and large osmotic changes in skeletal muscle occur during the initial minutes of exercise, an additional purpose was to carefully study the time course of plasma volume and ion changes over the first several minutes of exercise.

METHODS

Subjects. Four healthy males participated in the study. The characteristics of the subjects are as follows (means ± SE): body mass 76.9 ± 9.8 kg, height 182 ± 3 cm, age 22.5 ± 2.1 yr, maximal oxygen consumption ($Vo_{2\,max}$) 4.88 ± 0.34 l/min. The procedures and risks were explained to the subjects, and written informed consent was obtained. The studies were approved by the university's ethics committee.

Experimental protocol. Two trials of leg cycling exercise were performed at 75% of the subject's peak Vo_2 for identical durations; the trial in the GD state was performed 1–2 wk before the control trial. Three days before the GD trial the subject's peak Vo_2 was determined on a Monark cycle ergometer. This was immediately followed by continuous cycling at high and low intensities until exhaustion (60–80 min) to deplete muscle and liver stores of glycogen. The subjects then consumed a low-carbohydrate diet consisting of 3% carbohydrate, 51% fat, and 46% protein for 2.5 days and reported to the lab for the GD trial. At rest, just before starting the GD trial, muscle glycogen was 185 ± 19 mmol glucosyl units/kg dry muscle (24). Three days before the control trial subjects again exercised to exhaustion to reduce glycogen stores, and then consumed a diet consisting of 86% carbohydrate, 4% fat, and 10% protein. At rest, just before starting the control trial, muscle glycogen was 655 ± 71 mmol glucosyl units/kg dry muscle (24). Subjects were asked to abstain from caffeinated beverages and alcohol for at least 24 h before the study.

Catheters were inserted into the brachial artery and femoral vein as detailed previously (20, 24). Biopsy sites were infiltrated with 2% xylocaine (without epinephrine). The thigh of one leg was prepared for needle biopsy of the vastus lateralis with incisions of the skin through to the deep fascia. The subject sat for 15 min and a resting muscle biopsy and blood samples were obtained. The subject sat on an electronically braked cycle ergometer (Corival 400; Lode, The Netherlands) and exercised to exhaustion in the GD trial and to the GD exhaustion time in the control trial, at 75% of peak Vo_2.

Muscle. Biopsies were obtained from the vastus lateralis at rest, after 16 min of exercise, and at the GD exhaustion time in each trial. Biopsies were immediately frozen and stored in liquid nitrogen. The sample was weighed, freeze dried, and weighed again for determination of total tissue water (TTW). The muscle was then powdered to remove connective tissue and blood, and Lac^- was measured enzymatically (1).

Blood. Blood samples were simultaneously drawn from the brachial artery and femoral vein in lithium-heparinized plastic syringes (Sarstedt) at rest, 0.5, 1, 2, 5, 10, 15, 30, and 45 min of exercise and at exhaustion. Plasma was immediately separated from a 3-ml blood sample by centrifugation for 2 min at 15,900 *g* in high-speed centrifuges. A 200-μl aliquot of plasma was deproteinized in 400 μl of 6% perchloric acid and the supernatant analyzed for Lac^- (1). Plasma was analyzed for Na^+, Cl^-, and K^+ using ion-selective electrodes (AVL 983-S electrolyte analyzer). Hematocrit determinations were made on whole blood using heparinized microcapillary tubes centrifuged for 5 min at 15,000 *g* (IEC MB microhematocrit centrifuge). Hemoglobin ([Hb]) was measured using an hemoximeter (Radiometer model OSM3, Copenhagen, Denmark). The mean differences between duplicate measurements were 0.3 ± 0.1 meq/l for $[Na^+]$, 0.6 ± 0.2 meq/l for $[Cl^-]$, 0.02 ± 0.02 meq/l for $[K^+]$, 0.2 ± 0.2 meq/l for $[Lac^-]$, 0.4 ± 0.3% for hematocrit, and 0.2 ± 0.2 g/dl for [Hb].

Calculations. The change in plasma volume (dPV) was calculated using *Eq. 1* of Gillen et al. (8) from rest (r) and exercise (e) measures of [Hb] and Hct. Hct (%) was multiplied by 0.96 and 0.91, giving H, to correct for trapped plasma and peripheral sampling, respectively.

$$\%dPV = 100 \times [(Hb_r/Hb_e) \times (100 - H_e)/(100 - H_r) - 1] \quad (1)$$

The change in red cell volume (dRCV) was calculated according to Dill and Costill (4)

$$\%dRCV = 100 \times [(Hb_r/Hb_e) \times (Hct_e/Hct_r) - 1] \quad (2)$$

The net flux (F) of ions across the exercising leg was calculated from measured plasma ion concentrations and accounted for the simultaneous transcapillary flux of water from the plasma compartment (J_v), according to Watson et al. (32)

$$F = (Q - J_v) \times [ion]_{fv} - Q \times [ion]_a \quad (3)$$

where $[ion]_{fv}$ and $[ion]_a$ are the femoral venous and arterial ion concentrations, and Q is leg plasma flow calculated as leg blood flow × (1 − arterial Hct). Leg blood flow (LBF) was estimated from measured ventilatory Vo_2 and leg arteriovenous oxygen difference (24) as described by Jorfeldt and Wahren (14).

The net transcapillary flux of water from/to the plasma compartment was calculated from

$$J_v = Q \times \{[(Hb_a/Hb_{fv}) \times (100 - H_{fv})/(100 - H_a)] - 1\} \quad (4)$$

where the term in parentheses is derived from *Eq. 2* from van Beaumont et al. (30) and is equivalent to (assuming no net gain or loss of protein from the vascular compartment) *Eq. 1* of Watson et al. (32)

$$J_v = Q \times (TP_a - TP_v)/TP_v$$

where TP is total plasma [protein].

Working muscle mass (WMM) of the legs was estimated assuming that 25% of total body muscle mass (45% of body mass) was active during leg cycling (3)

$$WMM = \text{body mass} \times 0.45 \times 0.25 \quad (5)$$

Equation 5 yielded an average WMM of 8.65 ± 0.67 kg.

Statistics. Results are expressed as means ± SE. Data were analyzed using two-way analysis of variance (ANOVA) with repeated measures to compare measures over time and between conditions. When a significant *F* ratio was obtained, the Tukey test was used to compare means. Significance was accepted at $P \leq 0.05$.

RESULTS

The time to exhaustion in the GD trial was 49.7 ± 11.6 min (range: 30–60 min). In the control trial subjects exercised for the same duration as in the GD trial. Exercise was associated with increases in [Hb] and hematocrit (Table 1), and these data were used to calculate the plasma J_v and the change in plasma and red cell volumes.

Estimated blood flow at rest was 3.1 ± 1.4 and 3.5 ± 0.5 l/min in the control and GD trials, respectively. Plasma flow (Fig. 1) and blood flow (not shown) increased rapidly and peaked at 2 min. After 5 min of exercise plasma flow remained at 6–7 l/min until the end of exercise. There were no differences between the two groups.

Transcapillary water flux. The greatest rate of fluid shift occurred during the first 30 s of exercise with peak values of [Hb] and hematocrit occurring between 2 and

Table 1. *Hematocrit and [Hb] during cycling exercise at 75% of peak V_{O_2}*

	Hematocrit, %		[Hb], g/100 ml	
	Arterial	Venous	Arterial	Venous
Rest				
Control	44.7 ± 0.8	43.8 ± 0.7	14.0 ± 0.1	14.0 ± 0.2
GD	46.0 ± 0.6*	46.2 ± 1.0*	14.7 ± 0.2*	14.7 ± 0.2*
0.5 min				
Control	46.6 ± 0.3	47.8 ± 0.6	14.9 ± 0.1	15.1 ± 0.1
GD	48.7 ± 1.0*	49.4 ± 1.0*	15.4 ± 0.2*	15.6 ± 0.3*
1 min				
Control	46.5 ± 0.5	47.3 ± 0.4	15.1 ± 0.1	15.3 ± 0.1
GD	48.9 ± 1.0*	50.3 ± 0.9*	15.8 ± 0.3*	15.9 ± 0.2*
2 min				
Control	47.5 ± 0.7	48.1 ± 0.9	15.3 ± 0.2	15.4 ± 0.1
GD	49.1 ± 0.9*	50.5 ± 0.9*	15.9 ± 0.2*	16.1 ± 0.2*
5 min				
Control	48.0 ± 0.7	49.6 ± 0.5	15.3 ± 0.1	15.6 ± 0.1
GD	49.5 ± 1.0	50.2 ± 1.1	15.8 ± 0.3	15.9 ± 0.3
10 min				
Control	48.3 ± 0.9	49.8 ± 0.9	15.7 ± 0.3	15.7 ± 0.1
GD	48.9 ± 0.8	49.8 ± 1.1	15.8 ± 0.2	15.8 ± 0.2
15 min				
Control	49.2 ± 0.5	51.1 ± 0.9	15.8 ± 0.2	15.8 ± 0.2
GD	49.1 ± 0.6	49.8 ± 0.9	15.7 ± 0.1	15.6 ± 0.3
30 min				
Control	48.3 ± 0.5	49.7 ± 0.6	15.5 ± 0.2	15.2 ± 0.2
GD	48.3 ± 0.3	48.9 ± 0.7	15.6 ± 0.2	15.6 ± 0.3
45 min				
Control	47.9 ± 0.8	48.5 ± 0.8	15.4 ± 0.2	15.3 ± 0.1
GD	48.0 ± 0.1	48.4 ± 0.2	15.4 ± 0.1	15.4 ± 0.1
Exh				
Control	47.8 ± 0.8	48.8 ± 1.0	15.8 ± 0.2	15.1 ± 0.2
GD	48.5 ± 0.8	48.6 ± 0.4	15.7 ± 0.2	15.6 ± 0.3

Values are means ± SE. V_{O_2}, oxygen consumption; GD, glycogen-depleted trial; control, control trial; Exh, exhaustion; Hb, hemoglobin. All exercise means are significantly different from rest mean. *Significantly different from control.

10 min of exercise (Table 1). In the GD trial, arterial and venous hematocrit were greater than in the control trial at rest and for the first 5 min of exercise. In controls, plasma J_v peaked at 5 min of exercise, decreased to no net flux at 30 min, and was followed by a net flux of water into the vascular compartment in the last few minutes of exercise (Fig. 2). In the GD trial, plasma J_v peaked at 2 min and rapidly decreased to no significant net flux by 10 min; the last minutes of exercise also showed evidence for net flux of water into the plasma compartment.

Plasma and red cell volume. In both trials plasma volume had decreased to 87.5% of initial rest values after 5 min of exercise (Fig. 3). In controls, plasma

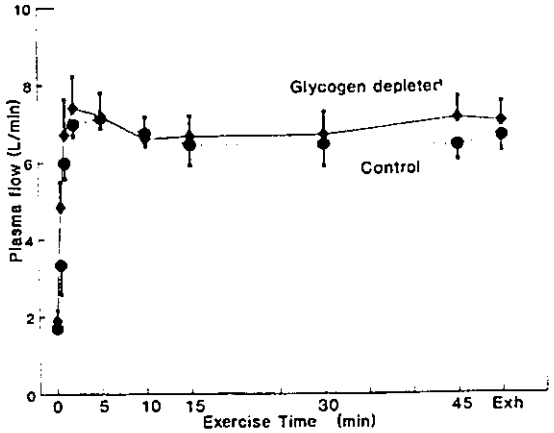

Fig. 1. Calculated plasma flow during exercise to exhaustion (Exh) in the glycogen depleted (GD) and control trials (to the GD exhaustion time). All exercise values are significantly different from rest (*time* 0).

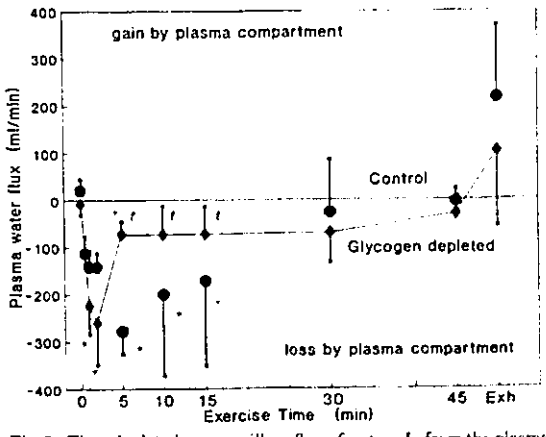

Fig. 2. The calculated transcapillary flux of water, J_v, from the plasma compartment during exercise. *Significantly different from *time* 0. †GD mean significantly different from control.

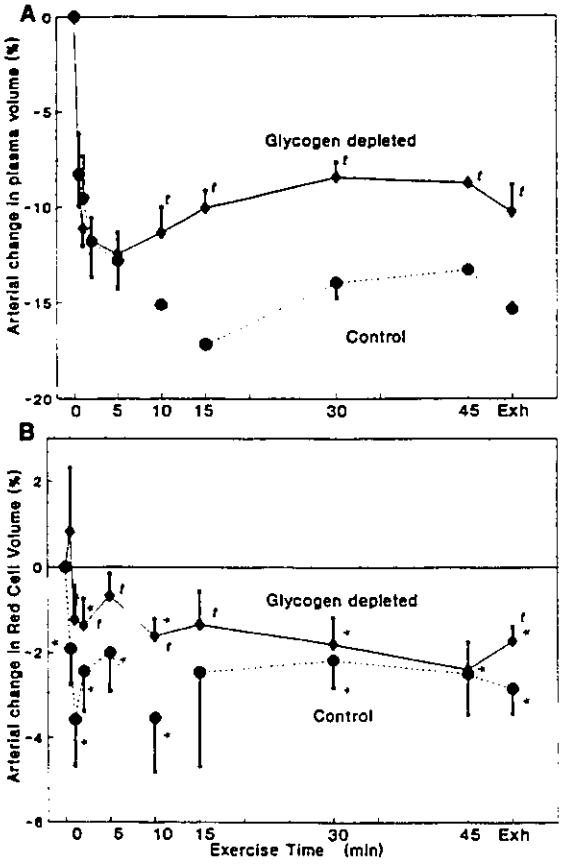

Fig. 3. The change in plasma volume (*A*; all exercise values are significantly different from rest) and red cell volume (*B*) during exercise. For details, see Fig. 2 legend.

volume decreased further to 83% of initial by 15 min, whereas in the GD trial plasma volume recovered to 90–94% of initial during the remainder of the exercise period.

In both trials arterial RCV decreased rapidly during the first 2 min of exercise and remained reduced by 1.5–4% during the exercise period (Fig. 3). The decrease in RCV during the first 15 min of the GD trial was significantly less than in controls.

An initial resting volume of RBCs of 2,380 ± 250 ml was calculated assuming that initial plasma volume was 38 ml/kg body mass (13) and blood volume = plasma volume × [100/(100 − Hct)]. The 1.9% (control) and 1.2% (GD) decreases in RCV at 1 min represent losses of 93 ± 36 and 24 ± 16 ml from the vascular compartment, respectively. In contrast, the corresponding losses from the plasma compartment were 282 ± 66 (control) and 344 ± 64 ml (GD).

Muscle TTW and lactate. In controls, muscle TTW increased significantly from 3.04 ± 0.17 ml/g dry wt at rest to 3.56 ± 0.07 (+17%) and 3.62 ± 0.15 ml/g (+19%) at 16 min and the GD exhaustion time, respectively. In the GD trial, TTW increased from 3.15 ± 0.02 ml/g dry wt at rest to 3.53 ± 0.10 (+12%) at 16 min and at exhaustion (3.47 ± 0.14 ml/g; +10%), which was significantly lower than control.

The decreases in plasma and red cell volumes only partially accounted for the increase in active muscle TTW. In the control trial, the 17.2% decrease in plasma volume and 2.5% decrease in RCV at 15 min translates to 535 ml (462 ml + 73 ml), using the calculated initial plasma volume of 2,920 ml and RCV of 2,380 ml. The increase in muscle TTW at 16 min translates to 1,110 ml, implying that 575 ml of fluid was transferred to the contracting muscles from nonvascular inactive tissues. Similarly, in the GD trial at 15 min the 243-ml (10.4%) decrease in plasma volume and 28-ml decrease in RCV (1.3%) was 519 ml less than the 790 ml increase in working muscle TTW.

Because blood and plasma flows were similar in both conditions, the osmotic contribution to the increases in muscle TTW between conditions warranted consideration. Muscle phosphocreatine, creatine, and inorganic phosphate were not measured in the present study (see DISCUSSION); therefore the analysis is limited to muscle lactate.

In the control trial, muscle lactate content increased ~31 μmol/g dry wt from 4.90 ± 1.68 μmol/g dry wt at rest to 36.5 ± 14.9 and 33.4 ± 21.9 μmol/g dry wt at 16 min and the GD exhaustion time. In contrast, in the GD trial muscle lactate increased significantly less (+12.3 μmol/g dry wt) from 2.42 ± 0.78 μmol/g dry wt at rest to 16.7 ± 11.3 μmol/g dry wt at 16 min, and then significantly decreased to 5.07 ± 1.09 μmol/g dry wt at exhaustion.

Muscle TTW increased as a power function of increasing muscle Lac^- content, with muscle Lac^- accounting for ~48% of the increase in TTW (Fig. 4). There was no relationship between blood flow or change in blood flow and TTW. However, the change in TTW (dTTW) was well described by the interaction between the change in lactate (dLac) and the change in blood flow (dBF)

$$dTTW = 0.0109 \pm 0.0561 + 0.0067 \pm 0.0022\ dLac + 0.0331 \pm 0.0084\ dBF$$

$r^2 = 0.7199$, $P < 0.0001$. The increase in muscle Lac^- contributed 50%, and the increase in blood flow 20%, to the increase in muscle TTW after 15 min of exercise, while 30% was unexplained.

Ion fluxes. Calculated plasma flow and J_v were used to calculate the net flux of ions between contracting tissues and the vascular compartment. The high resting values for plasma flow had minimal effect on the reported ion fluxes because of the small arteriovenous [ion] differences at rest.

The reduction in plasma volume is expected to increase the concentrations of plasma constituents if water moved independently of solute (ions) and proteins. The net addition or removal of solute from the vascular compartment can be determined by comparing, to the resting value, the amount of solute present within the plasma compartment at any point in time (Table 2).

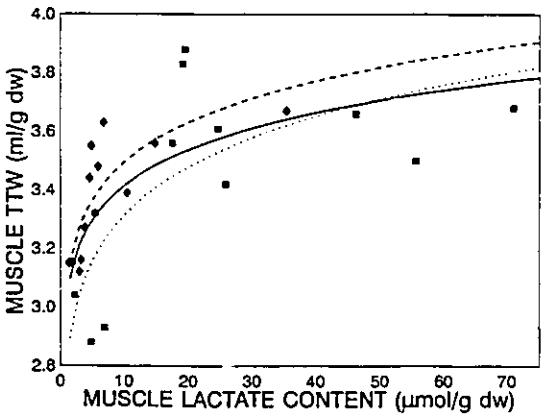

Fig. 4. The relationship between muscle lactate content and muscle total tissue water (TTW) in muscles sampled at rest, 16 min of exercise, and at the GD exhaustion time in the control trial (■) and GD trial (◆). The relationship in the GD trial is shown by the dashed line: ln(TTW) = 1.124 + 0.055 ln(lactate), $r = 0.721$, $P < 0.0001$. The relationship in the control trial is shown by the dotted line: ln(TTW) = 1.035 + 0.071 ln(lactate), $r = 0.741$, $P < 0.0001$. The combined relationship (solid line) is described by the equation ln(TTW) = 1.105 ± 0.027 + 0.052 ± 0.011 ln(lactate), $r^2 = 0.506$, $P < 0.0001$.

Exercise-induced increases in femoral venous [Lac^-] (Table 3) and Lac^- content (Table 2) were significantly greater than arterial during the first 1–2 min of exercise. Peak lactates occurred at 5 min, and this was followed by a gradual decline as exercise continued. Compared with the control trial, GD resulted in significantly lower peak [Lac^-] (Fig. 5, Table 3) and Lac contents (Table 2). In the control trial, Lac^- flux from muscle peaked at 1.8 ± 0.5 meq·kg^{-1}·min^{-1} at 1 min of exercise, while in the GD trial peak net efflux was only 0.94 ± 0.46 meq·kg^{-1}·min^{-1} at 1 min of exercise (Fig. 5). In both trials this was followed by a reduced arteriovenous difference (Table 4) and efflux.

At rest and during exercise arterial [Na^+] (Table 3) and [Cl^-] (Fig. 6) were higher in the control trial than in the GD trial. Despite the increased concentrations, both arterial and femoral venous Na^+ and Cl contents decreased significantly in both trials (Table 2), indicating a net loss of these ions from the plasma compartment. In the control trial, arterial Na^+ and Cl^- contents decreased rapidly during the first 5 min of exercise and then continued to decrease at a much slower rate. In the GD trial the trend was similar but the magnitude of decrease in arterial Na^+ and Cl^- contents was significantly less than in the control trial after the first 5 min of exercise (Table 2).

Net Cl^- influx to muscle peaked at 5 min (control) and then decreased to zero at the GD exhaustion time, while in the GD trial the net Cl^- influx peaked at 2 min and remained elevated until 45 min of exercise (Fig. 6). Therefore reliance on the arteriovenous differences for Cl^- for an interpretation of Cl^- flux across the leg would be erroneous, i.e., the negative values suggest net efflux from muscle.

Table 2. *Arterial plasma content of ions at rest and during cycling exercise at 75% of peak Vo_2*

	Na^+ Content, meq	K^+ Content, meq	Cl^- Content, meq	Lactate Content, meq
Rest				
Control	416 ± 32	12.2 ± 0.7	311 ± 22	6.3 ± 1.3
GD	413 ± 31	12.7 ± 1.1	306 ± 20	4.0 ± 0.8†
0.5 min				
Control	390 ± 34*	13.5 ± 1.1*	291 ± 25*	9.6 ± 2.5*
GD	381 ± 24*	13.4 ± 0.9*	285 ± 16*	4.2 ± 0.8†
1 min				
Control	387 ± 33*	14.5 ± 0.9*	289 ± 23*	18.4 ± 2.7*
GD	372 ± 25*†	14.2 ± 1.0*	278 ± 18*	10.9 ± 1.4*†
2 min				
Control	380 ± 27*	14.5 ± 0.9*	28[illegible] ± 19*	29.1 ± 4.2*
GD	371 ± 26*	14.4 ± 1.0*	276 ± 18*	21.8 ± 4.9†
5 min				
Control	374 ± 28*	13.5 ± 1.0*	277 ± 20*	34.4 ± 4.6*
GD	373 ± 26*	13.8 ± 1.0*	276 ± 18*	23.8 ± 5.9†
10 min				
Control	364 ± 22*	12.9 ± 0.8*	268 ± 17*	31.9 ± 6.0*
GD	374 ± 27*	13.8 ± 1.1*†	279 ± 20*†	20.7 ± 6.2*†
15 min				
Control	356 ± 25*	12.6 ± 0.8*	262 ± 18*	32.7 ± 6.3*
GD	377 ± 28*†	14.3 ± 1.2*†	281 ± 19*†	19.9 ± 4.6*†
30 min				
Control	370 ± 31*	13.6 ± 1.0*	274 ± 21*	32.7 ± 5.9*
GD	387 ± 29*†	15.0 ± 1.1*†	290 ± 23*†	17.0 ± 5.1*†
45 min				
Control	373 ± 32*	13.6 ± 1.0*	275 ± 23*	26.8 ± 3.3*
GD	389 ± 36*†	15.3 ± 1.3*†	288 ± 25*†	11.0 ± 2.7*†
Exh				
Control	366 ± 35*	13.6 ± 1.2*	270 ± 25*	31.6 ± 6.3*
GD	376 ± 23*†	15.1 ± 0.8*†	282 ± 17*†	16.0 ± 5.1*†

Values are means ± SE. Calculation of content assumes a plasma volume of 38 ml/kg body mass at rest in both trials (see text). *Exercise mean significantly different from rest. †GD mean significantly different from control mean.

Table 3. *Plasma concentration of ions at rest and during cycling exercise at 75% peak Vo_2*

	[Na^+]		[K^+]	[Cl^-]	[Lac^-]
	Arterial	Venous	(Venous)	(Venous)	(Venous)
Rest					
Control	142.5 ± 0.4	144.0 ± 0.3	4.30 ± 0.14	106.3 ± 0.6	2.13 ± 0.30
GD	141.2 ± 0.5	141.2 ± 0.6†	4.30 ± 0.10	105.3 ± 0.8	1.04 ± 0.07†
0.5 min					
Control	145.0 ± 0.3*	147.4 ± 0.8*	5.59 ± 0.08*	106.7 ± 0.7	5.23 ± 1.04*
GD	142.7 ± 0.5*†	146.0 ± 0.9*	5.55 ± 0.13*	106.9 ± 0.7*	2.95 ± 0.69*†
1 min					
Control	145.9 ± 0.7*	147.8 ± 1.4*	5.80 ± 0.10*	106.5 ± 0.6	9.60 ± 0.14*
GD	143.6 ± 1.4*†	146.2 ± 1.7*	5.81 ± 0.13*	106.3 ± 0.8	5.93 ± 1.28*†
2 min					
Control	147.8 ± 0.4*	150.0 ± 0.7*	5.73 ± 0.09*	106.5 ± 0.5	12.2 ± 1.03*
GD	145.7 ± 1.1*†	147.3 ± 1.8*†	5.91 ± 0.12*†	106.0 ± 0.7	9.13 ± 1.48*†
5 min					
Control	146.7 ± 0.7*	149.1 ± 1.0*	5.39 ± 0.07*	105.7 ± 0.6	13.4 ± 1.96*
GD	145.2 ± 1.1*	146.2 ± 1.5*†	5.64 ± 0.20*†	105.5 ± 0.5	10.1 ± 2.72*†
10 min					
Control	147.2 ± 0.5*	149.2 ± 0.8*	5.29 ± 0.05*	105.8 ± 0.4	13.5 ± 1.97*
GD	144.3 ± 1.0*	146.8 ± 1.0*	5.45 ± 0.16*†	105.0 ± 0.5	8.09 ± 2.31*†
15 min					
Control	147.4 ± 0.4*	149.1 ± 0.7*	5.43 ± 0.16*	105.2 ± 0.7	13.8 ± 1.89*
GD	144.3 ± 1.1*†	145.9 ± 1.2*†	5.43 ± 0.21*	105.0 ± 0.7	7.80 ± 1.81*†
30 min					
Control	146.8 ± 0.9*	148.7 ± 1.2*	5.44 ± 0.10*	105.5 ± 0.7	12.6 ± 1.97*
GD	144.7 ± 0.8*†	146.0 ± 1.2*†	5.54 ± 0.13*	105.1 ± 0.8	6.33 ± 1.68*†
45 min					
Control	146.1 ± 0.5*	148.6 ± 0.3*	5.48 ± 0.10*	105.3 ± 0.2	10.8 ± 0.71*
GD	144.9 ± 0.7*†	145.0 ± 1.4*†	5.69 ± 0.15*†	104.7 ± 1.1	4.22 ± 0.97*†
Exh					
Control	147.2 ± 0.9*	149.8 ± 0.7*	5.59 ± 0.19*	105.8 ± 0.5	12.3 ± 2.16*
GD	145.0 ± 0.8*†	146.2 ± 1.2*†	5.70 ± 0.07*†	105.2 ± 0.9	5.90 ± 1.70*†

Values are means ± SE. Units are meq/l. Lac, lactate. *Exercise mean significantly different from rest. †GD mean significantly different from control.

There was a net flux of Na^+ from the plasma compartment to the leg musculature during the first 2 min (GD) to 15 min (control) of exercise. This was followed by no significant net Na^+ flux in both trials (Fig. 7). As with Cl^-, the flux data are in contrast to the arteriovenous differences (Table 4), which are suggestive of net Na^+ efflux from muscle.

There were substantial increases in both femoral venous (Table 3) and arterial plasma [K^+] (Fig. 8) during the first 2 min of exercise, and elevated values were maintained throughout. Arterial plasma [K^+] increased significantly less in GD (1.25 ± 0.04 meq/l) than in controls (1.46 ± 0.05 meq/l) at 2 min despite peaking at 5.57 ± 0.12 in both trials. There were only minor differences in femoral venous [K^+] between trials (Table 3). After the first 2 min, arterial plasma [K^+] remained higher in GD than in controls for the remainder of exercise. Plasma K^+ contents were similar between trials during the first 5 min of exercise. However, between 10 min and exhaustion the K^+ content was significantly greater in the GD trial than in controls (Table 2). Net K^+ efflux was significantly greater in the GD trial, compared with control, at 0.5, 2, 5, and 10 min (Fig. 7). In the control trial net efflux of K^+ had ceased within 5 min; however, in the GD trial net efflux occurred for at least the first 10 min of exercise. In the control trial a significant net K^+ uptake at 5 and 10 min was followed by no net flux between 15 and 30 min and net efflux during the last several minutes of exercise. In the GD trial there was a small net influx of K^+ to muscle after the first 10 min of exercise.

DISCUSSION

This study appears to be the first to have reported the volume and ionic responses of both arterial and femoral venous blood during prolonged submaximal leg exercise in humans; most previous studies have used progressive exercise and measured responses occurring in venous blood draining inactive tissues. A unique finding of the present study is that during the onset of exercise there was a rapid flux of water from the vascular compartment to contracting tissues associated with rapid decreases in plasma volume. Furthermore, exercise in the GD state attenuated the reductions in plasma volume and RCV after the first 2 min of exercise and the net plasma J_v was nearly abolished after the first 5 min of exercise. It is suggested that the attenuated water flux after the first 2 min in the GD trial, compared with control, is related to metabolic-hormonal differences that resulted in altered osmotic gradients between contracting muscle and the vascular compartment. It is proposed that the attenuated changes in J_v, plasma and red cell volumes, and muscle TTW in the GD trial are related to the reduced accumulation of osmolytes such as Lac^- within contracting muscle.

Methodological limitations. The variables leg muscle mass, leg blood flow, and initial plasma volume were

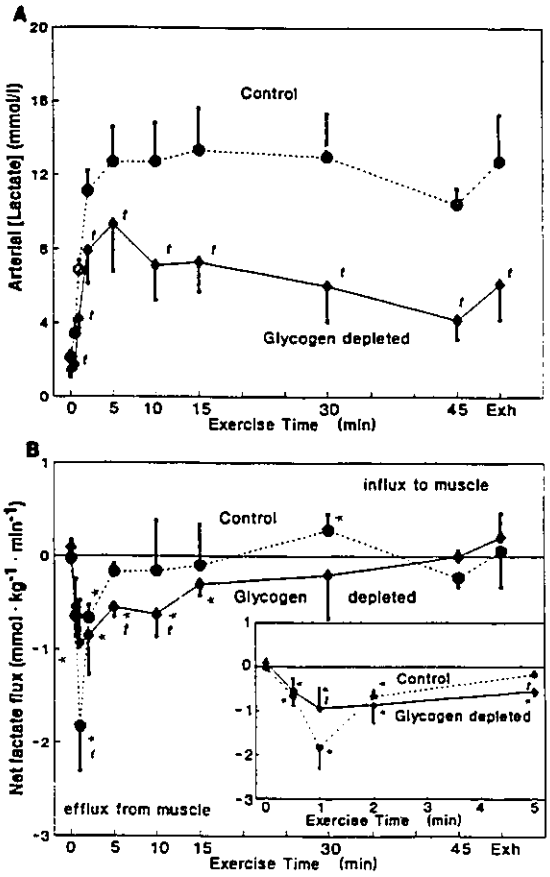

Fig. 5. Arterial [Lac^-] (*A*; all exercise values significantly different from *time 0*) and net Lac^- flux (*B*). *Inset*: net flux during the first 5 min. For details, see Fig. 3 legend.

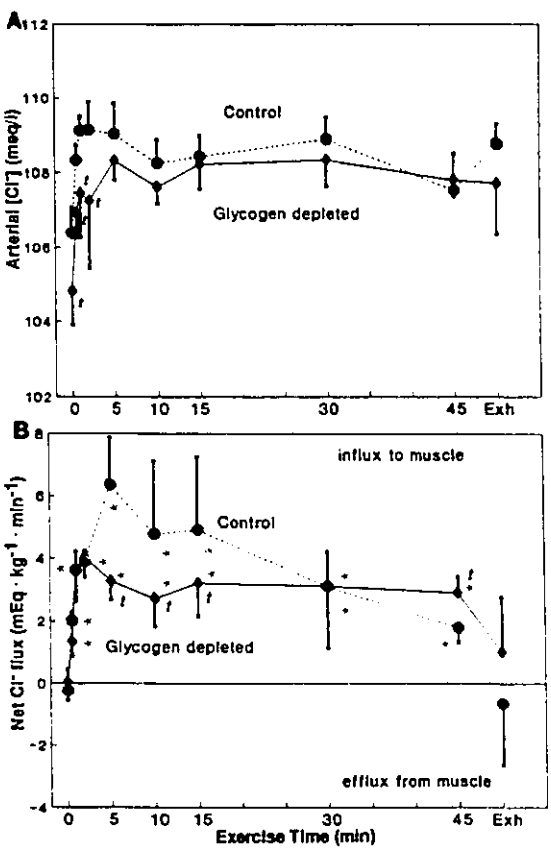

Fig. 6. Arterial [Cl^-] (*A*; all exercise values significantly different from *time 0*) and net Cl^- flux (*B*). For details, see Fig. 2 legend.

estimated from measured values (see METHODS) for each subject individually. Potential errors in the estimation of leg blood flow arise from the ±4% variation for anthropometric estimates of leg muscle mass (3) and the ±4% variation in estimating leg V_{O_2} from whole body V_{O_2} with leg cycling exercise at ~75% of peak V_{O_2} (14). These two estimates, while quantitatively affecting the calculated variables plasma flow, J_v, and ion fluxes, do not affect the qualitative interpretation of the results, since all subjects responded with similar rapidity and magnitude with respect to both measured and calculated variables. Similarly, the within-subject variability,

Table 4. *Arterial-femoral venous differences for plasma [Na^+], [K^+], [Cl^-], and [Lac^-] during cycling exercise at 75% of peak V_{O_2}*

	a-v [Na^+], meq/l		a-v [K^+], meq/l		a-v [Cl^-], meq/l		a-v [Lac^-], meq/l	
	Control	GD	Control	GD	Control	GD	Control	GD
Rest	−1.5 ± 0.4	0.1 ± 1.1	−0.12 ± 0.17	0.03 ± 0.02	0.1 ± 0.4	−0.4 ± 0.7	−0.04 ± 0.19	0.39 ± 0.35
0.5 min	−2.5 ± 0.6	−3.3 ± 0.5*	−0.56 ± 0.06*	−0.53 ± 0.04*	1.7 ± 0.7*	0.0 ± 0.4†	−1.83 ± 0.52*	−1.25 ± 0.63*†
1 min	−1.9 ± 0.9	−2.6 ± 0.7*	−0.30 ± 0.09*	−0.32 ± 0.07*	2.6 ± 0.5*	1.2 ± 0.6*†	−2.76 ± 0.58*	−1.71 ± 0.82*†
2 min	−2.2 ± 0.5	−1.6 ± 0.7*	−0.16 ± 0.04	−0.34 ± 0.11*†	2.7 ± 0.4*	1.3 ± 1.4*†	−1.07 ± 0.07*	−1.28 ± 0.39*
5 min	−2.4 ± 0.6	−1.0 ± 0.5	−0.08 ± 0.05	−0.25 ± 0.11*†	3.3 ± 0.6*	2.9 ± 0.3*	−0.70 ± 0.14*	−0.78 ± 0.20*
10 min	−2.1 ± 0.6	−2.5 ± 0.6*	−0.07 ± 0.05	−0.13 ± 0.10*†	2.4 ± 0.3*	2.6 ± 0.5*	−0.75 ± 0.46*	−0.97 ± 0.46*
15 min	−1.7 ± 0.7	−1.6 ± 0.5	−0.20 ± 0.27	−0.04 ± 0.07	3.2 ± 0.3*	3.3 ± 0.7*	0.49 ± 0.38*	−0.51 ± 0.22*†
30 min	−1.9 ± 0.5	−1.3 ± 1.0	−0.01 ± 0.01	0.07 ± 0.05	3.4 ± 0.7*	3.3 ± 0.9*	−0.39 ± 0.38	−0.29 ± 0.52
45 min	−2.5 ± 0.9	−0.1 ± 1.3	−0.14 ± 0.03	0.01 ± 0.05†	2.2 ± 0.1*	3.1 ± 0.5*	−0.34 ± 0.14*	−0.04 ± 0.08†
Exh	−2.6 ± 0.7	−1.5 ± 1.1	−0.11 ± 0.12	0.07 ± 0.08†	3.0 ± 0.3*	2.5 ± 0.5*	0.49 ± 0.38*	0.24 ± 0.24

Values are means ± SE. a-v, Arterial-femoral difference. *Significantly different from rest. †GD mean significantly different from control.

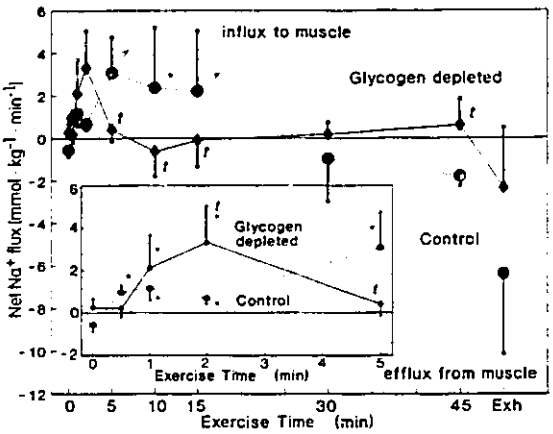

Fig. 7. Net Na^+ flux between plasma and muscle throughout the exercise. *Inset*: net flux during the first 5 min. Other details as in Fig. 2 legend.

~3–6% for measures of plasma volume (13), will influence the estimates of plasma ion contents.

The calculation of the water flux from the plasma compartment is highly dependent on the accuracy of measures of arterial and venous [Hb] (*Eq. 4*). Not evident from the means presented in Table 1 is that the difference between arterial and femoral venous [Hb] was consistent within and between individuals, and ranged between 0 and 0.8 g/dl depending on the time of exercise and the subject. An error of up to 50%, i.e., ±2 g/dl (reasonable), in the calculated plasma J_v quantitatively, but not qualitatively, alters the interpretation of ion fluxes.

Plasma volume. The present study appears to have reported for the first time that the reduction in plasma volume at the onset of exercise is very rapid and nearly complete within 2 min. The initial time point in previous studies is typically 5–10 min after the onset of exercise, by which time most of the change had occurred (9). The rapidity of the plasma volume decrease and associated J_v is consistent with increases in capillary pressure and surface area during the transition from rest to exercise (12, 14). It is also consistent with the rapid increase in intracellular osmolytes, primarily inorganic phosphate and creatine resulting from phosphocreatine hydrolysis and lactate accumulation, which pulls water into skeletal muscle at the onset of muscle contraction (10). In the present study, there was a high degree of correlation between the increase in muscle [Lac^-] and the increase in TTW.

The similar and rapid rates of decrease in plasma volume and plasma J_v during the first 2 min of exercise in both trials is proposed to result from similarly rapid initial rates of phosphocreatine hydrolysis in both trials. There is no reason to suspect that this relatively anaerobic phase during the transition from rest would differ between the two trials. After the initial 2 min of exercise the importance of muscle Lac^- in determining the rate of fluid movement between blood and muscle becomes evident (Fig. 4). Also, the reduction in J_v after the first 5 min of exercise was concurrent with the reduced Lac^- release from muscle, indicating reduced muscle production and accumulation and a decrease in muscle Lac^- between 16 min and the GD exhaustion time. In the GD

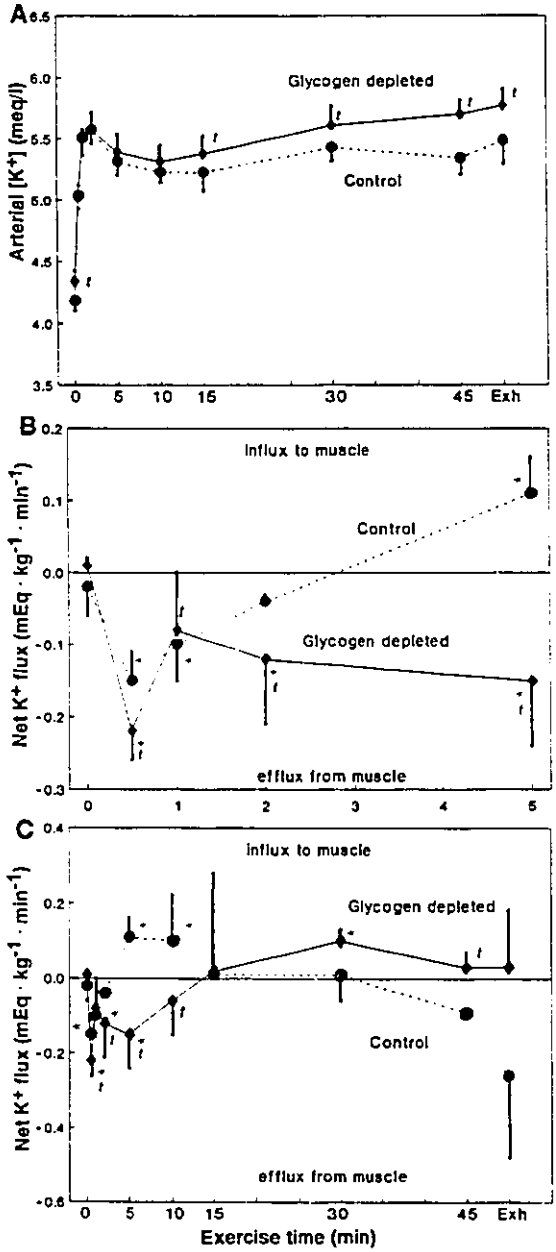

Fig. 8. Arterial [K^+] (*A*; all exercise values significantly different from *time 0*), net K^+ flux during the first 5 min of exercise (*B*), and net K^+ flux throughout exercise (*C*). For details, see Fig. 2 legend.

trial, in contrast to the control trial. the reduced muscle Lac^- release after the first 5 min of exercise was concurrent with a decrease in muscle Lac^- content between 15 min and exhaustion, indicative of a reduced Lac^- production and decreasing muscle osmolality. This is consistent with the partial recovery of plasma volume and low J_v in the GD trial compared with the control trial, where high muscle Lac^- maintained throughout exercise was associated with only a small recovery of plasma volume and higher J_v.

The decrease in plasma volume did not fully account for the increase in muscle TTW. The contribution of water from RBCs, through decreased RCV, was small, and therefore inactive tissues are believed to have contributed the balance. These results are consistent with other studies of humans exercising submaximally where decreases in RCV of 1–7% (4, 7, 9, 30) and inadequate decreases in plasma volume to account for increases in leg volume (21) have been reported. The relatively small decrease in RCV, compared with the 10–18% decreases in plasma volume, is in agreement with other human studies showing that RBCs regulate volume during exercise at submaximal (27, 30) and maximal (22, 23) intensities.

The contribution of sweat fluid losses to the decrease in plasma volume and changes in ion contents should be considered during prolonged exercise because resultant shifts of water from inactive tissues (9) may lead to intracellular dehydration of these tissues (18). Extrapolation from Harrison et al. (9) yields a predicted sweat volume loss of ~600 ml for the ~55 min of exercise in the present study. Volume decreases of ~500 ml in the vascular compartment have been accounted for by uptake into contracting muscle, in addition to a further 500–600 ml transferred from inactive tissues to contracting muscle (see RESULTS). The additional 600 ml that may have been lost through sweating would therefore further contribute to decreased intracellular volume of inactive tissues.

Potassium. Net K^+ flux between muscle and blood is dependent on the intensity of muscle contraction (28) and on the activity of the Na^+-K^+ pump (2). Similar to the present study, Sjogaard (28) and Vollestad and Sejersted (31) also reported a large arteriofemoral venous (a-fv) plasma $[K^+]$ difference (up to −0.6 meq/l) during the first few minutes of submaximal exercise (26, 28, 31). The persistent negative a-fv plasma $[K^+]$ of −0.1 to −0.2 meq/l after the first several minutes of submaximal exercise has led to the conclusion that there is a continuous net release of K^+ from contracting muscle (26, 28). Earlier studies, however, did not measure or account for the simultaneous transcapillary flux of water from plasma to muscle that contributes to the increase in plasma $[K^+]$, particularly in the femoral vein, during leg exercise. The calculation of ion fluxes in the present study was careful to account for the transcapillary flux of water into contracting muscle during exercise.

In contrast to the measured a-fv $[K^+]$, the net K^+ flux from contracting muscles ceased within 5 (control) to 15 (GD) min after the onset of exercise, and was in fact followed by periods of net uptake of K^+ from the plasma compartment. A similar result would have been missed in previous studies of submaximal exercise that did not account for the simultaneous flux of water from the plasma compartment. An exception is the study by Vollestad and Sejersted (31), which reported no significant a-fv $[K^+]$ after the first 4 min of exercise. In the present study, even a 50% overestimate of the plasma water flux does not qualitatively change the results.

The absence of a significant net K^+ and Lac^- release from muscle after the first minutes of exercise is difficult to reconcile with the persistent elevation in plasma $[K^+]$ (26, 28) and Lac^- (present study) seen through to the end of exercise, since inactive tissues have been shown to take up K^+ (16, 19, 22, 28) and Lac^- (16, 19, 22) from the circulation. The net flux of water from the plasma compartment decreased plasma volume by 450 ml in the control trial and contributed ~50% to the continued high plasma $[K^+]$ and $[Lac^-]$ during exercise. It is also probable that K^+ and Lac^- were taken up by inactive tissues and RBCs during the first few minutes of exercise, as has been shown for high-intensity exercise (16, 19, 22), and then were later slowly released back to the circulation. It is proposed that there is a process of continuous distribution of K^+ and Lac^-, initially released from muscle at the onset of exercise, through the tissues during exercise and recovery until whole body K^+ and Lac^- homeostasis is achieved.

Sodium and chloride. As has been shown in previous studies (27, 29, 30) the increases in arterial plasma $[Na^+]$ and $[Cl^-]$ in the present study were much less than could be accounted for by the decrease in PV, indicating the net loss of these ions from the plasma compartment. There is evidence that these ions may be taken up by RBCs (20, 22, 23), contracting muscle (17, 28), and inactive tissues (16, 19) during exercise. The results are consistent with Fenn's (6) early observation that the loss of K^+ from stimulated rat muscle was balanced by a gain of Na^+ and associated with a gain in Cl^- and water.

In the present and other studies (27, 32) it is apparent that the transcapillary fluxes of Na^+ and Cl^- may occur independently of concurrent water fluxes. The net flux of Na^+ and Cl^- into the interstitium of inactive tissues and contracting muscle would reduce plasma osmolality and reduce osmolality gradients between these tissues and the vascular compartment. The forces responsible for and the mechanisms regulating these ion fluxes are not understood at the present time and require further investigation.

Perspectives

Volume regulation. The present study has shown three phases in the plasma volume response to exercise: an initial rapid decrease that is nearly complete within the first 2 min, a slower decrease that ceases between 10 and 30 min, and then a period where plasma volume slowly increases. In prolonged, severe exercise where the ability to rehydrate is absent or inadequate, the third phase may be absent.

The initial (first 2 min) rapid and large net flux of water from the plasma compartment during the transition from rest to exercise in both trials suggests similar increases in capillary pressure and osmotic forces within contracting muscle (15, 21, 32). It is implicit that water has the potential to move independently of solute flux (32) and, in maximally vasodilated cat hindlimb perfusion studies, it has been shown that J_v is predominantly affected by changes in muscle osmolality (21, 32). The second phase of plasma volume decrease (and concurrent small decreases in RCV) is associated with increases in muscle lactate and other metabolites (10, 11, 24) together with no or minimal recovery of phosphocreatine (11), which leaves high concentrations of osmotically active creatine and inorganic phosphate in muscle. The third phase, recovery of plasma volume, reflects the ability of the body to defend blood volume and the decrease in intracellular osmolytes in contracting muscle (11, 34, present study). However, the defense against excessive reductions in plasma volume occurs at the expense of dehydration of noncontracting tissues. The cardiovascular implications of excessive decreases in blood volume on cardiovascular function have been well described (25).

Potassium regulation. The high initial rate of K^+ release from muscle during the transition from rest to exercise is believed to result from a delay in achieving maximal activation of Na^+-K^+ pumps in contracting muscles and inactive tissues (3, 5). Maximal activation of Na^+-K^+ pump activity appears to require ~10 min (5) and, in the present study, K^+ efflux from muscle decreased within 60 s (peak observed efflux at 30 s). This suggests that maximal in vivo activation of Na^+-K^+ pump activity occurs much more rapidly than in vitro. It also occurs much more rapidly than the increase in plasma catecholamines (7), in keeping with the minimal effect of catecholamines in further stimulating K^+ uptake in contracting rat skeletal muscle (5). In the present study the increased activity of the Na^+-K^+ pump was sufficient to produce a net uptake of K^+ by muscle contracting at ~75% of peak Vo_2 in both trials. Convincing evidence of the high rate of muscle Na^+-K^+ pump activity achieved during submaximal (28) or maximal exercise (17, 20) is shown by the precipitous decrease in arterial and femoral venous plasma [K^+] upon cessation of exercise. It is important to observe that the uptake of K^+ by contracting muscle is in direct contrast to the negative a-fv [K^+] difference seen in this and many other studies. It is recommended, therefore, that studies of muscle ion and metabolite flux also consider the simultaneous water flux.

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