Endurance exercise training attenuates leucine oxidation and branched-chain 2-oxo acid dehydrogenase activation during exercise in humans.

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ABSTRACT

Endurance exercise has been shown to both raise and lower leucine oxidation in studies in rodents. We studied the effects of a 38 d endurance exercise training program upon leucine turnover during a 90 min exercise bout at 60 % VO_{2peak} in 6 males and 6 females. Subjects were studied at both the same absolute (ABS) and relative (REL) exercise intensities post-training. Pre (PRE)- and post-training measurements were taken for analysis of: L-[1-^{13}C]leucine turnover, muscle branched-chain oxoacid dehydrogenase activity (BCOAD), muscle glycogen, phosphocreatine and ATP utilization, and resting enzyme activity of citrate synthase (CS) and NADH-cytochrome c-oxidoreductase (complex I-III). We also determined total substrate oxidation by indirect calorimetry, and plasma lactate, glucose, and insulin concentrations. The exercise training resulted in a significant increase in both CS ($P < 0.001$) and complex I - III ($P < 0.05$) activities. Leucine oxidation increased during exercise for the pre-training trial ($P < 0.001$), however, there was no increase for either the post-training ABS or REL trial. Leucine oxidation was significantly lower for females at all time points ($P < 0.01$). Total BCOAD activity was also significantly increased when comparing the PRE to both ABS and REL trials ($P < 0.001$). The % activation of BCOAD was significantly increased from t=0 to t=90 in both the PRE and REL exercise trials with the increase in PRE being greater ($P < 0.001$ (PRE), and $P < 0.05$ (REL)). Exercise RER was lower for females vs. males ($P< 0.05$). In addition, the ABS trial was significantly lower than PRE and REL ($P < 0.01$).

Plasma lactate was significantly lower at all time points for ABS vs. PRE ($P < 0.001$) and REL vs. PRE at t=30 min of exercise ($P < 0.001$). Resting muscle glycogen was higher for both ABS and REL vs. PRE ($P < 0.001$). In conclusion, we found that 38 d of endurance exercise training significantly attenuated both leucine oxidation and BCOAD activation during 90 min of endurance exercise at 60 % VO_{2peak} for both absolute and relative exercise intensities. In addition, females were also shown to oxidize a greater proportion of energy from lipid and a lesser amount from carbohydrates and proteins during exercise.

PREFACE.

The following is a list of abbreviations and operational definitions used throughout this manuscript.

AA – amino acid
ADP – adenosine diphosphate
ATP – adenosine triphosphate
B – breakdown
BCAA – branched chain amino acid
BCAAT – branched chain amino acid transferase
BCKAD – branched-chain keto acid dehydrogenase
BCOA – branched-chain oxo acid
BCOAD – branched-chain oxo acid dehydrogenase
BW – body weight
CHO – carbohydrate
CO_2 – carbon dioxide
Cr – creatine
CS – citrate synthase
DEXA – dual energy x-ray absorptiometry
DTNB - 5,5'-Dithio-bis(2-nitrobenzoic acid) 3-Carboxy-4-n
DTT – dithiothreitol
E_2 – estradiol
EDTA - Ethylenediaminetetraacetic acid
EPI – epinephrine
FFA – free fatty acid
FFM – fat free mass
G-6-P – glucose-6-phosphate
G6PDH – glucose 6 phosphate dehydrogenase
HCl – hydrochloric acid
HK – hexokinase
H_2O – water
IMTG – intramuscular triglyceride
KF – potassium fluoride
$KHCO_3$ – potassium bicarbonate
KIC – ketoisocaproate
KIV – ketoisovalarate
KOH – potassium hydroxide
LBM – lean body mass
Mg - magnesium
NaF – sodium fluoride
NAD – nicotinamide adenine dinucleotide

NADH – nicotinamide adenine dinucleotide, reduced form
NaOH – sodium hydroxide
NE – norepinephrine
NH_3 – ammonia
NH_4^+ - ammonium
NOLD – non-oxidative disposal
O_2 – oxygen
PCA – perchloric acid
PCr - phosphocreatine
PFK - phosphofructokinase
Pi – inorganic phosphate
PRO – protein
Q - flux
Ra – rate of appearance
Rd – rate of disappearance
RDA – recommended daily allowance
RER – respiratory exchange ratio
RNI – recommended nutrient intake
SNS – sympathetic nervous system
TCA – tricarboxylic acid cycle
TCr – total creatine
VCO_2 – carbon dioxide production
VO_2 – oxygen consumption
VO_{2peak} – maximum or peak oxygen consumption

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FOREWORD.

This thesis is presented in four chapters. The first chapter discusses the general adaptations to endurance training and their effect upon substrate utilization during prolonged endurance exercise. Chapter II examines the relationship between training and protein oxidation during exercise. Special emphasis has been placed upon factors affecting BCOAD regulation during endurance exercise. Chapter III is an exploration of possible gender differences that may exist during endurance exercise. Emphasis has been placed on differences in substrate utilization during exercise. Chapter IV is a presentation of the research paper in publication format. Following chapter IV are all references, tables, figures, statistics and relevant appendices.

Chapter 1. Adaptations to Training.

1.1. Introduction

For many years it was thought that the increase in the capacity for endurance exercise was solely a function of ventilatory and cardiovascular adaptations. Although these central training effects are important (55), one must not overlook the peripheral adaptations that occur within skeletal muscle. The course of muscle adaptation depends upon the stimulus that is presented. Disuse will result in a decrease in size and strength, whereas, resistance training will result in an increase in strength and size (55). The adaptations in skeletal muscle in response to endurance exercise training involve an increase in mitochondrial content as well as a number of other enzymatic adaptations that may contribute to the "enhanced" metabolic response to exercise in the trained state. For this thesis, adaptations in fat and carbohydrate metabolism will be discussed briefly whereas the training adaptation responses in protein metabolism will form the primary focus.

1.2. Substrate Metabolism.

1.2.1. Carbohydrate Oxidation.

It is well known that during prolonged endurance exercise (@ 60-85 % VO_{2peak}), muscle glycogen concentrations become depleted which correlates strongly with fatigue (44). The effect of endurance exercise training on this phenomenon was a focus of initial investigators. One of the first human studies to address this issue was conducted by Saltin and colleagues (95) who showed attenuated muscle glycogen depletion during

exercise in trained as compared to untrained individuals. Studies with rodents have yielded similar results, with liver glycogen being depleted less rapidly in trained animals (31). In rodents at different levels of training ranging from 10 to 120 minutes of running per day, there is an inverse relationship between the respiratory capacity of leg muscle and the total amount of glycogen utilized during the same exercise test (31). The slower utilization of carbohydrate during exercise of the same absolute intensity in the trained state is termed 'glycogen sparing' and is thought to occur due to a proportional increase in fat oxidation (1,59). This apparent increase in fat oxidation is reflected by a decrease in the respiratory exchange ratio (RER) as well as an up-regulation of the enzymes responsible for the transfer to, and oxidation of, free fatty acids (FFA's) by the mitochondria (c.f.)(22,55).

In addition to this 'sparing' of muscle glycogen utilization, endurance training has also been shown to increase the intramuscular glycogen stores, thus enabling one to sustain a given workload for a longer duration (41,42).

1.2.2. Fat Oxidation.

One of the most important metabolic adaptations to submaximal exercise, induced by endurance training, is an increased reliance on fat as an energy substrate. Since the training induced increase in muscle mitochondrial content (56)(discussed in greater detail to follow) are comparable to increases in the capacity to oxidize fat and CHO, one may ask why trained individuals oxidize proportionately more fat and less CHO than do untrained persons? This question may be partially answered by the glucose-fatty acid cycle as first proposed by Randle and colleagues (88). They showed that glucose uptake,

glycolysis, glycogenolysis, and pyruvate oxidation are inhibited in heart muscle by oxidation of fatty acids. This inhibition is thought to be mediated, in part, by the accumulation of citrate, which inhibits PFK activity and results in the accumulation of G-6-P, which inhibits HK. Despite Randle's work, numerous investigators have failed to find these inhibitory effects in skeletal muscle and thus concluded that this cycle was limited to heart muscle (c.f.)(55). Subsequent experiments examined the issues surrounding this theory and showed that enhanced FFA oxidation inhibited glucose uptake and glycolysis and had a glycogen-sparing effect in skeletal muscle of both rodents and humans, particularly in the rested state (21,52,91).

From these studies it can be seen that oxidation of FFA reciprocally inhibits glucose uptake and glycolysis and has a glycogen-sparing effect in skeletal muscle. It therefore seems probable that the greater oxidation of FFA's in the trained compared to the non-trained state contributes to the subsequent decrease in glycogen utilization.

The next question then becomes; what are the mechanisms behind an increased oxidation of FFA's in the trained state? Considering that exercising at the same absolute intensity in the trained state attenuates the rise in plasma FFA concentration, it does not appear that increased plasma FFA concentration causes the increased whole body oxidation of fats (23,114). If the rate at which muscle oxidizes FFA's is not directly determined by plasma FFA concentration, other factors must be considered. Training increases the capacity for β-oxidation, the mitochondrial process responsible for the dehydrogenation oxidative phosphorylation of FFA's and their delivery to the TCA cycle as acetyl-CoA (56). One possible explanation for the greater fat oxidation during

exercise, as suggested by RER measurements, is enhanced utilization of intramuscular triglycerides (IMTG's). A study conducted by Hurley et al. in 1986 (59) examined the effect of training on fat oxidation. Subjects were strenuously endurance trained for 12 weeks and subjected to a 2 h cycle ergometry trial @ 60 % of their initial VO_{2peak} , before and after training. Following the training program, muscle glycogen utilization decreased by 41 %, fat utilization increased by ~ 61% and the decrease intramuscular triglyceride concentration was twice as great during absolute exercise.

A more recent study by Phillips and colleagues (86) examined the effects of endurance exercise training on substrate turnover and oxidation during exercise using stable isotope infusions. Seven healthy males were cycle ergometer trained for 2 h/d @ 60 % of their VO_{2peak} for 31 days. Stable isotopes of glucose, glycerol and palmitate were used to assess the oxidation and turnover of fat and carbohydrate. Exercise testing (90 min cycle ergometry @ 60 % of initial VO_{2peak}) was performed pre-training, at 5 d and at 31 d of training. It was found that after 5 d of training there was a 10 % increase in fat oxidation, a 16 % decrease in glycogen utilization and a 63 % increase in intramuscular triglyceride oxidation. At 31 d, total fat oxidation was further increased by 58 % (in addition to 5 d values) while glycogen utilization and intramuscular triglyceride oxidation were significantly decreased and increased, respectively, from both pre and 5 d values. Although this study showed similar results to Hurley and colleagues, comparing the two studies must be done with caution. In order to conclude that the increases in fat oxidation truly exist, both kinetic, direct muscle, and indirect calorimetry measurements must all show similar results. The fact remains that these two studies did show similar

results using different methods thus lending support to the increase in fat oxidation and decrease in CHO oxidation post-training.

1.2.3. Muscle fibre type.

Skeletal muscle adaptations may be characterized by changes in morphological, biochemical, and molecular variables. Due to the focus upon the biochemical adaptations in this thesis, only a brief mention will be made of the morphological changes.

In untrained humans, it has been shown that type I muscle fibres have the greatest content of mitochondrial enzymes with approximately twice the content as type II fibres (79). On average, the majority of individuals have a relatively even proportion of 50 % type II: 50 % type I fibres in vastus lateralis, whereas specialized athletes such as elite endurance, have a greater percentage of type I compared to type II and the opposite is true for elite sprint or short duration power athletes (42).

Fibre type conversion has been shown in both extreme endurance training in rodents (47) as well as chronic electrical stimulation in rabbit skeletal muscle (74). Typically, there is an increase in type I oxidative fibres and an increase in conversion of type IIb to IIa fibres, thus enhancing overall muscle oxidative potential. A study by Baldwin et al, found a 4 to 8 fold difference in mitochondrial enzyme activities between type IIb and IIa, before and after 8 weeks of treadmill training. Humans are also capable of this IIb to IIa fibre-type conversion with training. Jansson et al in 1977 found conversion of type IIb to IIa fibres with a strenuous endurance training stimulus. Furthermore, the mitochondrial content of the type II fibres tends to increase to a greater

extent than that of type I fibres in response to very strenuous endurance training, thereby decreasing the mitochondrial density difference between type I and II fibres.

1.2.4. Muscle mitochondria.

Considering the fact that oxygen delivery to, and utilization by, muscle is not increased during the same absolute exercise intensity after endurance training, compared to pre-training, would suggest that there are other mechanisms involved that produce a training effect in muscle (55).

Some of the earliest evidence for mitochondrial adaptations to endurance training were elucidated by Holloszy et al in 1967 (53). The capacity of the mitochondrial fraction from the gastrocnemius muscle to oxidize pyruvate, doubled in rodents that had adapted to a 2h/day running program. Succinate dehydrogenase, NADH dehydrogenase, NADH-cytochrome c reductase, and cytochrome oxidase activities per gram of mitochondrial protein, increased approximately twofold in hind-limb muscles in response to the training stimulus. The actual concentration of cytochrome c was also increased suggesting an increase in mitochondrial protein. Furthermore, the mitochondrial protein content of the muscle fractions analyzed increased ~ 60 % from pre- to post-training (53).

Other authors have since confirmed that respiratory capacity and mitochondrial enzyme concentration increase in response to endurance training (6,43). Analysis of isolated mitochondria and whole muscle homogenates has shown that rodent skeletal muscle responds to training by increasing the capacity to oxidize fatty acids (5,82) and pyruvate (5). The enzymes responsible for activation, transport and β-oxidation of long-chain fatty acids (5,82), the enzymes of the citric acid cycle (54), and the components of

the electron transport chain involved in oxidation of NADH and succinate (53,54) all increase with training. Increased concentrations of marker enzymes for fatty acid oxidation, the respiratory chain (22,62), and the tricarboxylic acid cycle (20) are also elevated in skeletal muscle of endurance trained humans compared to sedentary subjects.

Endurance exercise training has therefore been conclusively shown to increase both the amount of skeletal muscle mitochondrial protein concentration, as well as the activity of key regulatory enzymes responsible for substrate metabolism, thus emphasizing the importance of skeletal muscle adaptations in addition to central cardiovascular and neural adaptations.

1.3. Hormonal Adaptations.

Various hormones are involved in the regulation of metabolic processes at rest and during exercise. Catecholamines and glucocorticoids (specifically epinephrine (EPI) /norepinephrine (NE) and cortisol, respectively) are two classes of hormones that exert such regulatory effects. The next two subsections examine the normal metabolic function and responses of these hormones to endurance exercise and training.

1.3.1. Catecholamines

Catecholamines are amino-acid based hormones that are released from the adrenal medulla and sympathetic nerve endings (100). In general, these hormones stimulate lipolysis, glycogenolysis, and may have anabolic effects in human skeletal muscle (36,48). Plasma concentrations of EPI and NE increase in a dose response to exercise and the magnitude of their increase is positively correlated to exercise intensity (13,37). During moderate intensity exercise, EPI does not increase until approximately 30 min

after the onset of exercise. The delayed increase in EPI is thought to act as a backup mechanism for plasma glucose maintenance (37). Tarnopolsky and colleagues (99) have confirmed that catecholamines steadily increase during prolonged endurance exercise. In addition, gender differences were found showing that males had a higher plasma EPI concentration at 90 min of endurance exercise. Such a gender difference may help to explain why males oxidize relatively more CHO than do females during prolonged endurance exercise (99).

It is well known that for a given submaximal workload both plasma NE and EPI are lower in endurance-trained individuals as compared with sedentary controls (80). This attenuation of catecholamine production in response to endurance exercise is most likely due to a diminished SNS stimulation due to chronic training stimulation as well as local metabolic adaptations enabling individuals to more efficiently maintain blood glucose such as enhanced catecholamine sensitivity (80).

1.3.2. Cortisol.

Cortisol is a steroid-based hormone secreted from the adrenal cortex in response to stimulation of adrenocorticotropic hormone (ACTH), which itself is released from the anterior pituitary in response to cortisol-releasing hormone (CRH) form the hypothalamus (100). Cortisol has regulatory effects on CHO, PRO and fat metabolism (48). The best known effect of cortisol is that of hepatic gluconeogenesis. Cortisol interacts with hepatocytes to up-regulate the enzymes responsible for gluconeogenesis as well as directly affecting AA liberation from skeletal muscle (48). The exact mechanism of muscle protein degradation is poorly understood but has been shown to involve the AA

alanine. Studies in rodents have shown that as plasma cortisol increases (stimulated by exercise or temperature), there is also a temporal increase in alanine efflux from skeletal muscle (109).

It is generally known that during endurance exercise, there is an increase in plasma cortisol concentration (13,34,100), however, the effects of endurance training on this response are not as well characterized. Keizer and colleagues examined the effects of endurance training on cortisol response during exercise in males and females (65). Subjects were trained over an 18 – 20 month period in which they were studied on three separate occasions in either 15, 25 or 42.2 km road races. Results showed that basal and post-exercise plasma cortisol concentrations were unchanged by training. Friedmann and colleagues (34) studied the effects endurance exercise on regulatory hormones in trained and untrained men and women. Results showed an increase in plasma cortisol, over the duration of the 50 min exercise bout at approximately 75 % VO_{2peak}, with no differences between gender or trained vs. untrained individuals. Duclos and colleagues (29) have recently found there to be no difference in plasma cortisol response to various durations and intensities of exercise between trained and untrained men.

Endurance training has also been shown to attenuate plasma cortisol concentration when performing exercise at the same absolute intensity. Both Duester (24) and Tabata (98) have conducted well controlled studies showing that plasma cortisol concentration is decreased when performing endurance exercise at the same absolute intensity as pre-training. In addition, Deuster (24) showed no change in catecholamine or cortisol response to exercise at the same relative intensity.

In summary, plasma EPI/NE and cortisol levels increase during endurance exercise. Endurance exercise training results in an attenuation of EPI and NE at the same absolute intensity of exercise while exercise performed at relative intensity results in similar hormonal response as in the untrained individual. Cortisol, on the other hand, has been shown to be unaffected by endurance training when tested at an intensity relative to final VO_{2peak} , however, some evidence does exist suggesting an attenuation of cortisol production when exercising at the same absolute intensity post-training. Whether this may or may not be a factor in attenuation of proteolysis after training is not clear. Further research could explore the effects of well controlled longitudinal training studies upon the interactions between glucocorticoids and protein oxidation.

Chapter II. Protein Metabolism.

2.1. Introduction.

Before discussing the relevant literature pertaining to protein metabolism, it is essential to begin with a review of the basic concepts in this area. The component parts of protein (amino acids) can enter the body's free amino acid pool (encompassing body fluids and tissues) following digestion from ingested foods (I) or from the breakdown of body protein (B). Removal from the AA pool occurs via two main routes, namely, protein synthesis (S) and/or oxidation (O) (this will be discussed in subsequent sections). Over 24 h under normal conditions, breakdown and synthesis are in equilibrium such that the degradation of body proteins are replaced by proteins synthesized from the free AA pool. When not enough protein is ingested, there is an insufficient amount of free pool AA's to replace those lost through protein degradation. Over time this can lead to loss in

muscle size, strength and thus physical performance. In contrast, when protein is in excess, the surplus of AA's can be directly oxidized by the muscle and liver or may supply the necessary carbon skeletons for CHO and fat storage (c.f)(78).

There are 20 different amino acids, 9 of which are considered to be essential or indispensable amino acids (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine) (84) that can only be obtained through dietary intake or breakdown within the body. The body of a 70 kg male contains about 12 kg of protein and 200-220 g of free (protein) amino acids (110). There is a continuous exchange of amino acids between pools as proteins are being degraded and synthesized simultaneously, this process represents protein turnover and flux.

2.2. The branched-chain amino acids (BCAA's)

Human skeletal muscle is able to oxidize at least 7 amino acids (alanine, asparagine, aspartate, glutamate, isoleucine, leucine, and valine) (39). Among these, 3 branch-chain amino acids (BCAA; isoleucine, leucine, and valine) are preferentially oxidized during exercise or catabolic situations such as starvation (39). The catabolic pathways of the BCAA have several features in common. The metabolism of BCAA's begins with a transamination and the formation of glutamate and an oxo (keto) acid. The glutamate formed can then donate a nitrogen group to pyruvate and form alanine. The second step is the irreversible oxidative decarboxylation via the enzyme branched-chain oxo (keto) acid dehydrogenase (BCOAD). Leucine, for example, is catabolized to acetyl-CoA and acetoacetate which may alternatively result in the formation of ketone bodies or oxidation within the TCA cycle. Leucine is therefore considered to be a purely ketogenic

AA because it's decarboxylation products can be used to form ketone bodies. Isoleucine forms both acetyl-CoA and succinyl-CoA and is therefore both ketogenic and glucogenic (its carbon skeleton can also be used to form glucose via gluconeogenesis). Valine, by contrast, produces only succinyl-CoA and is thus purely gluconeogenic (13).

2.3. Amino acid oxidation during exercise.

Dohm et al (25) investigated the effects of acute exercise on free amino acid concentrations in rodent muscle, liver and plasma. AA's such as phenylalanine, tyrosine, and the BCAA's were increased in the muscle and liver, while glutamate and glutamine levels were decreased. Similar results for glutamine and glutamate were found by Rennie et al. (90) in the skeletal muscle of human subjects.

The BCAA's as well as alanine, glutamate and aspartate appear to be oxidized in skeletal muscle to a greater extent than previously believed (40). Thus, these are the only AA's that contribute to the energy utilized by muscular contraction during exercise. Alanine is oxidized at the highest rate in terms of absolute concentration throughout the body which accounts for a 30-70 % higher rate than leucine oxidation (40). Despite this greater absolute oxidation rate, the BCAA's have received a great deal of attention for a number of reasons. Firstly, early work by Ahlborg et al. (4) showed that during 4 hours of cycle ergometry @ 30 % VO_{2peak} , the BCAA's were released from the splanchnic bed and taken up by active muscle. In contrast, all of the other AA's assayed for were taken up by the liver. This was some of the earliest evidence indicating the ability of skeletal muscles to oxidize BCAA's. Secondly, BCAA's transaminase (BCAAT) activities are greater than BCOAD activities in skeletal, diaphragm, and heart muscle, thus allowing

maximal BCAA oxidation to occur without being limited by BCAAT activity (97). Thirdly, under catabolic conditions such as fasting and prolonged exercise, the BCAA's are oxidized at an increased rate whereas the metabolism of the majority of other AA's are not affected (40). Some of the aforementioned research indicates that under conditions of reduced substrate availability, elevated protein breakdown or depressed protein synthesis, the BCAA's may serve as required energy sources for muscle as well as providing a pathway for gluconeogenesis (2,40). The metabolism of BCAA's is therefore uniquely initiated in skeletal muscle as opposed to the liver. Of the 3 BCAA's, leucine has been investigated to the greatest degree and will thus be discussed further in the forthcoming section.

2.4. Leucine Oxidation.

Leucine is an essential AA. Thus, in the post-absorptive state, plasma leucine must be derived from the catabolism of various body proteins. Leucine is unique as a purely ketogenic AA and when oxidized yields acetyl CoA and ultimately CO_2; therefore, relative to other AA's, the oxidation of leucine is easier to study (12).

An early study by Brooks and White (1981)(113) showed an increased AA oxidation during exercise using an *in vivo* model. They compared the oxidation rates of ^{14}C labeled, glucose, alanine and leucine during treadmill exercise in rodents. This particular study was designed to further examine the findings of previous work (16,40) where an increased leucine decarboxylation was observed using a rodent *in situ* preparation (113). Using ^{14}C infusions, White and Brooks (113) found that, with light exercise, leucine oxidation was increased above resting levels and with more intense

exercise they observed an even higher oxidation rate. In addition, a higher $^{14}CO_2$ excretion was seen following a ^{14}C-alanine injection as compared to the ^{14}C-glucose injection, thus suggesting that alanine can be disposed of independently of the glucose-alanine cycle.

To date, no study has longitudinally examined the effects of endurance training on amino acid metabolism during exercise in humans. In rodents, Henderson et al.(51) observed that training increased whole-body leucine turnover and oxidation during exercise at a given exercise intensity (easy (17 m/min) and moderate (25 m/min)). This could be due to a training-induced increase in muscle branched-chain oxo-acid dehydrogenase (BCOAD), which is the rate limiting enzyme for branched chain amino acid metabolism. In contrast, Hood and Terjung (57), found that training reduced the relative contribution of leucine oxidation to VO_2 in the electrically stimulated, perfused rat hindquarter preparation. These authors speculated that BCOAD may have been activated less in trained muscle during exercise due to smaller perturbations of energy homeostasis (i.e. higher ATP:ADP ratio). The authors therefore suggested that the increase in whole body protein oxidation previously reported by Henderson (51) must have been due to an increase in liver BCOAD activity and not skeletal muscle. However, the extent of the increase in leucine oxidation found in rodents was much less than the increase found in various human studies (49,116,117). Human studies have shown that leucine oxidation can increase up to 3.7 fold above rest during submaximal exercise (116), whereas the studies performed in rodents have shown modest 1.45 fold increases (51). The lesser amount of protein oxidation in rodents may be accounted for by their

relatively smaller proportion of muscle mass to body weight (~45 % in rodents and ~ 60 % in humans) as well the differences in relative tissue distributions of BCOAD enzyme, with rodents distribution being ~ 10 % in muscle and 70 % liver whereas humans are ~60 % in muscle and 30 % in liver (68).

Although the effects of endurance training on protein metabolism in humans has yet to be examined longitudinally, cross-sectional data does exist. Lamont et al (72) have recently examined leucine kinetics in endurance trained and sedentary humans. Sedentary controls and trained athletes were matched for age, gender, and body weight. Kinetic studies (L-[1-^{13}C]leucine and L-[^{15}N]lysine infusions) were then performed during 3 h of rest, 1 h of cycle ergometry exercise @ 50 % VO_{2peak} (i.e. relative intensity) and 2 h of recovery. Results showed an increase in leucine oxidation for both groups, from rest to exercise, when expressed per body weight or lean body mass. Trained subjects had a greater leucine rate of appearance and an increased leucine oxidation during exercise and recovery as compared with their sedentary counterparts on the basis of body weight. However, all between-group differences were eliminated when leucine kinetics were corrected for fat-free tissue mass. Therefore, the correction of leucine kinetics for fat-free mass may be necessary to compare groups when examining cross-sectional data.

It seems clear that leucine oxidation is increased during exercise (49,90,92), thus implicating an increase in net protein breakdown. Alternatively, protein breakdown may occur to fuel the amino nitrogen needs of protein synthesis post exercise. Interestingly, while protein breakdown is increased in response to exercise, rate of urea appearance

does not appear to increase acutely from rest to exercise (18,116,117). These data seem to contradict one another considering one would expect that with an increase in leucine oxidation one would also see an increase in urea production. A possible explanation for this discrepancy may lie in the synthesis of acute phase plasma proteins that increase during and after endurance exercise (19). The increased alanine and thus nitrogen flux seen during exercise may be accounted for, in part, by incorporation into these acute phase proteins (19) (fibrinogen and fibronectin) rather than urea (116,117). It can then be deduced that acute phase proteins may in fact be the amino nitrogen sources responsible for protein synthesis following exercise (106).

Despite this apparent lack of increase in plasma urea concentration with endurance exercise, numerous investigators have found increases in 24 h urine urea levels when collected on days involving a bout of prolonged endurance exercise.(27,28,99). The greater urea N excretion suggests that the AA's released from protein breakdown are oxidized and/or used as gluconeogenic precursors (4).

2.5. Branched-chain oxo-acid dehydrogenase (BCOAD).

2.5.1. Introduction.

Principle:

$$RCOOCO_2H + NAD^+ + CoASH \xrightarrow{(BCOAD)} RCOSCoA + NADH + H^+ + CO_2 \quad (11)$$

BCOAD (also referred to as branched-chain oxo acid dehydrogenase) is a multi-enzyme complex located In the inner surface of the inner mitochondrial membrane. A single enzyme exists which oxidatively decarboxylates all 3 branched-chain 2-oxo acids

derived from leucine, isoleucine and valine. BCOAD activity is regulated by phosphorylation/dephosphorylation, with phosphorylation causing inactivation. In many tissues (including skeletal muscle) BCOAD is a key enzyme in the degradation pathway of the branched-chain amino acids: following an initial (reversible) transamination reaction (BCAAT), the resultant BCOA's undergo an irreversible oxidative decarboxylation reaction according to the above equation. Some of the BCOA's are transported from the muscle to the liver where they are oxidized, because activity of the transaminase is much higher than the activity of BCOAD in muscle (63). The activity of BCOAD is much greater in the liver than in the muscle under normal conditions in rodents, and thus when the muscle activity of BCOAD is low, the BCOA's produced by the transaminase reaction are oxidized by the liver (86). However, when muscle BCOAD activity is high, the BCOA's are oxidized by the muscles. Availability of substrate (BCOA's) and changes in the activity of BCOAD determine the oxidation rate of the BCAA's in a given tissue and condition (75).

2.5.2. BCOAD activation: Dietary, exercise and training effects.

BCOAD activity is affected by a variety of factors, yet a complete understanding of its regulation is still in question. The role of exercise has been studied most extensively, demonstrating that activation of BCOAD increases in proportion to exercise duration. Kasperek et al (63) were among the first to determine BCOAD activity in rodent heart, liver and muscle during a 120 min bout of treadmill exercise. Percent activation in skeletal and heart muscle increased by 10 and 5 fold, respectively, by the end of exercise while significant increases were also reported 30 min into exercise. No

increase was found in liver. The results from this study furthered the idea that protein oxidation during exercise played a greater role than once thought.

Taking the study one step further, Kasperek et al (64) examined the effect of the same bout of exercise (2 hr @ 27 m/min) upon skeletal muscle and plasma BCAA and BCOA concentrations while also measuring muscle BCOAD activation. All measurements were taken at the end of exercise (2 hr) and 10 min post exercise. All values for BCAA's and BCOA's were found to be elevated in both muscle and plasma at both time points except for muscle BCOA's at 2 hr. It had previously been thought that a possible trigger for BCOAD activation was the accumulation of intramuscular BCAA's and thus BCOA's, yet these results suggest the contrary. In addition, an inverse correlation was found between BCOAD activation and muscle ATP concentration, such that, as BCOAD activation increased at the 2 h point, ATP was decreased and *vice versa* for the post 10 min time point. Unfortunately, BCOAD activity in this study was measured as total activity and not actual activity (% active in dephosphorylated form) which calls into question the generalizability of their results to other studies that have measured both activities.

One of the first studies to examine exercise BCOAD activation in humans was performed by Wagenmakers et al (112). Trained males were exercised on a cycle ergometer at ~70 % VO_{2peak} for 2 h. Skeletal muscle BCOAD activity was measured at t=0, 30, and 120 min. An increase in activity was seen at t=30 and t=120 (9.9 % and 17.5 % respectively) as compared to t=0. Increases in exercise induced BCOAD activity have been reported to occur as soon as 5 min into exercise (94). The group hypothesized that a

decrease in the ATP:ADP ratio or an increase in muscle BCOA's could be possible mechanisms for the increase in BCOAD activity.

Dietary manipulation has also been studied to increase our understanding of BCOAD regulation. Some early work done by Block et al (9) examined the effect of various protein diets (50, 25 and 9 % of total calories) on BCOAD activation. The results showed an increase in resting skeletal muscle BCOAD activation (~10 % and 16 % respectively) after a 25 and 50 % protein meal, while the 9 % protein meal did not cause any BCOAD activation (~2 %) above resting levels. Subsequent research began to investigate the role of both dietary modification and exercise bouts upon muscle BCOAD activation.

Wagenmakers et al (111) examined the effect of muscle glycogen concentration upon BCOAD activation in human skeletal muscle. They found an increase in BCOAD activation (3.6 fold) after 2 h of exercise at ~70 % VO_{2peak} in a glycogen depleted trial with no increase in BCOAD activation reported during the same exercise trial in a glycogen loaded and CHO supplemented trial. From these data, an inverse relationship has been shown to exist between muscle glycogen and BCOAD activation. No significant decrease in muscle ATP was noted in this study (contrary to other authors (64)) thus implicating other possible mechanisms for BCOAD activation.

Jackman et al (60) further enhanced our understanding by showing a combined effect of nutritional modification and exercise upon BCOAD activation. Six healthy subjects were exercised at ~ 75 % VO_{2peak} until exhaustion on three separate conditions: low muscle glycogen, low muscle glycogen plus BCAA supplementation during exercise

and high muscle glycogen. The results were concordant with Wagenmakers et al (111) in that the greatest muscle BCOAD activation occurred in the low glycogen plus BCAA supplementation while activation was attenuated in the high glycogen situation (43 and 12 % respectively). These results suggested that both BCAA's and muscle glycogen status (cellular energy status) have an effect upon the regulation of BCOAD activation during exercise.

To further examine this issue, van Hall and colleagues (108) studied the effects of low muscle glycogen and BCAA supplementation upon BCOAD activity in skeletal muscle during exercise. Ten male subjects were studied at rest and after a 90 min bout of one-leg knee-extensor exercise at 65 % of their maximal one-leg power output. Five subjects were studied after a BCAA bolus (308 mg•kg body wt^{-1}) and 5 were studied in the normal and low muscle glycogen states. Results showed similar findings to previous research (60) in that BCOAD activation was significantly higher in the low glycogen leg after exercise and that BCAA supplementation caused an increase in both resting and post-exercise BCOAD activation. The authors concluded that the mechanisms by which low muscle glycogen and BCAA supplementation regulate BCOAD are different. Grounds for this conclusion were primarily based on the inability of low muscle glycogen to stimulate BCOAD activity at rest, whereas, BCAA supplementation was able to increase activation both at rest and during exercise.

The most recent study examining BCOAD regulation was published by Bowtell and colleagues (10). Subjects were placed on a high (1.8 g protein•kg^{-1}•day^{-1}) and low (0.7 g protein•kg^{-1}•day^{-1}) protein diet to examine their whole body protein metabolism

before, during and after a 2 hr bout of treadmill walking @ 60 % VO_{2peak} . Exercise induced a 2 fold increase in both leucine oxidation and BCOAD activation for both groups, however, leucine oxidation was greater before (+ 46 %) and during (+ 40 %, $P < 0.05$) the first hour of exercise in subjects consuming the high rather than low protein diet, with no concordant increase in BCOAD activation. One would inherently expect a correlation between the amount of protein oxidized and a parallel increase in BCOAD activation. No clear explanation for this apparent discrepancy was offered in the discussion of this paper. One can therefore infer that there exists the possibility of an increase in hepatic leucine oxidation without a proportional increase in BCOAD activation, thus implicating other mechanisms for the increased leucine utilization. Perhaps an increase in hepatic BCOAD activation could have accounted for this difference despite the normal decrease in hepatic blood flow seen during exercise.

To date there have been no studies examining the effect of endurance training upon muscle BCOAD activation during exercise in humans. However, one could speculate that there would be a lesser metabolic perturbation, combined with an increased muscle glycogen concentration, which may cause an attenuation of the % BCOAD activation post-training (41,42,57). According to the current literature, it is evident that the regulation of BCOAD is complex and multi-faceted. It is known that, as protein oxidation during exercise increases, so does the percent activation of skeletal muscle BCOAD in rodents and humans (10,63,112). In addition, various authors have also implicated an increase in muscle BCOA's (112) and a decrease in the ATP:ADP (64)

ratio as possible triggers for BCOAD activation, however, these studies have been unable to definitively isolate the mechanisms by which activation occurs.

2.6. Endurance training and habitual protein intake

In mid 1970's Gontzea et al. (1974)(45), using the nitrogen balance technique, found that at least for the first 20 days of an endurance training program, daily protein needs are as high as 1.5 $g \cdot kg^{-1} \cdot day^{-1}$ (188 % RDA). Tarnopolsky and colleagues (105) found that the protein intake required to remain in positive nitrogen balance to be 1.37 $g \cdot kg^{-1} \cdot day^{-1}$ (171 % RDA) for highly trained endurance athletes. In a similar study, Meredeth et al (81) recommended a protein intake of 1.26 $g \cdot kg^{-1} \cdot day^{-1}$ (158 % RDA) for actively endurance trained men. In addition to these studies, various other authors have found similarly elevated protein requirements with endurance training (15,33).

In contrast to these findings, data from several studies have shown that protein requirements may become less with endurance training (17,46,107). One main factor for this discrepancy may be the intensity of exercise at which subjects were trained and/or tested. In addition, the studies that observed an increase in protein requirement had a much higher exercise intensity and energy expenditure than those that did not find an increase.

2.6. Stable isotope methodology

A stable isotope refers to the form of an element that differs in its number of neutrons (atomic weight) while its protons (atomic number) remain the same. Common isotopes used for human experimental purposes are ^{13}C, and ^{15}N which occur naturally on

earth with natural abundance's of 1.11 % and 0.37 % respectively (117). Such molecules do not undergo spontaneous radioactive decay and are thus safe to use in human subjects.

The most common tracer used in assessing protein turnover is L-[1-^{13}C]-leucine (87,104,116). The single pool model for assessing protein turnover utilizes the aforementioned tracer. In this type of analysis, the body is considered to be a single pool, whereas in more complex methods the body is divided into separate compartments and the exchange between these different pools is measured. For the purposes of this review, the topic will be restricted to the relevant single pool model. Practically speaking, the isotope is administered via a primed-constant infusion, meaning that L-[1-^{13}C] -leucine is dissolved in a sterile saline solution and is administered as a bolus followed by a constant infusion. The infusion is 'primed' to achieve an isotopic steady state in less time and is referred to as the primed-constant infusion technique (115). At isotopic steady state, the rate of appearance (Ra) of the unlabelled leucine (tracee) into the sampling pool is equal to the rate of disappearance (Rd) of the tracee out of the sampling pool. Ra in the single pool model represents endogenous production (B) and exogenous intake (I) of unlabelled leucine, while Rd represents the sum of the rate of irreversible tissue uptake (i.e. protein synthesis) (S) and loss by other avenues such as oxidation (O) (115). The single pool model is defined by the equation: $Q = S + O = B + I$; where Q = flux rate; S = the rate of incorporation of label into protein (non-oxidative leucine disposal (NOLD)); O = the rate of oxidation of amino acid or oxidation of the chosen amino acid; B = the rate of protein breakdown or endogenous Ra, and I = exogenous amino acid intake (i.e. food). This method is used to determine whole body protein turnover and, when combined with

expired CO_2 collection, can be utilized to ascertain estimates of whole body protein synthesis as the non-oxidative leucine disposal (NOLD) (115).

Chapter III. Gender Differences.

3.1. Introduction.

Gender differences in substrate utilization during endurance exercise is an area of study that began in the 1970's with researchers such as Costill et al. (22) who examined the differences in fat metabolism between genders during exercise. Over the past 20 years there has been an impressive shift in technology which has allowed researchers to examine gender differences in greater detail. Numerous studies within the past 15 years (35,58,59,87,99,105) have used stable isotopes and other techniques (i.e. direct muscle measurements, indirect calorimetry) to more fully understand the oxidation kinetics of substrates such as fats, carbohydrates (CHO's) and proteins (PRO's) during exercise. The following represents a critical review of the current literature outlining the most validated techniques and reliable research protocols for studying substrate utilization differences between the genders. Of particular interest to the current review is the effect of gender upon the differences in substrate utilization during endurance exercise and the determination of whether or not any differential training effects are present.

3.2. Substrate utilization.

Throughout the literature there exists consistent methodological problems between studies making them difficult to interpret. When examining substrate metabolism one, must be aware of how the experimenters matched their male and female subjects. Current training status and past training history must be included to equally

match genders. These precautions are taken to control for individuals who may naturally have relatively high aerobic capacities (VO_{2peak}) which are not due to training (i.e., genetic factors). According to Brooks et al. (14), substrate "crossover" (the point at which CHO oxidation predominates over fat oxidation) is partially dependent upon one's training status. In other words, two subjects may have the same VO_{2peak} kg FFM and yet one may be much more highly trained than the other and will thus experience a substrate "crossover" and/or ventilatory threshold later than the other lesser trained subject. Intra-group variation must also be controlled for. Males and females must also be matched to other members of the same gender within each group. If the variability within groups is too high then any data pooled within a gender is less representative of what is actually happening because of the large intra-group variability.

The importance of matching for training status can be illustrated in a study done by Hurley et al (59) who examined the effects of training upon metabolic responses to sub-maximal exercise. Subjects were tested before and after a 12 week aerobic training program during a 90 min, 65 % VO_{2peak} cycle ergometer test. Results showed that plasma FFA/ glycerol concentrations and RER values during exercise were all lower after training (although plasma FFA and glycerol still increased during exercise, but their responses were blunted after training). The proportion of caloric expenditure derived from fat during the bout of exercise (calculated from RER data) increased from 35 % before training to 57 % after training. Glycogen utilization was 41 % lower and the decrease in quadriceps intramuscular triglyceride (IMTG), during exercise, was two times greater in the trained state. Hurley concluded that trained male athletes rely upon a

greater energy derivation from IMTG as compared to non-trained males. Although this study did not examine the differences between the genders, it did illustrate the point that matching for training status is vitally important due to the metabolic adaptations that can occur with training thus making improperly matched groups, not comparable to one another. To summarize, the goal of subject matching is to equate males and females in terms of VO_{2peak} /kg FFM, training status, and training history. Controlling for these variables will enhance the validity of any gender difference findings.

Perhaps the most accurate and comprehensive subject matching protocol cited in the literature is that of Tarnopolsky et al., 1990 (99). In this study subjects were matched according to VO_{2peak} /kg FFM, a 6-week pre-study training log (outlining running distance per week and duration per workout), and an exercise history (outlining the subjects length of participation in endurance running, level, and frequency of competition). Correcting VO_{2peak} to FFM is necessary because of the difference in fat mass between the genders. Males are typically leaner than females and thus if a male and female were to have the same VO_{2peak} /kg BW, their VO_{2peak} /kg FFM would not equate due to the difference in fat mass between the two. In this study, subjects completed a 15 km treadmill run. Throughout the exercise bout (~90 min @ 63 % VO_{2peak}), females had significantly lower RER values, indicating a lower proportion of total CHO oxidation. Consistent with the RER data, muscle glycogen utilization was significantly lower in females (approx. 68 and 41 % decrease from pre- to post-exercise values for males and females, respectively).

To further examine gender specific differences, Tarnopolsky et al. (101) published a study examining carbohydrate loading and metabolism during exercise in men and women. When trained males and females were tested during two separate randomized trials (one trial they consumed 55-60 %CHO for four days and the other trial they consumed 75 % CHO for four days prior to the exercise bout) males were shown to have a 4 % increase in muscle glycogen and a 45 % increase in performance time on an 85 % VO_{2peak} ride, while the females had a 0 % increase in muscle glycogen content and only 5 % increase in performance time. The females may have increased muscle glycogen because the absolute energy intake was less for the females as compared to the males, and/or there may be differences in glycogen synthase or hexokinase activities, or GLUT 4 concentration between males and females.

In the years prior to proper gender matching, many studies were done in an attempt to better understand the metabolic differences between genders. One of the first studies comparing substrate utilization between the genders was conducted by Costill et al. in 1979 (22). This study examined the differences in lipid metabolism between male and female endurance athletes. The results showed that during a 60 minute bout of exercise at 70 % VO_{2peak}, both males and females derived similar amounts of energy from the oxidation of lipids. Genders were matched by VO_{2peak} /kg BW (body weight) not per FFM (fat free mass), training distances per week were included but not duration's, there was no mention of female menstrual status, and finally, subjects between genders were not properly age matched, the males were significantly older than the females in this particular study. One of the first studies to more appropriately match subjects by VO_{2peak}

/kg FFM was conducted by Blatchford et al. in 1985 (8) who examined the plasma FFA responses to prolonged walking in untrained men and women. They matched their subjects according to VO_{2peak} kg FFM which is used to account for a greater, on average, fat mass in females (13). Subjects were then exercised at 35 % VO_{2peak} for 90 minutes. Females were found to have lower RER values (see below for explanation) and higher plasma free fatty acid (FFA) and glycerol concentrations at all time points during exercise compared to the males. Based upon these RER and plasma FFA/glycerol data, females were shown to oxidize proportionately more fats than males during mild prolonged exercise. Froberg et al. (35) published a study examining the sex differences in endurance capacity and metabolic responses to prolonged, heavy exercise. Normally active subjects were matched according to the duration and intensity of their leisure time and VO_{2peak} kg FFM. On two separate occasions the subjects were instructed to ride until exhaustion at 80 % and 90 % of their VO_{2peak}. Results indicated that at 80 % of their VO_{2peak}, females performed significantly longer than males with a significantly lower RER throughout the exercise bout. At 90 % VO_{2peak} there were no gender differences. This study suggested that female subjects performed better as a result of "a later occurrence of glycogen depletion than males". Several problems evident within this study may help explain as to why the women significantly out-performed the men at 80 % of VO_{2peak}. Firstly, there was no reference to the phase of menstrual cycle which may have provided the females with a greater circulating FFA concentration if tested in the luteal phase of their cycle (89), secondly, there was no muscle analysis to confirm their inferred opinion that females oxidized less glycogen during their 80 % bout, and thirdly,

RER is not an accurate indicator of substrate selection at 80 - 90 % VO_{2peak}. For most untrained individuals, an average ventilatory threshold is within the range of 65-75 % of VO_{2peak} (13) thus making the intensity of 80 % too high to rely upon RER data to calculate substrate usage (RER > 1.0).

3.3. Estrogen.

In recent years, animal research has provided new insight into the effects of estrogen upon metabolic responses, at rest, as well as during exercise. Kendirck et al. (67) studied the effect of 17-β-estradiol (E_2) upon tissue glycogen metabolism in exercised oophorectomized female rodents. Various concentrations of E_2 were administered to the rodents for 5 d/wk for 4 weeks, after which time the rodents were exercised to exhaustion on a treadmill. They found that in all E_2 conditions, glycogen was spared above control levels (i.e. there was a greater glycogen content in heart, muscle & liver after exercise). As E_2 concentration increased, so did the glycogen sparing effect until a plateau concentration of 10μg/100g BW was reached. In a follow-up to their first study, Kendrick et al (66) examined the effect of E_2 upon lipid availability and tissue glycogen metabolism. As in their last study, they found that after 3-6 days of E_2 administration exercise, the treated male rodents ran longer with less glycogen depletion. In addition, in a second experiment they administered E_2 for 5 days and exercised the rodents for 2 hours sub-maximally. They found that, within the E_2 group, there was an elevated plasma FFA concentration which may have been correlated with the decrease in tissue glycogen utilization during the bout of exercise. To further support Kendrick's data, Rooney et al. (93) showed that after a 5 day E_2 administration and a

subsequent 2 hour submaximal treadmill run, male rodents showed significant glycogen depletion between 90 & 120 minutes of exercise, whereas the control rodents experienced significant glycogen depletion after only 30 minutes. Once again, the plasma FFA concentration was greater in the E_2 treated groups and may have contributed to the observed glycogen sparing effect. To further validate this data it would be advisable to examine intramuscular triglyceride concentrations before and after exercise to account for the decrease in glycogen utilization. To summarize, these data suggested that E_2 was likely to be an important factor in promoting lipid oxidation while sparing carbohydrate (glycogen) in male and oophorectomized female rodents.

3.4. Menstrual cycle.

The female menstrual cycle is an essential variable to control for when conducting gender difference research. Females must be normalized to one phase of the cycle in order to decrease intra-group variability. Reinke et al. (89) studied the effect of menstrual cycle upon CHO and lipid metabolism in normal healthy females. At rest, females were found to have greater plasma FFA concentrations in the luteal phase of their cycle. Nicklas et al. (83) also found a discrepancy between menstrual phases. This study examined the effect of muscle glycogen and substrate responses to exercise at the midfollicular (days 4-11) and midluteal phases (days 18-25) of their cycles. Normal healthy females performed a 60 % VO_{2peak} glycogen depletion ride for 90 minutes and 3 days later cycled until exhaustion at 70 % VO_{2peak}. There was a trend ($P < 0.05$) for females to have greater endurance in the midluteal phase of their cycle and, in addition, they also showed a significantly greater muscle glycogen repletion. Nicklas concluded

that female responses to exercise are affected by the phase of their menstrual cycle. Lamont et al (70) reported that females who completed 60 min of cycle ergometry at 70 % VO_{2peak} had a greater urinary and total (urine + sweat) urea excretion during the midluteal phase of their menstrual cycle, as compared to the follicular phase. As a result of such research, recent gender studies, for the most part, normalize female subjects to the midfollicular phase of their cycle for exercise testing (87,99).

3.5. Protein Metabolism.

Much of the literature within this area is primarily focused upon male athletes (87,103-105,116). Very few studies have actually examined human gender differences in protein metabolism. Tarnopolsky et al. (99) found that during a 24 h period of urine collection on the day of a 65 % VO_{2peak} exercise bout, males had a significantly greater (by 32 % (relative to rest day)) urea N excretion than equally exercised females. The apparent lack of increase in urea N excretion would suggest that females did not oxidize significant amounts of protein during submaximal endurance exercise. Tarnopolsky suggested that this discrepancy may be related to the observed greater glycogen depletion in males which could have induced the greater protein catabolism via increased BCOAD activity. Data to support this explanation can drawn from a study published by Lemon et al. (76) showing that men on CHO depleted diets catabolized significantly more protein during submaximal endurance exercise than did the same men on CHO loaded diets. Furthermore, these results wold be predicted from the BCOAD literature presented above.

Further support for these findings have been published by Philips et al (87) who examined the gender differences in leucine kinetics and nitrogen balance in endurance athletes. Six males and six females were equally matched according to the same criteria outlined by Tarnopolsky et al. (99). Subjects were then adapted to their habitual diet for 10 days at the Canadian RNI (Recommended Nutrient Intake) for protein intake. After the 10 day adaptation, a 3 day (two days of rest and exercise day 3) nitrogen balance assessment was conducted. Results showed that both male and female athletes were in negative nitrogen balance when consuming the RNI for protein intake. Leucine kinetics (measured by a primed constant infusion of L-[1-^{13}C]leucine) showed that males oxidized a significantly greater amount of leucine (70 %) during the 90 min 65 % VO_{2peak} exercise bout than females. Explanations for these findings once again relate back to the greater oxidation of lipids in females thus allowing inducing glycogen sparing (99). Such a sparing effect would cause there to be a lesser reliance upon protein for fuel during exercise thus decreasing the amount oxidized during the exercise bout. Tarnopolsky et al. (101) also found that females excreted less urinary urea and had lower blood urea nitrogen concentrations when compared to men during submaximal endurance exercise. It is thought that, in addition to the glycogen sparing effect seen in females, branched-chain keto acid dehydrogenase (the rate-limiting step in branched chain amino acid oxidation) activity and/or regulation may be influenced by gender (112).

Overall, this review has demonstrated that fat and carbohydrate are the predominant fuels during sub-maximal endurance exercise in humans. Protein can also be oxidized, however, this usually only amounts to 2 – 8 % of the total energy cost of the

exercise. Chronic endurance training induces a shift in the selection of substrates from carbohydrate to lipid. There is some evidence that endurance training will result in an attenuation of the protein contribution to the total energy cost of exercise, however, this has been given much less attention than the adaptive responses of the other macro-nutrients. Females appear to derive a greater proportion of their energy from the oxidation of lipid, which in turn "spares" muscle glycogen. The glycogen "sparing" may explain the apparent attenuation of protein oxidation in females as compared to males. To date, no study has examined the effects of chronic endurance training upon: gender differences in metabolism nor adaptations in protein oxidation.

Hypotheses & Objectives.

Hypotheses:

1. Protein oxidation will decrease during exercise after endurance training.
2. BCOAD activation will be attenuated during exercise post-training.
3. The attenuation of protein oxidation during exercise will be correlated with intra-muscular glycogen concentrations following training.

Objectives:

1. To determine the effect of endurance training upon protein oxidation during exercise.
2. To determine the effect of endurance training upon BCOAD regulation during exercise.
3. To determine the effect of endurance training upon oxidative enzyme adaptations (i.e. citrate synthase, complex I-III).
4. To determine any gender differences within all outcome measures.

Chapter IV. The Research Paper.

Endurance exercise training attenuates leucine oxidation and branched-chain 2-oxo acid dehydrogenase activation during exercise in humans.

INTRODUCTION.

Endurance exercise results in an acute increase in leucine oxidation (30,71,87). Amino acid oxidation may contribute 3 – 5 % of the total energy cost during an acute bout of endurance exercise (30,87). For very well trained endurance athletes who are exercising at a high percentage of VO_{2peak} on a daily basis, this may result in a small increase in the requirement for dietary protein (34,77,105). Conversely, for relatively untrained individuals exercising at a more modest exercise intensity, there is no increase in amino acid requirements based upon 24h amino acid oxidation tracer studies (30). These observations are consistent with the fact that leucine oxidation is positively correlated with the intensity of exercise (77), yet are at odds with the phylogenetic logic that would predict that repeated physiological stressors (i.e., exercise training) should attenuate the oxidative flux through pathways in an attempt to preserve proteins that are essential to structure and/or function.

There is currently a controversy surrounding whether endurance exercise training increases, decreases or does not alter the oxidation of amino acids (51,57,72). From a whole body perspective, an early human study showed an attenuation of the exercise induced negative nitrogen balance with repeated exercise bouts (45). Lamont and co-workers have shown that endurance exercise training did not affect resting (73) nor the exercise-induced increase in (72) leucine oxidation. Two studies in rodents have

demonstrated that the trained animals actually oxidized more leucine as compared to untrained animals at the same absolute intensity (26,51). Conversely, Terjung and Hood found an attenuation in leucine's contribution to total energy consumption in trained rodents using and *in situ* hind limb model (57). This controversy is likely due to the use of different models (animal vs. human; *in situ* vs. *in vivo*), as well as the difficulty in matching the workload in trained vs. untrained muscle (i.e., absolute or relative intensity).

Leucine is oxidized in the mitochondria by the branched-chain 2-oxo acid dehydrogenase (BCOAD) enzyme (11). This enzyme is activated by de-phosphorylation (3) and is rate limiting to branched chain amino acid oxidation in primates (68). An acute bout of exercise increases skeletal muscle BCOAD activity by increasing the de-phosphorylated form from about 7 % to 25 % (64,96,112). This activation is correlated with a decrease in the phosphorylation potential, a decrease in pH, or a decrease in glycogen (64,111). Given that endurance exercise training attenuates glycogen utilization at the same absolute intensity of exercise (85), one would predict that this may lessen the activation of BCOAD and hence, attenuate the increase in leucine oxidation. An alternate hypothesis however, is that the increase in mitochondrial volume that occurs with endurance exercise (53) would lead to an increase in total BCOAD volume and abolish any beneficial effect conferred by a lessening of activation. To date, no study has determined the effect of endurance exercise training upon human muscle BCOAD activity.

In addition to the paucity of information on the effect of endurance exercise training upon BCOAD activity and amino acid oxidation, there is very little information on potential gender differences in amino acid metabolism during exercise (87,99). Furthermore, there is limited information on potential differences in the response to

exercise training (61). Studies have previously shown that exercise induced urinary urea excretion was lower in females as compared to males and this was correlated with a lesser glycogen utilization (99). A subsequent study demonstrated that L-[1-^{13}C]leucine oxidation was lower in females as compared to males at rest and during endurance exercise at 65 % VO_{2peak} (87). Furthermore, protein oxidation contributed less to total fuel oxidation during exercise, and nitrogen balance was less negative for the female as compared to male endurance athletes in the latter study (87). To date, there have been no studies that have examined whether there are gender differences in amino acid oxidation consequent to endurance exercise training and no studies have specifically looked for a gender difference in BCOAD activity.

Therefore, our primary purpose was to study the relationship between leucine oxidation and BCOAD activity during endurance exercise and to determine the adaptive response to a 38 day endurance exercise training program. A second major purpose was to determine the potential relationship between attenuated leucine oxidation in females and total BCOAD activity and/or the de-phosphorylated percent activity (% active form)

METHODS.

Subjects/Recruitment.

We recruited a total of 6 males and 8 females to participate in a longitudinal endurance exercise training study. The participants were young, healthy, and did not participate in any form of physical activity for more than 1 hour per week at the time of recruitment and had not participated in a formal endurance exercise training program for at least 6 months prior to the study. The females were all eumenorrheic for at least six months prior to the study and none experienced menstrual dysfunction consequent to the training program. Three of the females were taking oral contraceptives for at least six months prior to the study and continued to take them for the duration of the study. All

individuals completed 4-day weighed diet records to ensure that no individuals were on a restrictive energy intake and a medical history was taken to ensure that there were no medical contra-indications to participating in an exercise training study (i.e., anorexia, bulimia, diabetes, joint dysfunction). After this initial screen, the participants completed a maximal aerobic testing protocol on a cycle ergometer to determine VO_{2peak} and body composition was determined using dual energy X-ray absorptiometry (DEXA)(QDR 1000W, Hologic, Waltham, MA) scanning as described previously (69).

Of the original 14 participants, a total of six males and six females completed all aspects of training and analysis. One female was removed from final analysis since she became pregnant after four weeks of training, and one female dropped out after a week of training for personal reasons. The descriptive characteristics and habitual dietary analyses of the participants are found in Table 1. The most notable factor was the equivalence in maximal aerobic capacity (VO_{2peak}) between the genders, when expressed per kilogram of fat free mass (FFM).

Design.

i. Training.

The participants completed an initial pre-testing (PRE) trial (see below), followed by 38 ± 2 days of cycle ergometry training. The training was performed for 60 min per day (5 d/wk) at an intensity of approximately 60 % VO_{2peak}. Intervals were performed 1 d/wk for 30 min in the form of 1 min @ 100 % VO_{2peak} followed by 4 min @ 60 % VO_{2peak}. All training was performed on cycle ergometers (Monark, Varberg, Sweden). A heart rate monitor was used to ensure that individuals exercised at the target intensity. At the half-way point in the training study, all participants completed a second VO_{2peak} to re-confirm the heart rate:VO_2 relationship. Following completion of the training program, all subjects performed a final VO_{2peak} test. This was used to determine the effect of the

training upon aerobic power and also to determine the workload for the post-training test ride corresponding to 60 % VO_{2peak} (relative intensity trial (REL)). They also completed another post-training trial at the same absolute exercise intensity as the PRE-training (absolute intensity trial (ABS)). The order of the trials was randomly allocated and separated by four days. Prior to the post-training trials, the following regimen was adopted; end of training (day –5), test day (ABS or REL) (day –4), rest day (day – 3), exercise for 60 min @ 60 % VO_{2peak} (day – 2), rest day (day –1), and final test day (ABS or REL) (day 0). Diet records were also completed for four days in the final week of training, such that isoenergetic, isonitrogenous, flesh-free diets were provided to each individual. DEXA (QDR 1000W, Hologic Inc., Waltham) scans were completed on each subject at the mid-point between the two post-training trials (same time of day as for the pre-training DEXA scan). All females were tested in the mid-follicular phase of the menstrual cycle for PRE, ABS, and REL testing trials.

ii. Testing trials.

Each of the PRE, ABS, and REL testing trials were identical with the exception that only one post-training, pre-exercise muscle biopsy was performed as described below. Each subject was tested following a 10 h fast at the same time of the day between trials (0800 – 1200 h). Twenty-four hour urine collections were completed for the rest (-1) and exercise testing days and were stored at 4°C for 24 h, following this, a 10 ml aliquot was stored at –50°C for subsequent analysis of urea nitrogen and creatinine.

Upon arrival to the laboratory, subjects had their mass determined on a beam scale, followed by placement of a 20 Ga plastic catheter into an ante-cubital vein. A 5 ml sample (t= -120 min) was collected into a heparinized tube, spun immediately and the plasma was stored at – 50°C for subsequent analysis of baseline (α-ketoisocproic acid α-KIC) isotopic enrichment. Following this, a primed-continuous infusion of L[1-

^{13}C]leucine (99 % ^{13}C enrichment; MSD Isotopes, Pointe Claire, Quebec) was started (prime = 7 μmol•kg^{-1}; continuous = 7 μmol•kg^{-1}•min^{-1}). Shortly after this, a second plastic catheter was placed into a contra-lateral distal forearm vein and a heating pad was applied for the duration of the experiment to "arterialize" the blood.

After 60 min of isotope infusion, a muscle biopsy was taken from the *vastus lateralis* muscle using a modified Bergström needle (5 mm diameter) with suction modification. A piece approximately 30 mg was immediately removed and placed on a moistened saline tissue for immediate processing for the determination of branched chain oxo-acid dehydrogenase (BCOAD) activity as described below. Another piece (~ 75 mg) was immediately quenched in liquid nitrogen and stored at – 176°C for subsequent analysis of; NADH-cytochrome c oxidoreductase (Complex I-III) and citrate synthase (CS) activities and for glycogen, phosphocreatine, creatine and adenosine triphosphate (ATP) concentrations (see below). A second incision was also made 10 cm proximal to the first incision at this time for the pre-training and for the first of the two post-training trials for a post-exercise biopsy. For the first of the two post-training trials, the contra-lateral leg was sampled and for the second of the two post-training trials a post-exercise sample was taken from a randomly allocated leg. Thus, a total of five muscle biopsies were taken from each subject for the entire study.

After 105 min of isotope infusion, another blood sample was taken for α-KIC determination. The subjects were then transferred to an electronically braked cycle ergometer (Lode, Netherlands) and rested quietly for 15 min prior to a final resting blood sample (t=0). A 5 ml sample was collected into chilled heparinized tubes for determination of glucose and lactate concentration.

Resting (t=0) measurements of oxygen consumption (VO_2), carbon dioxide production (VCO_2), and respiratory exchange ratio (RER) were then completed over a 5

min period using an open circuit gas analysis system, as described previously (102). They then completed 90 min of cycle ergometry with respiratory and blood samples collected at 30, 60, and 90 min of exercise. The intensity for each ride was calculated for each of the three trials as described above (PRE, ABS, REL). At 90 min of exercise the subjects stopped cycling and a post-exercise biopsy was performed immediately for all three trials. This biopsy was used for the determination of BCOAD activity and glycogen, PCr, Cr, and ATP concentrations.

Sample Analyses. Skeletal Muscle.

Branched chain oxo-acid dehydrogenase activity.

Assay review.

Whole tissue homogenates, mitochondria, and extracted partially purified enzymes have all been used to make estimates of the degree of BCOAD activation in various tissues (68). For estimation of the activity present *in vivo* (i.e., "actual"; also called basal, initial or expressed activity); inhibitors of the BCOAD kinase (α-KIC, α-chloroisocaproate, ADP, dichloroacetate) and phosphatase (NaF or KF) are added to the homogenization, isolation and assay buffers. "Total" activity can only be measured after complete activation of the complex; this has been achieved by (i) pre-incubation of homogenates, mitochondria or precipitated enzyme fractions, in the presence of high Mg^{2+} concentration in order to activate the endogenous phosphatase of the BC-complex, or (ii) pre-incubation of a precipitated enzyme fraction with a broad-specificity protein phosphatase. Activities of BCOAD have been measured with and without activation

using both spectrophotometric and radiochemical assays with [1-^{14}C]- -KIC (2-oxo acid of leucine) or [1-^{14}C]- -KIV (2-oxo acid of valine) as substrate (38).

BCOAD activity was determined using method developed by Wagenmakers et al (112). Briefly, a 20 to 40 mg piece of wet muscle was immediately blotted and rapidly weighed before being homogenized in an ice cold buffer (0.25 M sucrose, 10mM tris base, and 2mM EDTA (~ 1:11.5, wt/vol; pH 7.4)). The homogenate was aliquoted into two halves and processed separately. The aliquot used for the determination of total enzyme activity and active (i.e. dephosphorylated) enzyme fraction, was pre-incubated with ADP (8.3 mM) and NaF (83.3 mM), inhibitors of the BCOAD kinase and phosphatase, respectively – while the aliquot used to determine total BCOAD activity was incubated with ADP only. After pre-incubation for 5 min at 37°C, 100 µl of 0.5 mM 2-oxo-[1-^{14}C]isocaproate (54.0 mCi/mmol; Amersham Life Science, Buckinghamshire, UK) was added to the sealed reaction vials. The samples were incubated for 10 min at 37 °C, and the reaction was terminated by injection of 3 M PCA. The $^{14}CO_2$ produced during the incubation was collected using ethanolamine-ethylene glycol (1:2, vol/vol) solution located in a plastic reservoir within the sealed reaction vials. After 90 min, the reservoir was removed and placed in a 20 ml scintillation vial with 10 ml of scintillation cocktail (Beckman, Fullerton California). The specific activity of the $^{14}CO_2$ ethanolamine-ethylene glycol was then determined in a liquid scintillation β-counter (Phillips, PW 4700 Liquid Scintillation, Holland), and the amount of CO_2 produced during the 10-min incubation period was calculated. Activities of BCOAD were calculated from the specific radioactivity of the substrate (10 000 dpm/nmol) and are

expressed in nanomoles of 2-oxoisocaproate decarboxylated per minute per milligram wet weight of muscle. The % active BCOAD was calculated by dividing the actual activity (activity of the dephosphorylated aliquot) by the total activity aliquot. Activities were determined in duplicate or triplicate, and a blank correction was subtracted (incubation of buffer without homogenate). The coefficient of variation (CV) for triplicate samples averaged 16.2 %.

Complex I-III and citrate synthase activity. A 10 – 30 mg piece of wet muscle was homogenized in 200 µl of buffer consisting of 5mM potassium phosphate, 1mM EDTA, 0.1 mM DTT; pH of 7.4. This homogenate was used for both of the following assays.

Complex I-III. One ml of reaction buffer (0.1 mM potassium phosphate and 1 mM Azide (pH ~ 7.0) was placed into each of two cuvettes. Thirty µl of cytochrome c (40 mg/ml) was then added with 5 µl of 1mM rotenone added to the rotenone-sensitive cuvette. The cuvettes were then mixed and 20 µl of muscle homogenate was added to each. Following and initial absorbance reading at 550nm (Shimadzu UV-1201, Japan), 10 µl of NADH (5 mg/ml) was added to each tube. The cuvettes were mixed and absorbances were read at 30, 60, 90, and 120 seconds. Rotenone sensitive complex I-III activity was calculated by subtracting the total-rotenone values (µmol cytochrome c reduced/mg wet muscle/min (µmol•min^{-1}•mg w.w.$^{-1}$)). The intra-assay CV was 7.6 %.

Citrate Synthase (CS). One ml of Tris buffer (0.1 M (pH 8.0)) heated to 37°C was added to a cuvette containing 10 µl of DTNB (4 mg/ml of Tris buffer) and 2 µl of acetyl CoA (30mM in H_2O). To this, 10 µl of muscle homogenate was added and mixed. The spectrophotometer (Shimadzu UV-1201, Japan) was zeroed and 10 µl of Oxalo-acetic-acid (6.6 mg/ml Tris buffer) was added to start the reaction. Absorbances

were taken, at 412 nm, at 30 s for 2 minutes. The activity was calculated and expressed as $\mu mol \cdot min^{-1} \cdot mg\ w.w.^{-1}$. The intra-assay CV was 5.0 %

iii. *Glycogen, PCr, Cr, ATP.* Approximately 40 mg of frozen wet muscle was lyophilized, powdered to dissect out non-muscle elements, and stored at –50°C. Approximately 5 mg of powdered muscle was extracted using 0.5 M PCA containing 1mM EDTA:$2H_2O$, neutralized using 2 M $KHCO_3$, and assayed for ATP, PCr and creatine using fluorometric procedures adapted from (50).

The intra-assay CV for ATP and PCr were 4.6 % and 4.0 % respectively. Similarly, the intra-assay CV for creatine was 6.2 %.

Muscle glycogen determination was made using a method modified from that described by Bergmeyer et al (7). One hundred and sixty µl of 0.1 M NaOH (solution 1(S1)) was added to 2 – 4 mg of powdered muscle. Samples were then incubated for 10 min at 80°C. After cooling, a combined solution of 0.1 M HCl, 0.2 M citric acid and 0.2 M disodium hydrogen phosphate was added in a ratio of 1:4 (reaction to combined solution) (pH 7.4 ± 0.4). Forty µl of amyloglucosidase was then added and allowed to sit at room temp for 80 min. Liberated glucose was then assayed for spectrophotometrically at 340 nm by adding 25 µl of sample and 250 µl of reagent that consisted of 100 mM Triethanolamine, 40 mM KOH, 30 mM $Mg(Ac)_2.2H_2O$, 1 mM EDTA.$Na_2.2H_2O$, 0.75 mM ATP, 1 mM DTT, and 1 mM NAD brought to a pH of 8.2 with KOH. Absorbances were then measured @ 340 nm. Four µl of a hexokinase (HK)/glucose-6-phosphate dehydrogenase (G6PDH) solution (prepared by diluting 228 units of G6PDH and 200 units of HK with 200 µl each and combining them into one solution) was then added.

The cuvette was then mixed and the absorbance was read after 15 min at endpoint. Calculations are based on standard curve data for bovine glycogen and expressed as $mmol \cdot kg\ dry\ wt^{-1}$. The intra-assay CV was equal to 3.8 %.

Urine analysis.

Urea nitrogen was determined colorimetrically using the urease/nitroprusside reaction (Procedure # 640, Sigma). Urine creatinine was determined using a picric acid method (Procedure # 555, Sigma). The intra-assay CV's were equal to 4.5 % and 1.8 %, respectively.

Plasma analysis.

Plasma glucose was determined colorimetrically using the glucose oxidase (kit # 315, Sigma).

Plasma insulin was determined a standard single antibody radioimmunoassy (Coat-A-Count # TKIN5, Intermedico).

Plasma lactate was determined using a lactate analyzer (Model 23L, YSI Scientific).

The intra-assay CV's for the 3 aforementioned assays were 5.3 %, 2.5 %, and 3.2 %, respectively.

Statistics.

Statistical significance was determined by analysis of variance with repeated measures design. When a significant main effect and/or interaction occurred, the location of pairwise differences was determined using Tukey post hoc analysis. The level taken to indicate significance was $P < 0.05$. All data in tables presented as ± SD, while all figures presented as ± SEM. Statistica 5.1 (Statsoft Inc., Tulsa) was used for all statistical analyses.

RESULTS.

By design, the exercise intensity in percentage of VO_{2peak} was the same for the exercise trial prior to training (PRE) and the post-exercise trial at the same relative (REL) intensity, and was similar between genders (Table 2). Similarly, the power output was

identical for the PRE and the post-exercise training trial at the same absolute (ABS) intensity and the absolute power output was higher for males and females at all time points (Table 2).

There was a significant increase in VO_{2peak} ($P < 0.001$)(both $l \cdot min^{-1}$ & $ml \cdot kg^{-1} \cdot min^{-1}$) following 38 days of endurance exercise training for both males and females. This was paralleled by an increase in the maximal enzyme activities for marker enzymes of the electron transport chain (NADH-cytochrome c oxidoreductase (Complex I-III))($P < 0.05$) and the tricarboxylic acid cycle (citrate synthase)($P < 0.001$) following endurance exercise training (Table 3).

We found a significant increase in leucine oxidation (Table 4) over resting values during the last 30 min of the 90 min exercise bout prior to the endurance exercise training program (PRE), ($P < 0.001$). Following the training program, there was no significant increase in leucine oxidation during the final 30 min of exercise for both the REL and ABS exercise intensities. Males showed higher leucine oxidation as compared to females both at rest and during exercise for all trials ($P < 0.01$). Both leucine flux and non-oxidative leucine disposal (NOLD) were significantly lower during exercise as compared to rest for all three trials (Table 4). Females showed a lower leucine flux as compared to males at all time points ($P < 0.05$).

Our analysis showed a significant increase total BCOAD activity (Table 5) when comparing the PRE to both ABS and REL trials ($P < 0.001$ & $P < 0.0001$, respectively). The % activation of BCOAD was significantly increased from t=0 to t=90 in both the PRE and REL exercise trials with the increase in PRE being greater ($P < 0.001$ (PRE), and $P < 0.05$ (REL)). There was no significant increase from t=0 to t=90 in the ABS exercise.

Regression analysis demonstrated a positive correlation ($r = 0.41$; $P < 0.001$) between % active BCOAD and protein oxidation (data not shown). A similar correlation was found when relating the delta change in % active BCOAD (the change between t=0 and t=90) to the delta change in protein oxidation, ($r = 0.44$; $P < 0.01$).

Analysis of urinary excretion indices (Table 6) showed a main effect for gender for both absolute urea N excretion ($g \cdot day^{-1}$) and creatinine excretion ($g \cdot day^{-1}$). For both outcome measures, males were significantly higher than females ($P < 0.05$, and $P < 0.01$ respectively). Regression analysis demonstrated a positive correlation between Urea N excretion ($g \cdot day^{-1}$) and leucine oxidation ($r = 0.46$; $P < 0.0001$) (data not shown).

Muscle PCr, Cr and ATP (Table 7) were all corrected to TCr. Analysis of PCr and Cr showed a main effect for time when collapsed across trials such that t=0 was significantly greater than t=90 at ($P < 0.05$). In addition, for ATP there was a main effect for trial when collapsed across time showing PRE to be significantly greater than REL ($P < 0.01$).

Plasma glucose was unchanged within any condition during exercise. Plasma insulin concentration showed a main effect for time such that a decrease throughout exercise with t=0 and t=30 being greater than all other time-points ($P < 0.001$, and $P < 0.05$ respectively). The time-points t=60 and t=90 did not differ from one another. No main effects for trial or gender were found (Figure 1).

Plasma lactate (Figure 1) responses showed a main effect for trial and time as well as a trial by time interaction. The ABS trial was significantly lower than both the PRE and REL trials at all time-points ($P < 0.01$). The lactate concentration at t=30 was significantly greater as compared to t=0 during all trials ($P < 0.001$), and was greater at this time for PRE vs. REL and ABS ($P < 0.001$).

Muscle glycogen concentration (Figure 2) was significantly lower at t=90 as compared to t=0 during all trials ($P < 0.001$). In addition, the ABS and REL trial glycogen concentrations (collapsed across time) were significantly higher versus the PRE trial values ($P < 0.001$).

Exercise RER (Table 8) showed main effects for all variable. Males had a significantly higher RER than females ($P < 0.05$), ABS trial RER was significantly greater than both PRE and REL ($P < 0.05$), and t=30 was significantly higher than all other time-points ($P < 0.05$).

The % energy derived from CHO and fat during exercise (as calculated using RER and Zuntz tables)(Table 9) showed a main effect for gender with males using proportionately more CHO and females using proportionately more fat during exercise ($P < 0.05$). A main effect for trial showed % substrate utilization (CHO and fat) was decreased in the ABS trial when compared to PRE and REL ($P < 0.01$).

DISCUSSION.

The major finding of this study was that a 38 day endurance exercise training program resulted in a significant attenuation of the exercise induced increase in leucine oxidation. Furthermore, there was an attenuation of BCOAD % activation following training. Finally, females oxidized proportionately more lipid, less carbohydrate and less protein during exercise at 65 % VO_{2peak} as compared to males, both before and after endurance exercise training. Although this last point is not a novel finding it does support previous research finding similar results (8,32,35,99).

Several previous studies have shown acute increases in leucine oxidation during endurance exercise similar to those in the current study (49,90,92). To date, however, no study has examined the effects of a longitudinal endurance training program upon protein

oxidation in humans. Previous authors have investigated the effects of endurance training on rodents and have found equivocal results. Henderson and colleagues (51) observed that training substantially increased whole body leucine turnover and oxidation at rest as well as during exercise at a given intensity. These authors suggested that a training induced increase in BCOAD enzyme and subsequent activation may have accounted for the increase in whole body leucine oxidation. In contrast to these findings, Hood and Terjung (57) were able to show that training reduced the relative contribution of leucine oxidation to VO_2 in electrically stimulated, perfused rat hindquarters. These authors speculated that BCOAD may have been activated less in trained muscle during exercise due to smaller perturbations of energy homeostasis (i.e. lower ADP, Pi). Hood and Terjung therefore suggested that that the training-induced increases in whole-body leucine oxidation observed previously (51) must have occurred in the liver, which is a plausible explanation considering rodents have a much higher distribution of BCOAD enzyme within liver than do humans (70% compare to 30% respectively)(68).

In humans, Lamont and colleagues (72) have recently examined the cross-sectional differences in leucine kinetics in trained and untrained individuals. Results showed an increase in leucine oxidation, for both groups, during exercise when expressed per kg body weight. However, all between group differences were eliminated when leucine kinetics were corrected for fat-free tissue mass. Our results were not altered by the expression of leucine kinetics relative to fat-free tissue mass. Although Lamont and colleagues did not show a decrease in leucine oxidation in trained individuals, they did not show the increase seen by Henderson et al (51) in rodents.

The mechanism for the attenuated leucine oxidation following training may relate to the attenuation of BCOAD activation. These variables showed some correlation, in that training induced attenuation of leucine oxidation was positively correlated to % BCOAD activation. The mechanism for this attenuation following training may relate to the higher muscle glycogen concentration observed in the current study following training (111), as compared to the lower pre-training basal muscle glycogen concentration(60). An increase in muscle glycogen concentration in the basal state has been observed by others following endurance exercise training (39,43). Although the absolute and percentage decrease in glycogen, across exercise, were similar before and after training in the current study, the absolute glycogen concentration post-exercise, following training was within the normal "basal" range in an untrained individual, and would not likely be limiting to energy homeostasis. As mentioned above, Kasperek and colleagues suggested that exercise training resulted in a lesser energy charge (ATP/ADP + Pi) alteration, and that this may attenuate BCOAD activation (64). Although we did not find that endurance training altered post-exercise ATP or phosphocreatine concentration in the current study, we cannot rule out local energy charge perturbation in the mitochondria as homogenate ATP and phosphocreatine concentrations do not reflect mitochondrial energy charge. Clearly, mitochondrial energy charge should bear a higher correlation with BCOAD activation given it's intra-mitochondrial location.

In addition to changes in protein oxidation and BCOAD activation, we also found mitochondrial enzyme changes following training. Both citrate synthase, cytochrome c oxidoreductase and total BCOAD enzyme activities, as well as VO_{2peak} levels, were

found to increase after training indicating an increase in the total mitochondrial oxidative potential and aerobic capacity (6,53,54). It is interesting that, although total BCOAD "potential" capacity increased following training, the percent in the active form was attenuated. This may represent an adaptive strategy for the organism to "spare" critical proteins in response to a metabolic stress, following exercise training. This finding may also partially explains the apparent paradox between studies finding that endurance training increases (81,105) and decreases (17,107) dietary protein requirements. For example, the elite athlete who has up-regulated mitochondrial potential to a large degree, would also have an increased BCOAD capacity and, if exposed to a rigorous training program, may increase leucine oxidation (105). On the other hand, more modest endurance exercise adaptations and stressors may not tax the enhanced BCOAD capacity. Another possible explanation for the observed decrease in leucine oxidation during exercise post-training may be an attenuation of cortisol and catecholamines. Since these measurements were not made in the current study, any relationship to leucine oxidation is speculative. It is well known that plasma EPI/NE and cortisol levels increase during endurance exercise (13,34,37,99,100). Endurance exercise training results in an attenuation of EPI and NE at the same absolute intensity of exercise while exercise performed at relative intensity results in similar hormonal response as in the untrained individual (80). Cortisol, on the other hand, has been shown to be unaffected by endurance training when tested at an intensity relative to final VO_{2peak} (24), however, some evidence does exist suggesting both, an attenuation (24,98), and no change

(29,34,65) in plasma cortisol concentration when exercising at the same absolute intensity post-training.

In the current study, females oxidized proportionally more lipid and less CHO and protein, as compared to males, both pre- and post-training. Similar results were found in recent studies by Horton and Freidlander who were able to show that females utilized a greater proportion of lipid, during exercise, post-training (32,58). Considering that these differences were observed before and after training, one cannot imply that the genders were not properly matched, therefore, the current study offers the best evidence yet for showing gender differences in exercise substrate utilization. Studies with properly matched males and females have shown similar increases in lipid and decreases in protein oxidation during endurance exercise (87,99).

Females oxidized less protein than males at rest as well as during exercise. This result is identical to that shown by Phillips and colleagues (87) in a study that examined leucine kinetics of male and female endurance athletes during exercise. Even though protein oxidation was lesser for females at rest and during exercise, this lower level of oxidation was not due to differences in the % BCOAD activation in skeletal muscle. However, the difference in protein oxidation could be a result of differential activation of hepatic BCOAD enzyme, such that females may have a lesser activation than seen in males. In addition, considering that females had a lower RER at all time-points as compared to males implies a greater lipid and lesser CHO utilization. The fact that females did not significantly differ from males in their in their muscle glycogen

utilization (% decrease) during exercise implies that any CHO sparing may be hepatic (differences in glycogenolysis/gluconeogenesis) and/or a greater capacity to utilize lipid.

Finally, a recent study by Bowtell and colleagues (10) pooled male and female protein oxidation data on the basis that they believed any gender differences in protein oxidation were due to dietary intake of protein. Citing a previous study by Phillips et al (87), Bowtell rationalized that the gender differences in protein oxidation at rest and exercise was the result of a mismatched protein consumption of the males and females in the study (0.94 and 0.8 $g \cdot kg^{-1} \cdot day^{-1}$, respectively). Using data directly from Bowtell's study, it can be shown that the increase in rate of protein oxidation accounted for by each increment of 0.1 $g \cdot kg^{-1} \cdot day^{-1}$ of protein intake is 0.45 $\mu mol \cdot kg^{-1} \cdot h^{-1}$, which translates to a maximum of 1.8 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ at rest and 7.5 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ during exercise, when using the difference of 0.4 $g \cdot kg^{-1} \cdot day^{-1}$ ((1.6 $g \cdot kg^{-1} \cdot day^{-1}$ (males)) – ((1.2 $g \cdot kg^{-1} \cdot day^{-1}$ (females)) found between males and females in the current study. According to these findings, dietary protein intake accounts for approximately 10 % of the variance in protein oxidation, clearly not enough to account for the observed gender differences in protein oxidation.

In summary, the present study has provided some of the best evidence to date for training induced gender differences in substrate oxidation during exercise. On the basis of indirect calorimetry and infusion data, females were shown to oxidize more lipid and a lesser amount of CHO and protein than their male counterparts. When considered together, both genders exhibited an increase in protein oxidation during exercise as well as increase in BCOAD activation. In addition, due to the longitudinal nature of this

training study we were able to show an attenuation of both protein oxidation and BCOAD % activation during both post-testing scenarios (ABS and REL intensity). Further studies should examine, in greater detail, the effect of training on lipid and CHO kinetics in males and females.

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Tables and Figures

Table 1. *Subject Characteristics (Pre-training)*

		Males(N=6)	Females(N=6)	Significance
Age, yr		26.9 ± 3.4	23.7 ± 1.8	NS
Height, cm		176.5 ± 6.6	163.1 ± 65.8	$P < 0.01$
Weight, kg		78.8 ± 12.1	59.0 ± 9.0	$P < 0.01$
Body fat%		16.5 ± 4.3	25.2 ± 6.3	$P < 0.05$
FFM, kg		65.4 ± 8.3	43.7 ± 4.0	$P < 0.001$
VO_{2peak}:				
$l \cdot min^{-1}$		3.6 ± 0.5	2.3 ± 0.3	$P < 0.001$
$ml \cdot kg^{-1} \cdot min^{-1}$		45.9 ± 4.4	37.7 ± 6.1	$P < 0.05$
$ml \cdot kg\ FFM^{-1} \cdot min^{-1}$		54.9 ± 3.1	53.4 ± 6.4	NS
Diet	Energy (kcal)	3150 ± 629	1926 ± 275	$P < 0.01$
	kcal/kg	41.1 ± 11.8	33.4 ± 7.7	NS
	% PRO	15.8 ± 2.8	14.8 ± 3.2	NS
	% FAT	30.5 ± 7.2	28.8 ± 3.7	NS
	% CHO	48.3 ± 8.6	54.5 ± 5.3	NS
PRO ($g \cdot kg^{-1} \cdot day^{-1}$)		1.6 ± 0.4	1.2 ± 0.3	$P < 0.01$

Values are means ± SD. FFM, fat free mass. * Main effect for gender, males > females

Table 2. *Testing characteristics before and after training*

	Males(N=6)	Females(N=6)
Exercise Intensity, % VO_{2peak}		
PRE	61.8 ± 3.7	59.9 ± 9.4
ABS	56.7 ± 1.9	55.9 ± 9.8
REL	59.5 ± 2.9	58.7 ± 5.0
Exercise Intensity, watts		
PRE	132.5 ± 22.1	80.8 ± 17.2*
ABS	132.5 ± 22.1	80.8 ± 17.2*
REL†	160.5 ± 23.8	110.5 ± 15.1*

Values are means ± SD. PRE, ABS, REL – see text for definition. * Main effect for gender for average exercise intensity (females < males, $P < 0.001$).
† Main effect for exercise intensity, REL > ABS & PRE, ($P < 0.0001$).

Table 3. *Maximal mitochondrial enzyme activities and aerobic capacity*

		Pre	Post
NADH-Cytochrome c oxidoreductase (Complex I-III) ($\mu mol \cdot min^{-1} \cdot mg\ w.w.^{-1}$)	Male	1.6 ± 0.9	2.6 ± 0.9*
	Female	1.4 ± 0.4	2.1 ± 0.9*
Citrate Synthase (CS) ($\mu mol \cdot min^{-1} \cdot mg\ w.w.^{-1}$)	Male	12.0 ± 3.9	16.1 ± 3.2†
	Female	11.9 ± 0.6	15.1 ± 1.4†
VO_{2peak} ($ml \cdot kg^{-1} \cdot min^{-1}$)	Male	45.9 ± 4.4	51.4 ± 6.2‡
	Female	37.7 ± 6.1	44.6 ± 6.3‡,§
($l \cdot min^{-1}$)	Male	3.6 ± 0.5	3.9 ± 0.6‡
	Female	2.3 ± 0.3	2.6 ± 0.1‡§

Values are means ± SD (N=6 males, N=6 females, for VO_{2peak} and N=5 males, N=5 females for CS & Complex I-III). w.w.; wet weight. *,†,‡ Main effect for training (pre-training (Pre) to post-training (Post)), * for Complex I-III ($P < 0.05$); † for CS ($P < 0.001$), and ‡ for VO_{2peak} ($P < 0.001$). § Main effect for gender, males > female ($P < 0.05$).

Table 4. *Leucine turnover*

		PRE		ABS		REL	
		Rest	Ex	Rest	Ex	Rest	Ex
A. Oxidation	Male	43.9 ± 12.1	85.2 ± 21.2[b]	47.6 ± 8.8	39.6 ± 27.3	48.5 ± 19.1	59.8 ± 24.4
($\mu mol \cdot kg^{-1} \cdot h^{-1}$)	Female[a]	20.4 ± 8.1	38.8 ± 24.7[b]	25.8 ± 7.9	21.5 ± 14.7	29.0 ± 10.6	28.5 ± 14.4
B. Flux	Male	238.7 ± 129.1	227.5 ± 54.0[d]	195.2 ± 38.4	170.4 ± 27.6[d]	204.1 ± 27.8	185.9 ± 29.6[d]
($\mu mol \cdot kg^{-1} \cdot h^{-1}$)	Female[c]	164.5 ± 22.6	148.4 ± 20.2[d]	170.5 ± 30.3	144.3 ± 20.9[d]	172.6 ± 43.6	143.8 ± 26.0[d]
C. NOLD	Male	194.9 ± 118.8	142.3 ± 50.8[e]	147.6 ± 32.2	129.1 ± 23.2[e]	155.5 ± 17.7	126.2 ± 39.5[e]
($\mu mol \cdot kg^{-1} \cdot h^{-1}$)	Female	144.0 ± 23.8	109.6 ± 37.4[e]	144.6 ± 31.9	122.8 ± 24.6[e]	143.6 ± 40.1	115.3 ± 19.4[e]

Values are means ± SD (N=6 males; N=6 females). PRE, REL, ABS – see text for definition; Ex = exercise; NOLD = non-oxidative leucine disposal. a, c Main effect for gender (males > female)([a]$P < 0.01$)([c]$P < 0.05$). b Interaction (Trial X Time), Ex > Rest only in PRE trial ($P < 0.001$), while PRE Ex is significantly greater than all trials and times ($P < 0.05$). d, e Main effect for time (Rest > Ex collapsed across trials)([d]$P < 0.01$ & [e]$P < 0.001$).

Table 5. *Total and % Active BCOAD activity pre and post training*

		PRE		ABS		REL	
		0	90	0	90	0	90
Total Activity	Male	2.5 ± 0.7	3.3 ± 1.5	3.2 ± 0.9*	3.6 ± 0.9	3.2 ± 0.9*	3.5 ± 1.3
(nmol·min^{-1}·mg w.w.$^{-1}$)	Female	2.4 ± 0.7	2.6 ± 1.2	4.6 ± 1.9*	2.8 ± 1.1	4.6 ± 1.9*	3.2 ± 1.0
% Active	Male	8.9 ± 0.6	21.8 ± 6.0†	8.4 ± 1.6	10.9 ± 1.9	8.4 ± 1.6	11.0 ± 4.4†
(A.Act/T. Act)	Female	6.4 ± 1.4	24.9 ± 7.1†	6.1 ± 1.3	10.5 ± 4.5	6.1 ± 1.3	14.0 ± 6.8†

Values are mean ± SD (N=6 males; N=6 females). A.Act = actual activity ; T. Act = total activity (nmol·min^{-1}·mg w.w.$^{-1}$). PRE, ABS, REL – see text for definition. * Main effect for trial for Total Activity (PRE, ABS, REL), PRE significantly differs from ABS and REL ($P < 0.001$ & $P < 0.0001$) respectively. † Significant increase between t=0 & t=90 for PRE & REL. Increase for PRE > REL, ($P < 0.001$ & $P < 0.05$ respectively).

Table 6. *Urinary excretion measurements*

		PRE		ABS		REL	
		Rest	Ex	Rest	Ex	Rest	Ex
A. Urea N	Male*	10.0 ± 3.0	10.8 ± 4.2	8.4 ± 2.6	8.2 ± 2.3	9.2 ± 3.7	9.7 ± 4.0
($g \cdot day^{-1}$)	Female	6.4 ± 1.5	7.7 ± 2.0	6.6 ± 1.8	5.5 ± 2.0	6.5 ± 2.5	5.9 ± 1.3
B. Urea N	Male	0.12 ± 0.03	0.13 ± 0.04	0.11 ± 0.03	0.11 ± 0.02	0.12 ± 0.04	0.13 ± 0.04
($g \cdot kg^{-1} \cdot day^{-1}$)	Female	0.11 ± 0.03	0.13 ± 0.04	0.11 ± 0.04	0.09 ± 0.03	0.11 ± 0.04	0.1 ± 0.02
C. Creatinine	Male†	1.4 ± 0.5	1.5 ± 0.7	1.1 ± 0.3	1.3 ± 0.4	1.2 ± 0.5	1.6 ± 0.7
($g \cdot day^{-1}$)	Female	0.9 ± 0.2	0.8 ± 0.3	0.9 ± 0.1	0.7 ± 0.2	0.9 ± 0.1	0.7 ± 0.2

Values are means ± SD (N=6 males; N=6 females). PRE, ABS, REL – see text for definition. Ex – exercise. *,† Main effect for gender across trials (male > female)(*$P < 0.05$)(†$P < 0.01$).

Table 7. *High energy phosphate compounds*

		PRE		ABS		REL	
		0	90	0	90	0	90
A. PCr	Male	67.9 ± 19.9*	61.1 ± 30.0	78.5 ± 12.7*	64.0 ± 20.2	77.0 ± 17.5*	42.0 ± 20.6
	Female	76.6 ± 21.8*	56.1 ± 26.3	75.9 ± 10.4*	68.3 ± 14.2	75.5 ± 4.4*	63.4 ± 11.0
B. Cr	Male	47.6 ± 15.4*	54.7 ± 23.6	46.9 ± 6.8*	61.3 ± 25.7	45.8 ± 8.5*	80.7 ± 35.7
	Female	61.6 ± 17.1*	81.9 ± 38.8	48.3 ± 8.4*	55.7 ± 15.0	48.7 ± 10.7*	60.8 ± 17.6
C. ATP	Male	21.4 ± 2.1†	21.9 ± 2.6	23.1 ± 1.8	23.6 ± 2.0	23.8 ± 1.5†	23.1 ± 3.4
	Female	21.7 ± 1.0†	20.1 ± 1.5	22.3 ± 3.2	19.6 ± 1.4	22.3 ± 2.9†	22.7 ± 2.8

Values are adjusted to TCr and are means ± SD (N=6 males; N=4 females). PCr, phosphocreatine; Cr, creatine; TCr, total creatine; ATP, adenosine triphosphate, all values expressed as mmol·mg dry wt^{-1}. * Main effect for time (t=0 > t=90, collapsed across trials) ($P < 0.05$). † Main effect for trial collapsed across times (PRE < REL ($P < 0.01$)).

Table 8. *Exercise RER*

		PRE	ABS	REL
Male *				
	30 min	0.95 ± 0.04‡	0.91 ± 0.03†‡	0.95 ± 0.05‡
	60	0.92 ± 0.02	0.88 ± 0.03†	0.94 ± 0.04
	75	0.91 ± 0.02	0.88 ± 0.02†	0.93 ± 0.04
	90	0.91 ± 0.02	0.87 ± 0.02†	0.92 ± 0.04
Female				
	30 min	0.92 ± 0.04‡	0.91 ± 0.03†‡	0.93 ± 0.04‡
	60	0.89 ± 0.03	0.87 ± 0.04†	0.89 ± 0.03
	75	0.89 ± 0.03	0.86 ± 0.03†	0.88 ± 0.02
	90	0.9 ± 0.06	0.85 ± 0.04†	0.88 ± 0.03

Values are means ± SD (N=6 males; N=6 females). * Main effect for gender, males > females ($P < 0.05$). † Main effect for trial, ABS < PRE & REL ($P < 0.05$). ‡ Main effect for time, t=30 > all other time-points ($P < 0.05$).

Table 9. *Substrate utilization*

		PRE	ABS	REL
A. CHO	Male*	86.5 ± 4.6	78.8 ± 3.6†	88.5 ± 7.5
(% expenditure)	Female	80.6 ± 8.5	73.6 ± 9.0†	81.0 ± 4.9
B. Fat	Male*	13.5 ± 4.6	21.2 ± 3.6†	11.5 ± 7.5
(% expenditure)	Female	19.4 ± 8.5	26.4 ± 9.0†	19.0 ± 4.9

Values are means ± SD (N=6 males; N=6 females). * Main effect for gender, males > females ($P < 0.05$). † Main effect for trial, ABS < PRE & REL ($P < 0.01$)

Figure 1.

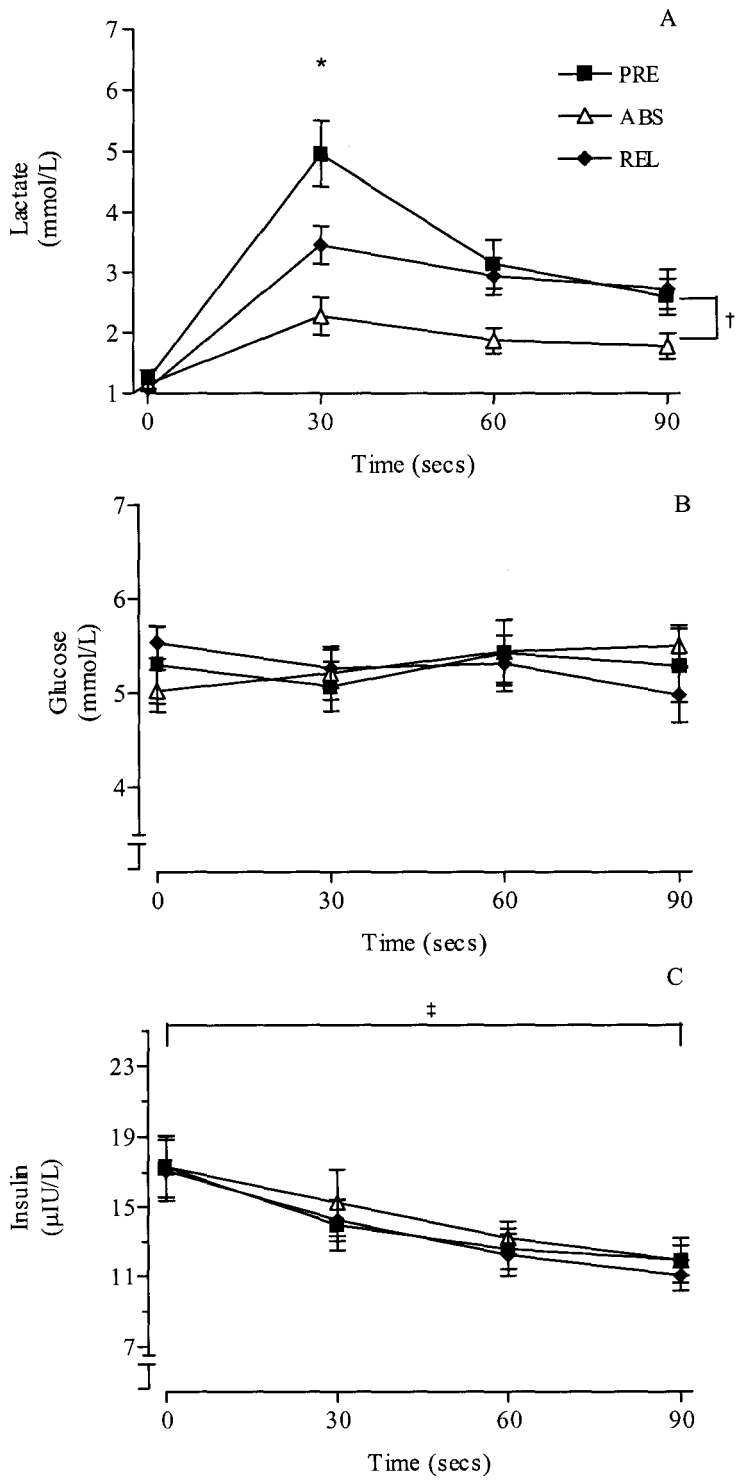

Fig. 1. Effects of exercise training upon plasma lactate, glucose and insulin responses during exercise. Values are means ± SEM (N = 12, (6 male and 6 female pooled)). * Interaction of Trial X Time, t=30 PRE > t=30 REL and ABS ($P < 0.001$). † Main effect for trial, ABS < PRE and REL at all time points ($P < 0.01$). ‡ Main effect for time, t=0 and t=30 > all other time-points ($P < 0.001$, and $P < 0.05$, respectively).

Figure 2

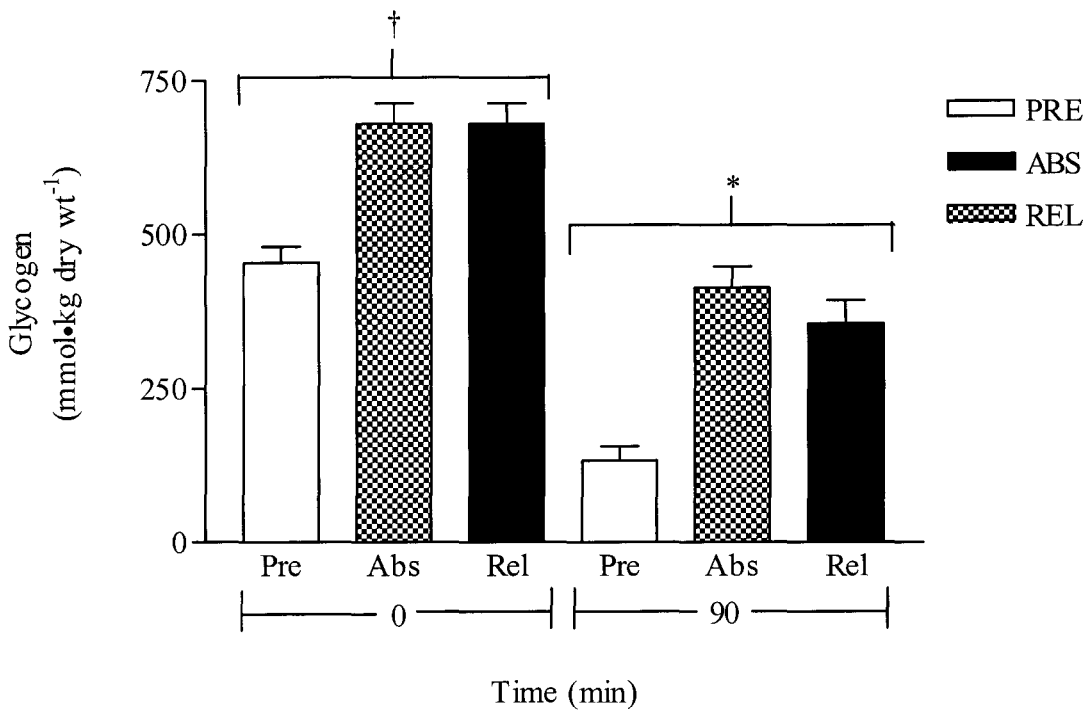

Fig. 2. Effects of exercise training on muscle glycogen content during exercise. Values are means ± SE (N = 12, (6 male and 6 female pooled)). * Main effect for time, t=90 < t=0 during all trials ($P < 0.001$). † Main effect for trial (collapsed across trial), PRE < ABS and REL ($P < 0.001$).

Appendix I: ANOVA Tables.

Legend

Trial

PRE – Pre trial (P)
PST – Post training
ABS – Absolute Trial (A)
REL – Relative Trial (R)

Time

0 – Time 0 min before exercise
90 – Time 90 min end of exercise
REST – Rest day before exercise trial day
EX – Day exercise was performed

Marked Effects: $P < 0.05$ (indicated by **bold** numbers)

1.0. Age, 1-Way ANOVA

1-GENDER

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	30.0833	10	7.21667	4.16859	0.06845

1.1. Weight, 2 – Way ANOVA, Between (GENDER), within (PRE/PST).

1-GENDER, 2 - PRE/PST

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	**1**	**2114.25**	**10**	**201.911**	**10.4712**	**0.00893**
2	1	8.88167	10	2.26218	3.92615	0.07569
12	1	6.38602	10	2.26218	2.82295	0.12385

1.2. Height, 1 – Way ANOVA

1-GENDER

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	**1**	**533.333**	**10**	**38.6333**	**13.805**	**0.004**

1.3. Body fat %, 2 – Way ANOVA, between (GENDER), within (PRE/PST).
1-GENDER, 2 - PRE/PST

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	**1**	**436.907**	**10**	**60.2322**	**7.25371**	**0.02257**
2	**1**	**5.80167**	**10**	**0.24817**	**23.3781**	**0.00069**
12	1	0.16667	10	0.24817	0.67159	0.4316

1.4. Fat free mass (kg), 2 – Way ANOVA, between (GENDER), within (PRE/PST).
1-GENDER, 2 - PRE/PST

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	**1**	**2550.28**	**10**	**74.1177**	**34.4086**	**0.00016**
2	1	1.64327	10	2.88108	0.57036	0.46753
12	1	7.1286	10	2.88108	2.47428	0.1468

1.5. VO_{2peak} ml•kg^{-1}•min^{-1} , 2 – Way ANOVA, between (GENDER), within (PRE/PST).
1-GENDER, 2 - PRE/PST

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	**1**	**332.55**	**10**	**61.2969**	**5.42524**	**0.04211**
2	**1**	**229.325**	**10**	**6.43072**	**35.6609**	**0.00014**
12	1	3.26102	10	6.43072	0.5071	0.49268

1.6. VO_{2peak} ml•kg FFM^{-1}•min^{-1} , 2 – Way ANOVA, between (GENDER), within (PRE/PST).
1-GENDER, 2 - PRE/PST

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	21.261	10	33.611	0.63256	0.44488
2	**1**	**235.175**	**10**	**18.6197**	**12.6304**	**0.00523**
12	1	0.70647	10	18.6197	0.03794	0.84946

1.7. PRE Energy intake (Kcal), 1 – Way ANOVA

1-GENDER

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	**1**	**4494528**	**10**	**235548**	**19.0812**	**0.0014**

1.8. % Calories from fat, 1 – Way ANOVA

1-GENDER

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	8.48401	10	32.5458	0.26068	0.62073

1.9. % Calories from CHO, 1 – Way ANOVA

1-GENDER

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	117.813	10	51.0702	2.30689	0.15976

2.0. % Calories from PRO, 1 – Way ANOVA

1-GENDER

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	3.22403	10	9.09333	0.35455	0.56478

2.1. Energy intake (Kcal/kg), 1 – Way ANOVA

1-GENDER

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	177.188	10	99.1963	1.78624	0.211

2.2. Protein intake ($g \cdot kg^{-1} \cdot day^{-1}$), 2 – Way ANOVA, between (GENDER), within (PRE/PST).

1-GEND, 2 – PRE/PST

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	**1**	**1.7779**	**10**	**0.161**	**11.042**	**0.0077**
2	1	0.0297	10	0.1105	0.2685	0.6156
12	1	0.1596	10	0.1105	1.4451	0.257

2.3. Exercise intensity (% VO_{2peak}), 2 – Way ANOVA, between (GENDER), within (P/A/R).

1-GENDER, 2 - P/A/R

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	12.5622	10.000	80.748	0.15557	0.70155
2	2	60.8586	20.000	18.362	3.3144	0.05712
12	2	1.15443	20.000	18.362	0.06287	0.93925

2.4. Exercise RER, 3 – Way ANOVA (repeated measures) between (GENDER), within (Trial by Time).

1-GENDER, 2-PAR, 3-30/60/75/90.

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	**1**	**0.021949**	**10**	**0.003975**	**5.522058**	**0.040647**
2	**2**	**0.018365**	**20**	**0.003272**	**5.613109**	**0.011617**
3	**3**	**0.010309**	**30**	**0.000523**	**19.72363**	**2.97E-07**
12	2	0.001123	20	0.003272	0.343288	0.713533
13	3	0.000442	30	0.000523	0.845877	0.479686
23	6	0.000329	60	0.000385	0.85448	0.533509
123	6	0.000359	60	0.000385	0.931895	0.47893

2.5. Exercise intensity (watts), 2 – Way ANOVA, between (GENDER), within (P/A/R).

1-GENDER, 2 - P/A/R

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	**1**	**23511.1**	**10**	**1088.48**	**21.6**	**0.00091**
2	**2**	**3325.44**	**20**	**44.6444**	**74.4873**	**5.4E-10**
12	2	2.77778	20	44.6444	0.06222	0.93986

2.6. Citrate synthase ($\mu mol \cdot min^{-1} \cdot mg\ w.w.^{-1}$), 2 – Way ANOVA, between (GENDER), within (PRE/PST).

1-GENDER, 2 - PRE/PST

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1.0000	1.6148	8.0000	12.9495	0.1247	0.7331
2	**1.0000**	**65.1084**	**8.0000**	**0.9985**	**65.2061**	**0.0000**
12	1.0000	0.8470	8.0000	0.9985	0.8483	0.3840

2.7. Complex I-III ($\mu mol \cdot min^{-1} \cdot mg\ w.w.^{-1}$), 2 – Way ANOVA, between (GENDER), within (PRE/PST).

1-GENDER, 2 - PRE/PST

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	0.57378	8	0.8013	0.71606	0.42203
2	**1**	**3.22821**	**8**	**0.47988**	**6.72719**	**0.03193**
12	1	0.08833	8	0.47988	0.18407	0.67922

2.8. VO_{2peak} $ml \cdot kg^{-1} \cdot min^{-1}$, 2 – Way ANOVA, between (GENDER), within (PRE/PST).

1-GENDER, 2 - PRE/PST

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	**1**	**332.55**	**10**	**61.2969**	**5.42524**	**0.04211**
2	**1**	**229.325**	**10**	**6.43072**	**35.6609**	**0.00014**
12	1	3.26102	10	6.43072	0.5071	0.49268

2.9. Leucine oxidation ($\mu mol \cdot kg^{-1} \cdot h^{-1}$), 3 – Way ANOVA (repeated measures) between (GENDER), within (Trial by Time).

1-GENDER, 2 - P/A/R, 3 - REST/EX

	Df Effect	MS Effect	df Error	MS Error	F	p-level
1	**1**	**12887.3**	**10**	**760.976**	**16.9353**	**0.00209**
2	**2**	**1095.42**	**20**	**250.342**	**4.3757**	**0.02653**
3	**1**	**1682.1**	**10**	**197.249**	**8.52777**	**0.01529**
12	2	346.603	20	250.342	1.38452	0.27343
13	1	481.275	10	197.249	2.43993	0.14934
23	**2**	**2027.66**	**20**	**183.321**	**11.0607**	**0.00058**
123	2	271.185	20	183.321	1.47929	0.25168

3.0. Leucine Flux ($\mu mol \cdot kg^{-1} \cdot h^{-1}$), 3 – Way ANOVA (repeated measures) between (GENDER), within (Trial by Time).

1-GENDER, 2 - P/A/R, 3 - REST/EX

	Df Effect	MS Effect	df Error	MS Error	F	p-level
1	**1**	**38600.3**	**10**	**7103.96**	**5.43363**	**0.04198**
2	2	3926.69	20	2431.31	1.61505	0.22376
3	**1**	**7855.54**	**10**	**584.871**	**13.4312**	**0.00435**
12	2	4352.28	20	2431.31	1.7901	0.19268
13	1	143.098	10	584.871	0.24467	0.63154
23	2	240.907	20	801.968	0.30039	0.74381
123	2	33.9573	20	801.968	0.04234	0.95863

3.1. NOLD ($\mu mol \cdot kg^{-1} \cdot h^{-1}$), 3 – Way ANOVA (repeated measures) between (GENDER), within (Trial by Time).

1-GENDER, 2 - P/A/R, 3 - REST/EX

	Df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	6686.23	10	6246.06	1.07047	0.32521
2	2	1175.86	20	1979.82	0.59392	0.56161
3	**1**	**17117.1**	**10**	**758.121**	**22.5783**	**0.00078**
12	2	2342.73	20	1979.82	1.1833	0.32681
13	1	124.625	10	758.121	0.16439	0.69369
23	2	833.509	20	947.694	0.87951	0.43043
123	2	194.029	20	947.694	0.20474	0.81655

3.2. BCOAD % Active (A. Act/T. Act), 3 – Way ANOVA (repeated measures) between (GENDER), within (Trial by Time).

1-GENDER, 2 - P/A/R, 3 - 0/90

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	1.02814	10	27.0089	0.03807	0.84922
2	**2**	**287.8**	**20**	**14.7052**	**19.5713**	**2E-05**
3	**1**	**1237.88**	**10**	**13.749**	**90.0341**	**2.6E-06**
12	2	5.57004	20	14.7052	0.37878	0.6895
13	**1**	**82.6**	**10**	**13.749**	**6.00772**	**0.0342**
23	**2**	**254.441**	**20**	**17.3015**	**14.7063**	**0.00012**
123	2	6.03522	20	17.3015	0.34883	0.70972

3.3. BCOAD Total activity ($nmol \cdot min^{-1} \cdot mg\ w.w.^{-1}$), 3 – Way ANOVA (repeated measures) between (GENDER), within (Trial by Time).

1-GENDER, 2 - P/A/R, 3 - 0/90

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	0.36693	10	5.52881	0.06637	0.80193
2	**2**	**6.28877**	**20**	**0.54502**	**11.5385**	**0.00047**
3	1	0.90814	10	1.38797	0.65429	0.4374
12	2	1.41734	20	0.54502	2.6005	0.09911
13	**1**	**10.2826**	**10**	**1.38797**	**7.40836**	**0.02149**
23	2	2.17394	20	0.74147	2.93192	0.07645
123	2	1.45116	20	0.74147	1.95713	0.16739

3.4. Urea N ($g \cdot day^{-1}$), 3 – Way ANOVA (repeated measures) between (GENDER), within (Trial by Time).
1-GENDER, 2 - P/A/R, 3 – REST/EX

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	**1**	**159.307**	**10**	**26.3391**	**6.04829**	**0.03372**
2	2	14.682	20	6.58005	2.23129	0.13344
3	1	0.26077	10	1.65876	0.15721	0.70007
12	2	2.52161	20	6.58005	0.38322	0.68656
13	1	1.2829	10	1.65876	0.77341	0.3998
23	2	4.53635	20	1.86396	2.43371	0.11324
123	2	1.03256	20	1.86396	0.55396	0.58324

3.5. Urea N ($g \cdot kg^{-1} \cdot day^{-1}$), 3 – Way ANOVA (repeated measures) between (GENDER), within (Trial by Time).
1-GENDER, 2-P/A/R, 3-REST/EX

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	**1**	0.001794	10	0.003079	0.58283	0.462836
2	**2**	0.002073	20	0.001291	1.605281	0.225654
3	**1**	8.13E-06	10	0.00038	0.021369	0.886683
12	**2**	0.000297	20	0.001291	0.230033	0.79658
13	1	0.000232	10	0.00038	0.609721	0.452981
23	2	0.001123	20	0.000464	2.421113	0.114392
123	**2**	0.000473	20	0.000464	1.019729	0.378696

3.6. Creatinine ($g \cdot day^{-1}$), 3 – Way ANOVA (repeated measures) between (GENDER), within (Trial by Time). creatinine
1-GENDER, 2 - P/A/R, 3 – REST/EX

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	**1.000**	**5.770**	**10**	**0.621**	**9.294**	**0.012**
2	2.000	0.179	20.000	0.116	1.549	0.237
3	1.000	0.036	10.000	0.053	0.670	0.432
12	2.000	0.124	20.000	0.11576	1.073	0.36085
13	**1.000**	**0.667**	**10.000**	**0.053**	**12.472**	**0.005**
23	2	0.01457	20	0.04581	0.31818	0.73109
123	2	0.04908	20	0.04581	1.07153	0.36135

3.7. Phosphocreatine (mmol•mg dry wt^{-1}), 3 – Way ANOVA (repeated measures) between (GENDER), within (Trial by Time).
1-GENDER, 2 - P/A/R, 3 - 0/90

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	57.6	8	477.438	0.12064	0.7373
2	2	224.382	16	275.103	0.81563	0.45993
3	**1**	**4764.47**	**8**	**609.478**	**7.8173**	**0.02334**
12	2	92.2838	16	275.103	0.33545	0.71992
13	1	184.356	8	609.478	0.30248	0.59735
23	2	214.027	16	407.381	0.52537	0.60119
123	2	411.767	16	407.381	1.01077	0.38603

3.8. Creatine (mmol•mg dry wt^{-1}), 3 – Way ANOVA (repeated measures) between (GENDER), within (Trial by Time).
1-GENDER, 2 - P/A/R, 3 - 0/90

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	0.16384	8	495.278	0.00033	0.98593
2	2	227.16	16	303.913	0.74745	0.4894
3	1	1351.25	8	356.782	3.78733	0.08752
12	2	724.861	16	303.913	2.38509	0.124
13	1	261.36	8	356.782	0.73255	0.41696
23	2	232.554	16	260.001	0.89444	0.42832
123	2	114.634	16	260.001	0.4409	0.65105

3.9. ATP (mmol•mg dry wt^{-1}), 3 – Way ANOVA (repeated measures) between (GENDER), within (Trial by Time).
1-GENDER, 2 - P/A/R, 3 - 0/90

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	26.7486	8	10.1869	2.62579	0.1438
2	**2**	**14.1017**	**16**	**2.40768**	**5.85694**	**0.01234**
3	1	4.61267	8	8.8795	0.51947	0.49159
12	2	3.97142	16	2.40768	1.64948	0.2232
13	1	6.95278	8	8.8795	0.78301	0.40203
23	2	1.29596	16	3.95215	0.32791	0.72515
123	2	5.88503	16	3.95215	1.48907	0.25523

4.0. Plasma insulin (uIU/L), 3 – Way ANOVA (repeated measures) between (GENDER), within (Trial by Time).
1-GENDER, 2 - P/A/R, 3 - 0/30/60/90

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	239.057	10	133.02	1.79715	0.20972
2	2	7.20221	20	31.1268	0.23138	0.79553
3	**3**	**219.094**	**30**	**7.42947**	**29.4899**	**4.4E-09**
12	2	18.1933	20	31.1268	0.58449	0.56663
13	3	20.339	30	7.42947	2.73762	0.06083
23	6	1.52401	60	8.49773	0.17934	0.98146
123	6	7.72303	60	8.49773	0.90883	0.49484

4.1. Plasma glucose (mmol/L), 3 – Way ANOVA (repeated measures) between (GENDER), within (Trial by Time).
1-GENDER, 2 - P/A/R, 3 - 0/30/60/90

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	7.17347	10	5.29057	1.3559	0.27128
2	2	0.00856	20	1.14574	0.00747	0.99256
3	3	0.2798	30	0.62813	0.44544	0.7223
12	2	0.76068	20	1.14574	0.66392	0.52581
13	3	1.54998	30	0.62813	2.46761	0.08126
23	6	0.59685	60	0.4535	1.31609	0.26404
123	6	0.34684	60	0.4535	0.7648	0.60046

4.2. Plasma lactate (mmol/L), 3 – Way ANOVA (repeated measures) between (GENDER), within (Trial by Time).
1-GENDER, 2 - P/A/R, 3 - 0/30/60/90

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	1.75563	10	5.40788	0.32464	0.5814
2	**2**	**18.0757**	**20**	**1.92136**	**9.40773**	**0.00132**
3	**3**	**35.6374**	**30**	**0.91975**	**38.7471**	**1.9E-10**
12	2	1.55943	20	1.92136	0.81162	0.45824
13	3	0.31456	30	0.91975	0.34201	0.7951
23	**6**	**4.15998**	**60**	**0.24462**	**17.0056**	**2.3E-11**
123	6	0.22364	60	0.24462	0.91422	0.49109

4.3. Muscle glycogen (mmol·kg dry wt^{-1}), 3 – Way ANOVA (repeated measures) between (GENDER), within (Trial by Time).
1-GENDER, 2 - P/A/R, 3 - 0/90

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	20836.2	10	29618	0.7035	0.42121
2	**2**	**461839**	**20**	**9045.21**	**51.059**	**1.4E-08**
3	**1**	**1655384**	**10**	**16946.4**	**97.6834**	**1.8E-06**
12	2	9144.85	20	9045.21	1.01102	0.3817
13	1	14965.4	10	16946.4	0.8831	0.3695
23	2	6485.7	20	5135.61	1.26289	0.30444
123	2	2314.52	20	5135.61	0.45068	0.64351

4.4. % energy from CHO during exercise, 2 – Way ANOVA, between (GENDER), within (P/A/R).
1 – GENDER, 2 – P/A/R

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	**1**	**340.5981**	**10**	**53.99134**	**6.308383**	**0.030825**
2	**2**	**257.303**	**20**	**39.77728**	**6.468593**	**0.006815**
12	2	4.09623	20	39.77728	0.102979	0.902621

4.5. % energy from fat during exercise, 2 – Way ANOVA, between (GENDER), within (P/A/R).
1-GENDER, 2-P/A/R

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	**1**	**340.5981**	**10**	**53.99134**	**6.308383**	**0.030825**
2	**2**	**257.303**	**20**	**39.77728**	**6.468593**	**0.006815**
12	2	4.09623	20	39.77728	0.102979	0.902621

APPENDIX II: Data Tables.

2.0. Subject Characteristics (Pre-training).

Gender	Age	PRE wt (kg)	PST wt (kg)	Ht. (cm)	PRE % FAT	PST % FAT	PRE FFM (kg)	PST FFM(kg)
1	26	74.94	71.04	170	15.4	15	63.41	60.41
1	32	76.57	77.1	169	13.6	11.8	66	68.03
1	25	89.75	85.79	186	15.3	14	75.99	73.77
1	24	57.34	58.63	174	11.5	10.5	50.75	52.50
1	24	86.07	82.08	180	19.9	20	68.95	56.64
1	30	87.93	84.47	180	23.3	22.8	67.46	65.38
2	26	55.91	57.97	164	19.4	18.6	45.06	47.2
2	25	49.15	48.36	152	24.5	23.6	37.13	36.96
2	24	67.31	68.59	167	32.8	31.5	45.2	46.97
2	23	51.07	50.82	163	17.1	16.1	42.35	42.62
2	23	71.79	68.93	168	31.5	31.1	49.2	47.52
2	21	58.55	58	165	25.9	23.4	43.38	44.45

1 = Male
2 = Female

Dietary Intakes

Legend

TC – Total Calories
GF – Grams from fat
CF – Calories from fat
%F – Percent from fat
GC – Grams from CHO
CC – Calories from CHO
%C – Percent from CHO
GP – Grams PRO
CP – Calories from PRO
%P – Percent from PRO

GENDER	PRE TC	Kcal/kg	PRE GF	PRE CF	PRE %F	PRE GC	PRE CC	PRE %C	PRE GP	PRE CP	PRE %P
1	2459	32.8	68.6	617.2	25.0	342.9	1371.6	56.0	100.6	402.4	16.0
1	4016	52.4	85.2	767.2	19.0	645.2	2580.8	62.0	127.6	510.4	12.0
1	3176	35.4	124.8	1123.2	35.0	358.2	1433.0	45.0	149.2	596.8	19.0
1	3326	58.0	115.8	1042.2	31.0	363.3	1453.2	43.0	118.5	474.0	14.0
1	2392	27.8	92.8	834.9	34.9	248.3	993.2	41.5	88.4	353.6	14.8
1	3528	40.1	152.7	1374.3	38.0	373.9	1495.6	42.0	166.5	666.0	19.0
2	1818	32.5	50.6	455.7	25.1	238.0	952.0	52.4	65.1	260.2	14.3
2	2146	43.7	81.4	732.7	34.0	274.8	1099.2	51.0	80.4	321.6	15.0
2	1636	24.3	48.7	437.9	25.0	265.2	1060.8	63.0	49.5	197.9	12.0
2	2124	41.6	77.7	699.3	32.0	307.6	1230.4	57.0	61.7	246.7	11.0
2	2227	31.0	68.7	618.0	27.8	310.4	1241.6	55.8	90.5	361.9	16.3
2	1602	27.4	52.4	471.8	29.0	194.6	778.4	48.0	80.2	320.7	20.0

Protein intake ($g \cdot kg^{-1} \cdot day^{-1}$)

GENDER	PRE GP	PST GP	PRE ($g \cdot kg^{-1} \cdot day^{-1}$)	PST ($g \cdot kg^{-1} \cdot day^{-1}$)
1	100.60	101.50	1.34	1.43
1	127.60	148.00	1.67	2.55
1	149.20	136.90	1.66	1.78
1	118.50	94.90	2.07	1.96
1	88.41	142.90	1.03	1.67
1	166.50	114.80	1.89	1.67
2	65.05	81.29	1.16	1.60
2	80.39	53.50	1.64	0.78
2	49.48	52.04	0.74	0.90
2	61.67	70.24	1.21	1.20
2	90.47	58.93	1.26	0.72
2	80.17	137.30	1.37	1.63

2.1. Testing Characteristics

	PRE % peak	ABS %peak	REL %peak	PRE RER	ABS RER	REL RER	PRE wattage	ABS wattage	REL wattage
1	65.92	59.43	58.67	0.89	0.91	0.99	135.00	135.00	155.00
1	57.42	54.95	55.70	0.92	0.87	0.90	120.00	120.00	155.00
1	62.04	56.99	61.16	0.96	0.90	0.97	165.00	165.00	200.00
1	62.74	58.09	64.33	0.92	0.88	0.93	105.00	105.00	133.00
1	65.07	56.84	59.48	0.92	0.88	0.89	150.00	150.00	175.00
1	57.41	54.14	57.96	0.93	0.89	0.93	120.00	120.00	145.00
2	57.14	58.33	52.63	0.90	0.88	0.93	75.00	75.00	90.00
2	60.56	58.97	55.64	0.90	0.87	0.87	90.00	90.00	101.00
2	48.58	39.75	62.81	0.91	0.92	0.90	60.00	60.00	110.00
2	60.40	54.81	57.74	0.87	0.88	0.90	110.00	110.00	135.00
2	55.75	54.00	56.77	0.85	0.82	0.91	75.00	75.00	112.00
2	76.80	69.95	66.41	0.96	0.85	0.88	75.00	75.00	115.00

2.2. Mitochondrial enzyme activities and aerobic capacity

Complex I-III ($\mu mol \cdot min^{-1} \cdot mg\ w.w.^{-1}$)

GENDER	PRE	PST
1	3.10	3.46
1	1.06	3.20
1	2.05	1.58
1	0.92	2.87
1	0.94	1.64
2	1.93	2.46
2	1.08	1.45
2	1.65	1.52
2	1.32	3.42
2	1.07	1.54

Citrate Synthase Activity ($\mu mol \cdot min^{-1} \cdot mg\ w.w.^{-1}$)

GENDER	PRE	PST
1	18.60	21.23
1	9.52	13.59
1	10.05	13.38
1	12.81	16.15
1	9.20	15.92
2	12.77	16.10
2	11.59	14.04
2	11.60	16.53
2	11.39	13.13
2	12.04	15.58

VO_{2peak} ($ml \cdot kg^{-1} \cdot min^{-1}$)

GENDER	PRE ($ml \cdot kg^{-1} \cdot min^{-1}$)	PST ($ml \cdot kg^{-1} \cdot min^{-1}$)	PRE ($ml \cdot kg\ FFM^{-1} \cdot min^{-1}$)	PST ($ml \cdot kg\ FFM^{-1} \cdot min^{-1}$)
1	48.80	56.91	57.40	68.58
1	44.44	56.38	51.97	61.74
1	49.37	55.18	57.90	64.25
1	50.56	51.13	57.73	57.75
1	39.19	40.93	51.00	53.46
1	43.08	47.59	53.74	63.59
2	39.87	42.41	50.60	52.75
2	43.22	50.76	57.37	66.74
2	32.02	37.94	62.61	55.57
2	45.02	51.71	55.18	62.37
2	29.82	37.27	44.25	57.31
2	36.40	47.78	50.51	61.27

R – Rest
Ex - Exercise

2.3. Leucine Turnover ($\mu mol \cdot kg^{-1} \cdot h^{-1}$)

Leucine Oxidation

GENDER	PRE R	PRE Ex	ABS R	ABS Ex	REL R	REL Ex
1	31.93	72.24	40.58	11.80	58.65	38.52
1	57.67	78.38	45.56	33.48	33.76	41.16
1	51.04	103.63	54.04	58.83	77.34	77.50
1	55.12	117.94	59.59	75.82	57.31	57.41
1	36.55	77.52	50.37	61.06	37.03	99.89
1	30.95	61.59	35.52	6.42	27.14	44.11
2	16.33	22.43	15.99	43.30	20.29	26.87
2	17.18	77.31	15.82	3.58	14.54	4.96
2	35.29	61.49	33.64	28.41	34.38	33.33
2	22.55	24.88	27.29	6.66	30.15	48.88
2	11.70	15.86	30.09	24.85	44.52	33.37
2	19.56	30.53	31.93	22.18	30.37	23.65

Leucine flux

GENDER	**PRE R**	**PRE Ex**	**ABS R**	**ABS Ex**	**REL R**	**REL Ex**
1	195.50	229.73	175.58	157.66	239.10	179.76
1	461.54	314.29	190.24	187.92	204.06	179.06
1	239.67	226.30	208.95	174.08	219.41	193.33
1	295.73	252.93	210.47	213.74	218.85	240.88
1	132.62	171.70	250.19	151.57	177.58	163.56
1	107.36	170.11	135.92	137.24	165.48	159.11
2	160.78	147.76	174.74	150.46	155.37	144.95
2	152.57	144.18	153.58	127.35	148.14	130.76
2	161.75	136.90	149.01	132.67	150.19	129.16
2	201.18	176.61	218.13	165.30	241.59	181.78
2	176.17	164.95	190.68	170.37	211.08	165.30
2	134.38	119.78	136.59	119.60	129.52	110.79

NOLD

GENDER	**PRE R**	**PRE Ex**	**ABS R**	**ABS Ex**	**REL R**	**REL Ex**
1	163.57	157.49	135.00	145.86	180.45	141.24
1	403.86	235.90	144.68	154.44	170.30	137.91
1	188.63	122.68	154.91	115.25	142.07	115.83
1	240.61	134.99	150.89	137.92	161.54	183.47
1	96.08	94.18	199.82	90.50	140.55	63.67
1	76.42	108.53	100.40	130.82	138.34	115.00
2	144.44	125.33	158.75	107.16	135.08	118.08
2	135.38	66.87	137.76	123.76	133.60	125.80
2	126.46	75.41	115.37	104.26	115.81	95.83
2	178.63	151.73	190.84	158.63	211.44	132.90
2	164.47	149.09	160.58	145.52	166.56	131.93
2	114.82	89.25	104.66	97.42	99.16	87.14

2.4. % and Total BCOAD activations

% BCOAD activation (A. Act/T. Act)

GENDER	PRE 0	PRE 90	ABS 0	ABS 90	REL 0	REL 90
1	5.06	10.38	9.10	8.47	9.10	10.88
1	9.94	19.70	8.48	9.36	8.48	7.85
1	5.16	23.98	7.47	10.85	7.47	13.08
1	10.14	25.59	9.97	10.82	9.97	15.68
1	14.72	25.45	9.72	13.62	9.72	16.23
1	8.12	25.63	5.82	12.43	5.82	4.97
2	6.58	16.96	3.97	8.37	3.97	21.10
2	6.98	35.64	7.31	7.44	7.31	8.69
2	7.48	17.45	4.99	17.03	4.99	19.96
2	5.30	20.20	6.48	6.78	6.48	7.87
2	7.72	30.02	6.59	15.70	6.59	20.84
2	4.26	28.90	7.20	8.04	7.20	8.24

Total BCOAD activation (nmol·min^{-1}·mg w.w.$^{-1}$)

GENDER	PRE 0	PRE 90	ABS 0	ABS 90	REL 0	REL 90
1	3.79	5.01	3.62	3.84	3.62	5.73
1	2.48	2.22	3.56	5.09	3.56	4.36
1	2.91	4.86	4.55	4.14	4.55	3.40
1	2.71	3.63	2.33	2.85	2.33	2.87
1	1.73	1.80	2.74	3.41	2.74	1.74
1	1.79	1.93	2.11	2.54	2.11	3.38
2	3.74	3.55	7.97	3.01	7.97	3.69
2	1.98	3.34	3.64	4.71	3.64	3.54
2	2.36	1.75	3.05	1.90	3.05	2.33
2	2.59	4.17	5.92	2.31	5.92	4.24
2	1.76	1.78	3.87	2.82	3.87	1.62
2	1.86	1.21	3.11	1.82	3.11	3.95

2.5. Urinary excretion measurements

Urea N ($g \cdot day^{-1}$)

GENDER	PRE R	PRE Ex	ABS R	ABS Ex	REL R	REL Ex
1	11.13	9.27	10.39	7.75	9.96	10.50
1	8.82	11.57	11.07	7.54	4.78	4.83
1	13.48	15.49	8.18	11.72	15.60	16.59
1	4.73	3.48	4.12	6.25	6.48	7.09
1	11.15	11.95	9.78	9.94	10.19	11.00
1	10.69	13.32	6.76	5.85	8.34	8.48
2	5.21	5.25	7.71	4.96	5.22	6.39
2	7.02	7.75	7.57	3.56	2.81	3.41
2	5.43	5.41	5.29	7.28	8.22	6.67
2	4.67	8.24	4.53	4.47	6.52	6.99
2	8.72	9.20	9.11	8.54	10.14	6.42
2	7.29	10.08	5.43	4.12	5.98	5.52

Urea N ($g \cdot kg^{-1} \cdot day^{-1}$)

GENDER	PRE R	PRE Ex	ABS R	ABS Ex	REL R	REL Ex
1	147.78	123.12	144.64	107.97	136.83	144.19
1	113.99	149.54	147.61	100.55	64.18	64.86
1	151.66	174.27	94.62	136.26	181.36	191.78
1	81.61	59.94	70.13	106.47	109.85	120.24
1	128.88	138.18	117.80	119.74	122.05	131.72
1	121.79	151.69	79.26	68.58	97.76	99.38
2	92.01	92.80	132.55	85.29	88.85	108.66
2	142.16	156.88	155.51	72.84	57.48	70.06
2	81.79	81.40	77.18	106.11	119.13	96.69
2	89.88	158.50	87.83	86.69	125.63	134.62
2	149.86	158.15	158.51	148.54	174.86	110.70
2	99.88	138.08	77.51	58.75	86.06	79.42

Creatinine ($g \cdot day^{-1}$)

GENDER	PRE R	PRE Ex	ABS R	ABS Ex	REL R	REL Ex
1	1.16	1.17	1.24	0.87	1.15	1.85
1	1.49	1.73	1.47	0.93	0.79	0.93
1	1.96	2.35	0.98	1.52	1.81	2.68
1	0.61	0.47	0.67	1.18	0.71	0.70
1	1.85	2.03	1.30	2.08	1.74	2.03
1	1.41	1.54	0.91	1.14	1.23	1.55
2	0.70	0.37	0.91	0.39	0.76	0.49
2	0.62	0.60	0.98	0.65	0.69	0.59
2	0.98	0.73	0.74	1.00	1.01	0.72
2	0.74	0.74	1.08	0.74	0.75	0.75
2	1.10	1.26	0.84	0.71	1.03	0.53
2	1.13	1.15	0.76	0.70	0.93	0.98

2.6. High energy phosphates ($mmol \cdot mg\ dry\ wt^{-1}$)(In muscle)

Phosphocreatine

GENDER	PRE 0	PRE 90	ABS 0	ABS 90	REL 0	REL 90
1	79.00	45.22	97.07	32.76	97.07	37.47
1	87.48	79.44	52.79	68.14	52.79	48.75
1	87.58	69.93	77.20	67.72	77.20	7.55
1	41.73	79.93	89.46	88.32	89.46	55.84
1	64.18	38.72	74.90	51.74	74.90	22.30
1	40.74	13.04	57.85	48.23	57.85	52.33
2	69.67	58.60	76.39	35.44	76.39	36.30
2	44.98	87.41	74.49	86.20	74.49	52.58
2	94.66	13.91	52.75	60.84	52.75	66.30
2	88.14	41.14	71.26	62.13	71.26	71.04

Creatine

GENDER	PRE 0	PRE 90	ABS 0	ABS 90	REL 0	REL 90
1	35.17	63.88	49.58	85.44	49.58	69.56
1	30.53	25.63	36.98	49.05	36.98	44.73
1	38.92	30.65	43.89	33.51	43.89	130.57
1	64.45	34.07	46.89	41.46	46.89	62.44
1	61.56	61.34	56.66	75.92	56.66	81.88
1	45.95	82.24	33.15	51.15	33.15	38.41
2	60.06	86.44	55.49	41.60	55.49	65.30
2	79.05	38.16	51.88	35.09	51.88	43.99
2	60.27	78.77	40.95	52.17	40.95	58.21
2	38.95	52.91	29.01	62.97	29.01	42.50

ATP

GENDER	PRE 0	PRE 90	ABS 0	ABS 90	REL 0	REL 90
1	22.91	23.42	24.30	21.52	24.30	20.37
1	24.40	20.29	24.39	24.98	24.39	21.07
1	20.48	18.78	20.61	18.76	20.61	20.05
1	19.67	17.92	24.34	22.89	24.34	22.48
1	18.41	18.07	21.04	21.81	21.04	19.55
1	19.14	19.65	24.18	19.33	24.18	19.55
2	18.75	17.99	19.19	18.19	19.19	18.11
2	22.57	21.25	20.53	20.66	20.53	19.45
2	22.36	21.00	19.11	19.75	19.11	18.90
2	20.75	14.97	21.14	19.16	21.14	23.06

2.7. Plasma lactate (mmol/L)

GENDER	PRE 0	PRE 30	PRE 60	PRE 90	ABS 0	ABS 30	ABS 60	ABS 90	REL 0	REL 30	REL 60	REL 90
1	1.20	6.05	3.60	2.70	1.30	3.30	2.40	2.20	1.30	3.40	3.05	3.25
1	2.30	3.85	2.20	2.65	1.65	1.70	1.25	1.45	1.25	3.26	2.65	2.60
1	0.85	4.80	2.55	2.30	0.90	1.70	1.25	1.25	0.95	2.80	2.10	2.15
1	1.65	3.45	2.15	1.85	1.15	1.20	1.10	1.00	1.85	3.05	2.70	1.85
1	1.20	7.05	4.00	3.20	1.75	5.10	3.05	2.95	1.05	4.40	3.00	3.15
1	1.05	4.75	2.40	2.10	0.85	2.20	2.25	2.35	1.00	5.85	5.75	5.70
2	0.85	2.50	1.60	1.40	0.65	1.40	1.30	0.80	1.25	2.30	2.15	1.70
2	1.15	7.40	4.70	3.20	1.05	1.75	1.30	1.25	1.00	4.90	4.00	3.75
2	2.10	7.20	4.75	3.45	0.90	3.00	2.60	2.45	0.75	3.90	3.20	2.65
2	0.45	3.65	2.65	2.40	0.95	2.05	2.40	2.40	0.65	2.30	2.50	2.30
2	1.10	6.60	5.85	4.90	1.30	2.35	2.55	2.25	0.70	2.80	2.30	2.00
2	0.85	2.25	1.30	1.05	1.45	1.55	1.05	1.05	1.15	2.55	1.85	1.60

2.8. Plasma glucose (mmol/L)

GENDER	PRE 0	PRE 30	PRE 60	PRE 90	ABS 0	ABS 30	ABS 60	ABS 90	REL 0	REL 30	REL 60	REL 90
1	6.17	5.20	6.22	6.33	3.26	3.49	8.09	5.76	5.23	4.71	6.07	5.54
1	4.91	3.89	4.01	4.02	5.44	4.26	3.78	4.31	5.01	4.79	4.64	3.38
1	5.89	5.26	5.17	4.53	5.49	6.25	5.83	5.49	6.61	5.92	7.53	6.26
1	4.90	4.06	4.05	4.65	4.66	4.03	5.07	5.31	6.15	4.00	4.94	5.32
1	8.55	6.93	8.04	8.92	5.21	6.15	6.26	6.48	6.22	6.15	6.38	6.41
1	4.22	4.75	6.55	5.16	6.09	6.38	6.70	5.52	5.88	6.21	5.99	5.41
2	4.97	5.34	5.00	3.73	5.09	4.79	5.47	4.88	5.00	5.67	4.79	4.97
2	5.84	5.71	6.17	5.81	5.41	6.34	4.46	7.16	5.70	5.57	4.74	5.97
2	5.27	5.30	5.01	5.27	3.81	4.89	4.92	5.25	5.55	5.30	4.46	3.96
2	5.63	5.27	5.52	5.47	5.40	4.86	4.87	5.83	4.78	5.15	3.95	4.41
2	4.88	3.55	5.45	4.95	5.33	5.87	4.62	5.03	5.01	5.44	5.72	4.40
2	2.41	5.63	3.97	4.69	5.12	5.29	5.30	5.02	5.32	4.27	4.58	3.74

2.9. Plasma insulin (uIU/L)

GENDER	PRE 0	PRE 30	PRE 60	PRE 90	ABS 0	ABS 30	ABS 60	ABS 90	REL 0	REL 30	REL 60	REL 90
1	10.15	9.17	9.51	6.34	12.57	8.69	7.69	5.37	8.39	8.43	6.86	5.73
1	12.37	11.59	9.55	12.43	9.26	9.22	9.66	10.13	7.25	8.76	8.14	8.53
1	21.66	17.03	13.97	16.20	18.08	18.60	14.26	14.60	21.15	17.29	13.40	14.29
1	14.57	11.26	14.31	13.51	18.87	15.78	13.04	11.23	18.81	16.55	12.38	11.61
1	16.00	8.17	8.76	8.17	9.35	12.73	16.27	14.38	19.55	20.26	19.26	13.64
1	20.47	11.74	11.15	10.13	13.64	17.01	12.55	11.07	16.70	14.69	10.54	11.38
2	9.34	8.12	7.57	8.69	14.15	10.62	7.45	8.45	8.98	8.54	6.39	6.88
2	13.72	14.72	13.02	10.91	23.17	15.12	14.09	14.93	22.55	16.01	17.10	15.89
2	13.55	12.52	14.52	11.51	26.97	32.19	16.66	14.75	20.53	18.14	16.86	11.66
2	23.80	20.13	15.89	21.52	20.90	19.76	15.42	14.85	26.18	17.95	15.41	12.43
2	27.10	23.49	22.44	16.59	25.85	7.85	16.32	11.26	15.38	13.02	9.97	8.70
2	25.25	19.41	10.29	7.03	15.16	15.36	15.19	11.80	19.87	11.30	10.49	11.72

3.0. Muscle glycogen (mmol·kg dry wt^{-1})

GENDER	PRE 0	PRE 90	ABS 0	ABS 90	REL 0	REL 90
1	468.85	91.49	714.71	291.88	714.71	221.15
1	487.62	271.27	672.87	342.06	672.87	332.00
1	500.51	137.89	777.50	492.22	777.50	180.64
1	309.15	43.29	497.45	452.35	497.45	481.00
1	621.65	188.57	617.62	310.09	617.62	262.74
1	402.28	67.31	751.39	390.99	751.39	318.53
2	473.80	271.63	887.77	508.76	887.77	619.94
2	574.20	135.99	680.64	575.88	680.64	471.82
2	394.33	109.39	643.22	549.83	643.22	429.09
2	433.50	75.15	709.06	262.18	709.06	238.34
2	359.31	198.49	458.15	268.94	458.15	297.38
2	438.20	16.74	757.76	539.26	757.76	438.09

3.1. % energy derived from CHO and FAT (Zuntz table calculations)

	% CHO			% Fat		
Gender	PRE	ABS	REL	PRE	ABS	REL
1.00	79.34	83.90	98.50	20.66	16.10	1.50
1.00	85.98	74.24	81.63	14.02	25.76	18.37
1.00	93.59	81.63	95.31	6.41	18.37	4.69
1.00	85.98	76.80	88.05	14.02	23.20	11.95
1.00	85.98	76.80	79.34	14.02	23.20	20.66
1.00	88.05	79.34	88.05	11.95	20.66	11.95
2.00	81.63	76.80	88.05	18.37	23.20	11.95
2.00	81.63	74.24	74.24	18.37	25.76	25.76
2.00	83.90	85.98	81.63	16.10	14.02	18.37
2.00	74.24	76.80	81.63	25.76	23.20	18.37
2.00	68.76	59.19	83.90	31.24	40.81	16.10
2.00	93.59	68.76	76.80	6.41	31.24	23.20

3.2. Exercise RER data

	PRE				ABS				REL			
	30	60	75	90	30	60	75	90	30	60	75	90
Gender	RER	RER	RER	RER	RER	RER	RER	RER	RER	RER	RER	RER
1	0.914	0.895	0.876	0.885	0.927	0.920	0.905	0.904	0.995	0.993	0.994	0.987
1	0.979	0.903	0.918	0.897	0.868	0.869	0.855	0.872	0.918	0.909	0.909	0.872
1	1.015	0.945	0.931	0.931	0.944	0.912	0.905	0.850	1.013	0.954	0.949	0.948
1	0.894	0.918	0.943	0.922	0.922	0.844	0.887	0.860	0.913	0.947	0.945	0.926
1	0.946	0.924	0.900	0.904	0.887	0.896	0.870	0.868	0.902	0.894	0.877	0.875
1	0.956	0.926	0.899	0.944	0.917	0.883	0.896	0.873	0.939	0.928	0.923	0.911
2	0.936	0.904	0.902	0.908	0.939	0.875	0.856	0.866	0.996	0.936	0.912	0.858
2	0.936	0.904	0.902	0.908	0.890	0.865	0.875	0.866	0.901	0.855	0.856	0.849
2	0.952	0.888	0.909	0.871	0.942	0.923	0.911	0.894	0.964	0.895	0.887	0.871
2	0.888	0.848	0.865	0.880	0.909	0.875	0.865	0.886	0.923	0.896	0.874	0.915
2	0.849	0.855	0.852	0.840	0.876	0.787	0.812	0.816	0.940	0.905	0.893	0.919
2	0.964	0.937	0.927	1.009	0.877	0.867	0.868	0.799	0.880	0.882	0.884	0.880

APPENDIX III: Assay Protocols

APPENDIX OF ASSAYS

ESTROGEN AND INSULIN (Radio-immunoassays kit #TKE21 and #TKIN5 respectively, Coat-A-Count®, Diagnostic Products Corporation, purchased through Intermedico of Mississauga 1-800-387-9643).

The Coat-A-Count® procedures are solid-phase radio-immunoassays, wherein ^{125}I-labeled hormone competed for a fixed time with the actual hormone in the patient sample for cites on hormone-specific antibodies. Because the antibody is immobilized to the wall of a polypropylene tube, simply decanting the supernatant suffices to terminate the competition and to isolate the antibody-bound fraction of the radio-labeled insulin. Counting the tube in a gamma counter then yields a number which converts by way of a calibration curve to a measure of the hormone present in the patient sample.

PLASMA GLUCOSE:

Glucose (Trinder) Product #315, Sigma Diagnostics, 1-800-325-0250.

Principle:

$$\text{Glucose} + H_2O + O_2 \xrightarrow{\textit{Glucose Oxidase}} \text{Gluconic Acid} + H_2O_2$$

$$H_2O_2 + \text{4-Aminoantipyrine} + \text{p-Hydroxybenzene Sulfonate} \xrightarrow{\textit{Peroxidase}} \text{Quinoneimine Dye} + H_2O$$

---

*The intensity of the color produced is directly proportional to the glucose concentration in the plasma sample.

UREA NITROGEN:

Procedure #640, Sigma Diagnostics, 1-800-325-0250.

Principle:

$$\text{Urea} + H_2O \xrightarrow{\textit{Urease}} 2NH_3 + CO_2$$

$$NH_3 + \text{2-Phenol-OH} \xrightarrow{\textit{Sodium Nitroprusside}} \text{Indophenol (blue)}$$

CREATININE:

Procedure #555, Sigma Diagnostics, 1-800-325-0250

DERIVATIZATION OF α-KIC
(Tarnopolsky et al., 1991)

Reagents and materials:

-Absolute ethanol
-O-phenylenediamine (OPDA) solution (2% in 4 M HCl) (SIGMA)
-Methylene chloride (dichloromethane)
-BSTFA with 1% TMCS[12] (PIERCE: 1-800-8PIERCE)

Procedure:

1. Place 5ml of absolute ethanol in 16 x 100mm disposable culture tube.
2. Add 500-750µl of plasma. Shake vigorously, centrifuge at 10,000 rpm for 10min at 4°C.
3. Remove supernatant to 16 x 100mm screw top culture tube.
4. Evaporate to dry under N_2 at 50°C.
5. Dissolve the residue in 1ml of 4x filtered water. Vortex.
6. React the solution with 1ml of OPDA (photosensitive, use immediately after preparation. Solution has deteriorated when it is no longer pink.).
7. Cap the tube and heat for 1hr at 90°C.
8. Extract with 2.5ml of methylene chloride (vortex each time), centrifuge at 2,500rpm for 2-5min at 4°C. Transfer lower layer to a clean tube. Repeat.
9. Dry under N_2 at room temperature.

GC/MS conditions:

1. Add 75µl of 1.1 BSTFA with 1% TMCS to each sample, heat at 100°C for 30min. (Use Hamilton syringe for transfer, rinse after use.)

2. Inject 0.3µl of derivatized sample into a 15 m fused silica capillary column (0.25mm I.D.).

3. Set oven to 120°C, ramp to 160°C (8°C per min), and ramp to 300°C (20°C per min). Hold at 300°C for 3min.

4. MS: monitor EI's 232.1 and 233.1 for α-KIC (m+1).

GLYCOGEN EXTRACTION PROTOCOL

Reference: Bergmeyer, Hans Ulrich. (1983). Methods of Enzymatic Analysis. New York, Academic Press, Inc. Vol. VI. pp. 11-18.

Pre-extraction Procedure:

(1) Turn on water-bath to 80°C. Remove samples from freezer and allow to equilibrate at room temperature.

(2) Prepare solutions **G5, G6, & G7.**

(i) **G5** - 0.1 M NaOH
(ii) **G6** - 0.1 M HCl (prepared from fisher A144P-500, **12.1M**)

			In Solution	gm/100ml
(iii)	**G7** -	0.2 M Citric acid(F.W.- 210.1)	$C_6H_8O_7.H_2O$	4.2
		0.2 M $Na_2.HPO_4$ (F.W.- 142.0)	$Na_2HPO_4.7H_2O$	5.36

(iv) Amyloglucosidase (**AGS**) (Boehr. 208 469; 2g).

(3) Combine solutions **G5** & **G6** in a ratio of 1:1 to see if they neutralize one another in the pH range of **6.2-7.8.** Normally, this pH range is not achieved by a 1:1 solution mix. Experiment with the volumes of HCl and NaOH to see what is needed to achieve the proper range.

(4) If **G5** & **G6** neutralize at 1 : 0.74 (for example), then for every 100 µl of **G5** added, there must be 74 µl of **G6** added to neutralize the solution.

- Now prepare the **G7** solution. Mix the aforementioned amounts of solute into 75-80 ml of DDI H_2O and dilute up to 100 ml volume (or desired volume). Use 10 M NaOH to pH the solution up to 5.0.

- According to the 1 : 0.74 neutralization example in step 4, **G5** will be added to a **G6+G7** solution in a ratio of 1 : 4 **during the solution addition of the extraction procedure** (described in the chart below). Therefore, for every 100 µl of **G5** added, 400 µl of the **G6+G7** solution will be added. In this case, within the 400 µl of **G6+G7** we want 74 µl to be solution **G6** and the remaining 326 µl to be **G7**. Therefore, **G6** and **G7** should be mixed 74 : 326 or 1 : 4.41.

(5) Now prepare a solution of **AGS** + **G7** with a concentration of 1mg **AGS**/per ml of **G7** (takes time to fully dissolve, mix by inversion DO NOT VORTEX ENZYMES).

Glycogen Standards Preparation

Table I

Glycogen STD. sol'n (µl)	DDI volume (µl)	Glycogen Conc. (nmol/µl)
3	47	75
4	46	100
8	42	200
16	34	400
32	18	800
50	0	1250

(1) Prepare the dilutions outlined in Table 1 within their respected eppendorf tubes.
(2) Now proceed with the reaction mixture additions outlined in Table II

*Glycogen powder **1mol = 162g***

- Add 81mg of glycogen powder to 5ml of DDI.
- Take 2.5 ml of this 5 ml and add to 7.5ml of DDI for a final volume of 10ml.
- Proceed with chart dilutions.

Calculations:

- 81mg/5ml = 16.2mg/ml therefore, 2.5 ml of a 16.2mg/ml solution contains 40.5mg/2.5ml.
- Now dilute this 40.5mg/2.5ml solution with 7.5ml to a total of 10ml. We now have 40.5mg in 10ml of sol'n which equals 4.05mg/ml.

Procedure:

Table II (Standards Preparation)

Muscle STD Solution	G5 (NaOH)(µl)	G6+G7 (HCl, Citrate)(µl)	AGS (µl)	Total (µl)
Prepared dilution (50µl total)	130	520	40	740

Table III (Muscle Preparation)

Dry Muscle(mg)	G5 (NaOH)(µl)	G6+G7 (HCl, Citrate)(µl)	AGS (µl)	Total (µl)
2.0 - 4.0	160	640	40	840

(1) Add the appropriate amount of **G5** to muscle samples or std. solution according to the table above. **Make sure that all muscle is reached by the solution by gently vortexing**

(2) Incubate the samples for 10 min at 80°C. This step destroys background glucose and hexose monophosphates.

(3) Allow samples to cool for 10-15 min. and proceed to neutralize the samples by adding the appropriate amount of **G6+G7** (from table) after allowing the samples to cool. Close Eppendorf tubes and gently shake or invert.

(4) Add **AGS** as outlined in the table, gently mix and incubate samples at room temperature for 80 min. Glycogen degradation occurs at this step.

(5) Freeze samples at -50°C (or colder) or measure glycogen (glucose) content directly.

Glycogen (Glucose Assay)

$$\text{Glucose} + \text{ATP} \xrightarrow{HK} \text{G-6-P} + \text{ADP}$$

$$\text{G-6-P} + \text{NAD}^{+} \xrightarrow{G6PDH} \text{6-P-Gluconolactone} + \text{NADH}$$

Table IV

No.	Reagents	Wt/Vol.	Stock Conc. mmol/l	Cuvette Conc. mmol/l	1 rxn (µl)	20 rxns (µl)	40 rxns (µl)	60 rxns (µl)	80 rxns (µl)
G1	Triethanolamine	7g/100ml	375	100	80	1600	3200	4800	6400
	KOH	0.8g/100ml	150	40					
	$Mg(Ac)_2.2H_2O$	2.4g/100ml	112.5	30					
	$EDTA.Na_2.2H_2O$	.14g/100ml							
	(pH to 8.2 with KOH)		3.75	1					
G2	ATP	27.7 mg/ml	45	0.75	5	100	200	300	400
G3	DTT	9.36 mg/ml	60	1	5	100	200	300	400
G4	NAD	19.9 mg/ml	30	1	10	200	400	600	800
DDI					150	3000	6000	9000	12ml

Enzymes:

(1) **G6PDH** - Glucose-6-Phosphate Dehydrogenase (100 NADP units or 228 NAD units), dilute in 200 µl of DDI (one unit will oxidize 1µM of d-glucose-6-P to 6-phsopho-d-gluconate per min in the presence of NAD @ pH 7.8 @ 30°C).

(2) **HK** - Hexokinase (200 units), dilute in 200 µl of DDI (one unit will phosphorylate 1µM of glucose/min @pH 7.6 @ 25°C).

(3) After enzyme reconstitution in separate vials, take all 200µl of the G6PDH and add it to the 200 µl HK vial. Next take an additional 400 µl to rinse out the G6PDH vial and add it to the HK.

Procedure:

NOTE **Be sure to centrifuge incubated samples before glucose analysis to eliminate any muscle sediment from the reaction mixtures.**

(1) Add 25 µl of sample, 25 µl of **DDI** and 250 µl of reagent to cuvette. Reagent will consist of solutions G1-G4 & DDI according the concentrations outlined in table II. Mix by gentle inversion.

(2) Measure background absorbance at 340 nm.

(3) Add 4 µl of **HK/G6PDH** enzyme mixture to cuvette. Gently mix contents every 5 min and measure absorbance after 15 min at 340 nm. **Ensure enzyme is mixed with sample by gentle inversion.**

- If samples are frozen, allow them to thaw and then centrifuge to pull sediment to tube bottom and ensure particle free extracts for the assay.

- **NOTE:** Given the variability in the measure of muscle glycogen content, two aliquots of a given muscle sample should be extracted and assayed separately. Acceptable variation between aliquots is 10%.

- **Be sure to multiply final glycogen concentrations by 2 to account for the 2X dilution of the muscle samples in step 1 from above.**

Expectations:

- Normal glycogen values range from **50-600 mmol/kg D.W. = 50-600 nmol/mg D.W.**

Reagent Stabilities

G5, G6, G7 - All stable in solution at room temperature for max of 3 months.

G1 - Can be stored at room temperature for several months.

G2 (ATP) - Can be solvated with DDI, frozen and stored at -50°C for several months. Avoid excessive freeze thaw cycles.

G3 (DTT) - Can be solvated with DDI, frozen and stored at -50°C for several months. Avoid excessive freeze thaw cycles.

G4 (NAD)- Same as above two solutions.

Enzymes:

G6PDH- **Can be frozen for two months at conc. of 1mg/ml. No more than one freeze thaw cycle is recommended. Can be frozen for 2 months, it is suggested to make up fresh each time.**

Hexokinase- **Can store frozen for 30 days at -50°C with repeated freeze thaw cycles.**

Lactate Analysis

Performed with Lactate analyzer Model 23L, YSI Scientific, Yellow Springs Instrument Co.

Principle and Function:

This instrument is a quantitative device for the discrete measurement of L-Lactate in whole blood, plasma, and cerebrospinal fluid.

When the L-Lactate in an injected sample diffuses through the outer membrane of the lactate probe and the following reaction occurs:

Reaction 1:

$$\text{L-Lactate} + O_2 \xrightarrow[\text{L-Lactate oxidase}]{\text{FAD}} H_2O_2 + \text{Pyruvate}$$

The H_2O_2 then diffuses through the inner layer of cellulose acetate and contact with a platinum anode. Reaction 2 now takes place at the platinum anode and produces a current linearly proportional to the concentration of lactate in the sample.

Reaction 2:

$$H_2O_2 \longrightarrow 2^{H+} + O_2 + 2e^-$$

The circuit is completed bye the silver reference cathode.

Reaction 3:

$$2AgCl + 2e^- \longrightarrow 2Ag + 2Cl$$

COMPLEX I-III ASSAY PROTOCOL

Reference: **Journal of Clinical Investigation 74(3):685-97**

Solution Preparation

Homogenization Buffer

5mM KPi
1mM EDTA
0.1mM DTT
pH to 7.4

For 25 ml Homogenization Buffer:

5mM KPi

GMM= 136.1g/mol
In 25ml, 3.4025g yields 1M
therefore for 5mM solution **17.0mg** is needed.

1mM EDTA

GMM= 372.2g/mol
In 25 ml, 9.305g yields 1M
therefore for 1mM solution, **9.3mg** is needed.

0.1mM DTT

GMM= 154.2g/mol
In 25 ml, 3.855g yields 1M
therefore for 0.1mM solution, **0.385** mg is needed.

*Prepare 38.55mg/ml and take **10µl (0.385mg)** and mix in 25ml with 17.0mg KPi and 9.3mg EDTA*

-for muscle biopsies 10-30mg (wet weight), homogenize on ice with 200 µl of buffer

Complex I-III Buffer

0.1M KPi
1mM Azide

0.1M KPi

GMM = 136.1 g/mol in 50 ml

$\frac{0.1 \text{ moles}}{\text{L}} = \frac{x \text{ moles}}{0.05 \text{ L}}$ $x = 5.0 \times 10^{-3}$ moles KPi

grams required in 50 ml:

$\# \text{ moles} = \frac{\text{mass}}{}$

GMM

5.0×10^{-3} moles KPi = $\frac{\text{mass}}{\text{GMM}}$

5.0×10^{-3} moles KPi = $\frac{\text{mass}}{136.1 \text{ g/mol}}$

mass = 0.6805 g = **680.5 mg KPi in 50 ml**

1 mM Azide Sigma S2002
GMM = 65.01 g/mol

in 50 ml:

Make 50ml 10mM Azide and dilute 10:1

$\frac{0.01 \text{ moles}}{\text{L}} = \frac{\text{x moles}}{0.05 \text{ L}}$ $\quad x = 5.0 \times 10^{-4}$ moles Azide

grams required in 50 ml:

moles = $\frac{\text{mass}}{\text{GMM}}$

5.0×10^{-4} moles Azide = $\frac{\text{mass}}{\text{GMM}}$

5.0×10^{-4} moles Azide = $\frac{\text{mass}}{65.01 \text{ g/mol}}$

mass = **32.5 mg Azide in 50 ml**

Take 5ml (10mM Azide) in 50ml volumetric flask with 680.5mg KPi and bring volume to 50ml.
pH to 7 with 5M NaOH

Reagents

Cytochrome c 40mg/ml
NADH 5mg/ml
Rotenone 1mM (dissolve in 100% ethanol)

Cytochrome c Sigma C7752
40 mg/ml

Measure in tared 10 ml plastic tube.
Stability: Good for several months in freezer -20°C

NADH BM 127 345 ***Light-Sensitive***
5 mg/ml
PREPARE FRESH EACH TIME

Measure 5 mg in tared eppendorf tube add 1ml dH_20

Stability: Good for 1 week at RT 100mg/ml

Store in aliquots at -70°C Good til expiry date

1 mM Rotenone Sigma R8875
GMM = 394.4 g/mol

in 10 ml:
Make 10 ml 10mM Rotenone and dilute 10:1

$\frac{0.01 \text{ moles}}{\text{L}} = \frac{\text{x moles}}{0.01 \text{ L}}$ $x = 1.0 \times 10^{-4}$ moles Rotenone

grams required in 10 ml:

$\# \text{ moles} = \frac{\text{mass}}{\text{GMM}}$

$1.0 \times 10^{-4} \text{ moles Rotenone} = \frac{\text{mass}}{\text{GMM}}$

$1.0 \times 10^{-4} \text{ moles Rotenone} = \frac{\text{mass}}{394.4 \text{ g/mol}}$

mass = **39.44 mg Rotenone in 10 ml 100% Ethanol**

Solubility: Chloroform: 50 mg/ml
Pure Ethanol: 10mg/ml

Measure 39.44mg in 10ml (10 mM Rotenone).

Add 100 µl 10mM Rotenone to 900 µl 100% ethanol to yield **1mM Rotenone**

Note: Vigorous mixing and warming solution will be required for complete solvation.

Procedure

1. Place 1 ml 0.1M KPi + 1mM Azide pH 7 in labelled **ROTENONE-SENSITIVE** and **REACTION** cuvettes.

2. Add 30µl oxidized cytochrome c (40 mg/ml) to both cuvettes.

3. *Add 5 µl rotenone to **ROTENONE-SENSITIVE** cuvette.*

4. Mix both cuvettes well with OWN CLEARLY LABELLED mixing stick/pipette tip.

5. Add 20 µl muscle homogenate to both cuvettes.

6. Mix well with OWN CLEARLY LABELLED mixing stick/pipette tip.
To both cuvettes:

7. Measure abs @ 550nm (max. abs. reduced cytochrome c). Use scale = 1
Equilibirate for approximately 1 minute.
Take Reading. t= 0 sec (blank reading)

8. Add 10 µl NADH (5 mg/ml)

9. Quickly mix with OWN CLEARLY LABELLED mixing stick/pipette tip. (Tip: use two 100µl pipettes to mix cuvettes at the same time)

10. Take readings @ 30, 60, 90 and 120 seconds.

Calculations:

$$\text{Activity} = \frac{?\text{abs/min}}{0.0185} \times \frac{\text{volume of buffer}}{\text{volume of homogenate added}} \times \frac{\text{volume of homogenization medium}}{\text{mass of wet muscle}} \times \frac{1}{1000}$$

$$= \frac{?\text{abs/min}}{0.0185} \times \frac{1000}{20} \times \frac{\text{volume of homogenization medium}}{\text{mass of wet muscle}} \times \frac{1}{1000}$$

- expressed in µmol cytochrome c reduced/mg wet muscle ($\mu mol \cdot min^{-1} \cdot mg\ w.w.^{-1}$)

Rotenone is added to one reaction cuvette to account for any non-specific reduction of cytochrome c not resulting from the oxidation of NADH through complex I-III. Therefore, any reduction of cytochrome c in the rotenone-sensitive cuvette is non-specific to the oxidation of NADH via complex I. The difference in the change of absorbance between the reaction and the rotenone-sensitive is predominantly due to the activity of complex I.

Complex I - III Assay Protocol

Rotenone-Sensitive	Reaction (without Rotenone)
1. Add **1 ml** of 0.1M KPi + 1 mM Azide pH 7	1. Add **1 ml** of 0.1M KPi + 1 mM Azide pH 7
2. a) Add **30 µl** oxidized cytochrome c (40 mg/ml)	2. Add **30 µl** oxidized cytochrome c (40 mg/ml)
2. b) *Add **5 µl** 1 mM Rotenone* *	3. Mix with own mixing stick/pipette tip
3. Mix with own mixing stick/pipette tip	4. Add **20 µl** of muscle homogenate
4. Add **20 µl** of muscle homogenate	**5.** Mix with own mixing stick/pipette tip and measure abs @ **550nm**
5. Mix with own mixing stick/pipette tip and measure abs @ **550nm**	6. (Use scale = 1) Equilibrate for approx.**1min** Take Reading t=0 (blank reading)
6. (Use scale = 1) Equilibrate for approx. **1min** Take Reading t=0s (blank reading)	7. Add **10 µl** NADH (5mg/ml)
7. Add **10 µl** NADH (5mg/ml)	8. Quickly mix with stirring stick/pipette tip and take readings at 30, 60, 90, 120 sec.
8. Quickly mix with stirring stick/pipette tip and take readings at 30, 60, 90, 120 sec.	

BRANCHED-CHAIN KETO ACID DEHYDROGENASE ASSAY

Principle:

$$RCOOCOOH + NAD^{+} + CoASH \xrightarrow{(BCKADH)} RCOSCoA + NADH + H^{+} + C0_2$$

GENERAL PREPARATIONS

1. DAY BEFORE TRIAL:

(a) Calculate total amount of substrate (HOT + COLD) required
(b) COLD substrate
(c) Acids/bases
(d) Homogenate buffer
(e) Total buffer
(f) Ethanolamine-ethylenglycol solution
(g) Dilute Na solution

2. DAY OF TRIAL:

(a) Add HOT substrate to COLD substrate
(b) Dilute substrate for actual activity (AA) and total activity (TA)
(c) Prepare co-enzyme buffers for AA and TA
(d) Other general preparations

DETAILS OF "DAY BEFORE TRIAL" PREPARATIONS:

A. Calculate total amount of substrate required:

1. Determine total volume of substrate (HOT + COLD) required:

- 100 µl of substrate is required per vile
- 2 vials are required per reading (AA and TA)
- 3 readings are performed per muscle homogenate (i.e., triplicates)

For example, 6 separate muscle samples would require:

-6 x 3 (readings/sample) x 2 (AA and TA) x 100 µl = 3600 µl

Each trial also requires:

- 2 sets of duplicate blanks for both AA and TA (2x400 800 µl)
- 2 specific activity (SPAC) readings for both AA and TA (400 µl)

Therefore, the *total* substrate volume required for 6 samples is 4800 µl (4.8 ml). Since 1 ml of substrate

(HOT + COLD) is diluted 1:4 prior the assay (to a total of 5ml), 1 ml of undiluted substrate is enough for approx. 6 muscle samples.

B Determine the amount of HOT KIC and COLD KIC required for sensitivity of assay: (a) for muscle samples which am greater than 35 mg, a concentration of 2.5 mM and SPAC of 5000 dpm/nmol is required; (b) for human biopsies and samples less than 35mg, concentration of 2.5 mM and SPAC of 10 000 dpm/nmol is required. Before the following sample calculations are presented, a brief review of radioactive labeling and counting is in order:

The basic unit of radioactivity decay is the curie. One curie (Ci) is defined as the number of disintegration's per second (dps) per gram of radium and equals 3.70×10^{10}. For most biological applications, quantities much less than one curie are normally used and the millicurie (mCi) or microcurie (μCi) is employed. Furthermore, in practice, a minute is the standard time unit - hence, 1 μCi = 2.22×10^6 disintegration's per minute (dpm). The curie, however, has been replaced by the becquerel as the SI unit of radioactivity. One becquerel (Bq) is defined as the activity of a quantity of radioactive material in which 1 nucleus decays per second (i.e., 1 dps). In standard units of time, therefore:

1 Bq = 60 dpm

It is difficult to determine the absolute number of disintegration's because radiation counters detect only a fraction of them. In practice therefore, activity is usually stated as "detected counts per minute" (cpm). Radioisotopes and isotopically labeled compounds are rarely isotopically pure, since the radio isotope is usually diluted by the presence of chemically identical non-radioactive isotopes. The relative abundance of a radioisotope is described by the specific activity (SPAC) that is, the disintegration rate (mCi, μCi, GBq, MBq) per unit mass (mmol, μmol, nmol)

a Sample calculations for 2.5 mM solution with a SPAC of 5.000 dpm/nmol:

We require an end specific activity of 5000 dpm/nmol this value must be converted to Bq/nmol in order relate it to units on the label of 2-KETO[1-^{14}C]ISOCAPROIC ACID (HOT KIC) obtained from Amersham (CFA671 B37L02).

-since 60 dpm = 1 Bq (see above)
-therefore 5000 dpm = 5000/60 = 83.3 Bq

The end SPAC required therefore is 83.3 Bq/nmol.

We also require an end concentration of 2.5 mM (i.e., 2.5 mmol/L).

2.5 mM = 2.5 mmol/L
= 2.5 μmol/ml
= 2500 nmol/ml

Therefore, the amount (mole mass) of α-KIC in 1 ml of the final solution is 2500 nmol

To calculate the required total activity in 1 ml of the final (undiluted) solution then, multiply the end SPAC required by the amount of α-KIC in 1 ml:

TA = end SPAC required x amount of α-KIC in 1 ml
= 83.3 Bq/nmol x 2500 nmol
= 208 250 Bq
= 0.208 MBq

Next, we must determine the volume of HOT KIC required for a TA of 0.208 MBq in the final solution. Refer to the activity listed on the label of the [1-^{14}C]KIC obtained from Amersham. 1 ml of HOT KIC has an activity of 1.85 MBq.

-since 1 .85 MBq corresponds to 8 vol of 1 ml HOT KIC
-therefore 0.208 MBq corresponds to a vol of:

0.208/1.85 = 0.112 ml HOT KIC

Therefore, the vol of HOT KIC required in 1 ml of substrate is 0.112 ml

The HOT KIC, however has a very low concentration of KIC, and contributes very little to the final concentration of 2.5 mM in the 1 ml of undiluted substrate. The final step, therefore, is to determine the concentration of COLD KIC which needs to be added to make up 1 ml of a 2.5 mM solution. Since there is already 0.112 ml of HOT KIC which will contribute to the total concentration, we must determine the amount of HOT KIC in 0.112 ml and subtract it from the total amount of substrate required. The specific activity listed on the label of HOT KIC from Amersham is 2.04 GBq/mmol.

-since 2.04 GBq (2040 MBq) corresponds to 1 mmol HOT KIC
-therefore 0.208 MBq corresponds to:

0.208 MBq/2040 MBq = 0.000102 mmol
= 0.102 μmol
= 102nmol

Therefore, the amount of HOT KIC already added is 102 nmol.

This value is subtracted from the 2500 nmol (total amount of KIC required in the 1 ml final solution) to determine the amount of COLD KIC which must be added:

2500-102=2398 nmol

Therefore, 2398 nmol of COLD KIC must be added to the HOT KIC.

Since 0.112 ml (HOT KIC) has already been added, the volume of COLD KIC which must be to make up a 1 ml final solution is

1-0.112 = 0.888 ml

Therefore, the concentration (molarity) of COLD KIC required is:

2398 nmol / 0.888 ml = 2700 nmol/ml
= 2.70 mM

The 2.70 mM solution of COLD KIC must be made up from its powder form. The COLD KIC which we use is Sigma K-0629 (MW 152.1). To determine the concentration (mass per volume) required:

0.002700 mol./L x 152.1 g/mol = 0.411 g/L
= 0.411 mg/ml
= 4.11 mg/10ml

Make up 50 ml solution (since it can be stored at 4°C for 3-4 weeks). Therefore, 20.55 mg of COLD KIC is added to 50 ml of dH_2O .

THE FINAL 2.5 mM (5.000 dpm/nmol) KIC SOLUTION THEREFORE CONTAINS 0.888 ml OF (2.70 mM) COLD KIC AND 0.112 ml OF HOT KIC.

(b) Sample calculations for 2.5 mM solution with a SPAC of 10000 dpm/nmol:

We require an end specific activity of 10,000 dpm/nmol -- this value must be converted to Bq/nmol in order relate it to units on the label of 2-oxo [1-^{14}C] ketoisocaproic acid (HOT KIC) obtained from Amersham.

- since 60 dpm = 1 Bq (defined above)
- therefore 10000 dpm = 10000/60 = 166.67 Bq

The end SPAC required therefore is 166.7 Bq/nmol.

We also require an end concentration of 2.5 mM (i.e., 2.5 mmol/l).

2.5 mM = 2.5 mmol/L
= 2.5 μmol/ml
= 2500 nmol / ml

Therefore, the amount (mole mass) of α-KIC in 1 ml of the final solution is 2500 nmol.

To calculate the required total activity in 1 ml of the final (undiluted) solution then, multiply the end SPAC required by the amount of α-KIC in 1 ml:

TA = end SPAC required x amount of α-KIC in 1 ml
= 166.67 Bq/nmol x 2500 nmol
= 416,675 Bq
= 0.417 MBq

Next, we must determine the volume of HOT KIC required for a TA of 0.217 MBq in the final solution. Refer to the activity listed on the label of the [1-^{14}C]KIC obtained from Amersham: 1 ml of HOT KIC has an activity of 1.85 MBq.

-since 1 .85 MBq corresponds to a vol of 1 ml HOT KIC
-therefore 0.417 MBq corresponds to a vol of:

0.417/1.85 = 0.225 ml HOT KIC

Therefore, the vol of HOI KIC required in 1 ml of substrate is 0.225 ml.

The HOT KIC, however, has a very low concentration of KIC, and contributes very little to the final concentration of 2.5 mM in the 1 ml of undiluted substrate. The final step, therefore, is to determine the concentration of COLD KIC which needs to be added to make up 1 ml of a 2.5 mM solution. Since there is already 0.225 ml of HOT KIC which will contribute to the total concentration, we must determine the amount of HOT KIC in 0.225 ml and subtract it from the total amount of substrate required. The specific activity listed on the label of HOT KIC from Amersham is 2.04 GBq/mmol.

-Since 2.04 GBq (2040 MBq) corresponds to 1 mmol HOT KIC
-therefore 0.417 MBq corresponds to:

0.417 MBq/2040 MBq = 0.000204 mmol
= 0.204 μmol
= 204 nmol

Therefore, the amount of HOT KIC already added is 204 nmol.

This value is subtracted from the 2500 nmol (total amount of KIC required in the 1 ml final solution) to determine the amount of COLD KIC which must be added:

2500-204 =2296 nmol

Therefore, 2296 nmol of COLD KIC must be added to the HOT KIC.

Since 0.225 ml (HOT KIC) has already been added, the volume of COLD KIC which must be to make up a 1 ml final solution is:

1 - 0.225 = 0.775 ml

Therefore, the concentration (molarity) of COLD KIC required is:

2296 nmol/0.775 ml = 2963 nmol/ml
= 2.96 mM

The 2.96 mM solution of COLD KIC must be made up from its powder form. The COLD KIC which we use is Sigma K-0629 (MW = 152.1). To determine the concentration (mass per volume) required:

.002963 mol/L x 152.1 g/mol = 0.451 g/L
= 0.451 mg/ml
= 4.51/mg per 10 ml

Make up a 50 ml solution is made up (since it can be stored at 4°C for 3-4 weeks Therefore, 22.55 mg of COLD KIC is added to 50 ml of dH_2O.

THE FINAL 2.5 mM (10000 dpm/nmol) KIC SOLUTION THEREFOR CONTAINS 0.775 ml OF 2.96 mM COLD KIC AND 0.225 ml OF HOT KIC.

B. COLD Substrate:

Make up the appropriate COLD KIC solution (Sigma K-0629), depending on the assay sensitivity required, as outlined in the sample calculations above.

(1) for a 2.70 mM COLD KIC solution (used to make a 2.7 mM substrate solution with an activity of 5,000 dpm/nmol):
- dissolve 20.55 mg COLD KIC in 50 ml dH_20

(2) for a 2.96 mM substrate solution (used to make a 2.5 mM substrate solution with an activity of 10000 dpm/nmol):
-dissolve 22.55 mg COLD KIC in 50 ml dH_2O

Store overnight at 4°C (may be kept @ -20°C for 3-4 weeks).

C. Acids/Bases:

Prepare the following solutions:

- 4N HCl (Fisher A144P-500 (12.1N))
- 3M PCA (51.3 ml in 200 ml dH_2O)
- 5M KOH

.

D. Homogenate Buffer;

Chemical	Company	Amount
Sucrose	Sigma S-9378	17.115g
Tris Base	Sigma T-1503	0.2422g
EDTA	Sigma ED2SS	0.1488g

Dissolve into 180 ml of dH_2O,. pH to 7.4 with 4N HCI, and bring to final volume of 200 ml with dH_2O. Preferably, this solution should be made fresh, but it can be stored at 4°C for 10 days.

E. Total Buffer:

Chemical	Company	Amount
Potassium Chloride (KCl)	Sigma P-3911	0.224g
Potassium Phosphate Monobasic (KH_2PO_4)	Fisher P285-500	0.272g
Tris Base	Sigma T-1503	1.454g
Sucrose	Sigma S-9378	1.712g

Dissolve into 70 ml of dH_2O, pH to 7.4 with 4N HCI and bring to final volume of 80 ml with dH_2 0. Preferably, this solution should be made fresh, but it can be stored at 4°

F. Ethanoloamine-Ethyleneglycol Solution

Chemical	Company	Amount
Ethanolamine	Fisher M251-1	10ml
Ethyleneglycol	Fisher E178-500	20ml

Store overnight at 4°C. This is a 1:2 v/v solution, which is added to eppendorf tube and functions to collect radio-labeled CO_2 from the decarboxylation reaction. It can be stored at 4°C for 2 weeks.

G. Dilute NaF Solution;

Dissolve 656 mg Sodium Fluoride (Fisher S299-100) into 50 ml dH_2O. Solution may be stored at 4°C for 3-4 weeks.

DETAILS OF "DAY OF TRIAL" PREPARATIONS:

A. Add HOT KIC to COLD KIC:

Remove HOT KIC from freezer and allow to thaw. Make up the 2.5 mM substrate solution by combining appropriate amounts of the HOT and COLD α-KIC solutions (refer to sample calculations for 5000 and 10000 dpm/nmol solutions). The following examples are based on a required amount of 1 ml undiluted substrate solution (enough for 6 muscle samples to be done in triplicate):

(1) for 1 ml of a 2.5 mM (5,000 dpm/nmol) substrate solution:

-0.888ml of a 2.70 mM COLD KIC solution
-0.112 ml of HOT KIC

(2) for 1 ml of a 2.5 mM (10000 dpm/nmol) substrate solution:

-0.775ml of a 2.96mM COLD DIC solution
-0.225ml of HOT α-KIC
Combine the HOT and COLD KIC solutions in a 10x75mm glass tube.

B. Dilute substrate for ACTUAL ACTIVITY and TOTAL ACTIVITY:

Divide the undiluted substrate solution in half (e.g., 2 x 0.5 ml), and dilute 1:4 with either dH_2O or NaF solution, respectively to make substrate solutions for "actual activity and "total activity":

1. For the ACTUAL ACTIVITY (AA) substrate solution:

   -combine 0.5 ml undiluted substrate with 2.0 ml dH_2O

2. For the TOTAL ACTIVITY (TA) substrate solution:

   -combine 0.5 ml undiluted substrate with 2.0 ml NaF solution

Put each solution in a 10 x 75 glass tube (clearly marked "AA" or "TA) and keep on ice.

C. Prepare co-enzyme buffers for ACTUAL ACTIVITY and TOTAL ACTIVITY:

Chemical	Company	Amount
ADP	Sigma A 6521	53.4mg
2-oxoglutarate (α-KG)	Sigma K 3752	3.7mg
L-carnitine	Sigma C 7518	8.0mg
Sodium Bicarbonate	Fisher S233-500	31.5mg
EDTA	ED2SS	42.8mg

Dissolve in 10 ml of Total Buffer pH to 7.4 with 5M KOH (1-2 drops), and bring to final volume of 15 ml with dH_2O.

[This solution must be made fresh immediately (~30 min) prior to the experiment. The chemicals, however, can be weighed out prior and stored DRY at 4°C for 3-4 weeks.]

To make the appropriate co-enzyme buffers for ACTUAL ACTIVITY and TOTAL ACTIVITY, divide the solution into 2 glass tubes:

1. For the ACTUAL ACTIVITY (AA) co-enzyme buffer:

   -combine 7 ml of the solution with 24.5 mg NaF solid

2. For the TOTAL ACTIVITY (TA) co-enzyme buffer:

   -use the remaining 8 ml of the solution (do not add anything)

D. Other General Preparations:

1. Turn on water bath (37°C).

2. Set up ice boxes for homogenizing equipment and vials.

3. Prepare muscle weighing area:
- homogenizing buffer chart (i.e., amount added per tissue wt)
- pre-tared petri dish
- forceps
- tweezers
- pipettes
- gauze pads
- homogenizing buffer, pestles, and glass tubes on ice.

4. Prepare eppendorf tubes cut cap, add 300μL of ethanolamine-ethyleneglycol solution to each (wipe end of pipette with Kim-wipe each time for consistency), and replace cap.

5. Prepare glass vials:

- label outside of vile
- place eppendorf holder inside

6. Prepare plastic scintillation viles:

- place 10 ml of scintillation fluid into each vile
- label caps

7. Prepare 1 ml syringes and PCA (to halt reaction).

8. Chronograph (to time incubation periods).

PROTOCOL:

1. Homogenization procedure:

(a) obtain muscle sample, dab in gauze, and weigh to 1 decimal place.
(b) transfer to glass tube containing 600 µl of homogenizing buffer.
(c) refer to chart and add specified amount of additional buffer.
(d) homogenize 5-6X with each pestle

The homogenization procedure is very critical. An appropriate method is to. (1) put the first pestle into the glass tube and push it to the bottom, ensuring that the muscle sample is in the buffer and in contact with the teflon end of the pestle; (2) rotate the pestle between the thumb and index finger (so that it spins in the tube) for 3-5 sec; (3) lift up the pestle so that the tip just clears the homogenate, then push it back in; (4) repeat these 2 steps 5-6X. and then remove the pestle by angling the glass tube (60°) and spinning the pestle as you remove it (mitochondria adhere to glass very easily, and thus it is important to keep as much of the homogenate as possible at the bottom of the tube; repeat the procedure using the second pestle.

****N.B. - HOMOGENATE IS STABLE FOR APPROXIMATELY 1/2 HOUR**

2. Add 300 µl of appropriate co-enzyme buffer (AA or TA) to each vile.

3. Add 100 µl of homogenate to each sample vile (alternating between AA and TA); add 100µl of homogenizing buffer to blanks. [Gently swirl the homogenate before pipefitting each time; this will prevent the mitochondria from settling at the bottom of the vile and therefore reduce variability in the assay].

4. Cap vile, gently swirl, and transfer to water bath (37°C) (no longer than 10 mm).

N.B. - TIMING IS ABSOLUTELY CRITICAL FOR STEPS 5-10!!!

[Remove all viles from bath before beginning step 5. Each sample must be incubated for precisely 10 min. To do this, it is best to process one vile every 30 sec (e.g., start chronograph at 0 Sec, draw 100µl of substrate into pipette at 5 sec, inject the 100 µl into vile at 15 sec cap vile and gently swirl, and place vile

into bath at 30 sec (repeat this sequence for every vile until all are in the bath). Note that if you are incubating more than 20 viles at one time, 2 people are required to run the assay since the reaction in vile 1 will need to be halted at the same time vile 21 must be placed into the water bath.

5. Add 100 µl of appropriate substrate (AA or TA).

6. Remove eppendorf cap and carefully drop into holder in glass vile.

7. Cap glass vile, gently swirl, and transfer to water bath (37°C) for 10 min.

8. After exactly 10 min, remove vile from bath and halt reaction by injecting 300 µl PCA through cap down side of vile. Gently swirl.

9. Place viles on ice for 1.5 hrs.

8 Remove from ice, transfer eppendorf tube from glass vile to plastic scintillation vile (containing 10 ml scintillation fluid), and shake well.

11. Let scintillation viles sit 1-2 hrs before counting in β-counter.

****N.B. - DON'T FORGET <u>SPECIFIC ACTIVITY</u> VILES FOR AA AND TA!!****

Add 100µl of appropriate substrate to vial containing 10 ml scintillation fluid. Prepare duplicate specific activity readings for both AA and TA.

<u>CALCULATIONS</u>:

1. Calculate average cpm values for blanks, AA viles, TA viles and specific activity readings (specific activity readings from β-counter are divided by 50 to obtain a value per nmol).

2. Calculate ACTUAL ACTIVITY and TOTAL ACTIVITY:

$$AA = \frac{\text{homogenate (dpm)} - \text{blank (dpm)}}{\text{specific activity (dpm/nmol)}} \times \frac{1}{10 \text{ min}} \times \frac{1}{\text{g w/w tissue}}$$

$$TA = \frac{\text{homogenate (dpm)} - \text{blank (dpm)}}{\text{specific activity (dpm/nmol)}} \times \frac{1}{10 \text{ min}} \times \frac{1}{\text{g w/w tissue}}$$

N.B.

(a) Be sure to use the appropriate homogenate, blank and specific activity values that correspond to AA and TA respectively.

(b) The "10 min" value refers to the time of incubation.

(c) The "g w/w tissue" refers to the weight of 100 µl of homogenate (in g), and is calculated by:

$$\frac{100}{\text{net biopsy w/w (mg)} + \text{buffer added } (\mu l)} \times 10^3$$

3. Divide AA by TA (x 100) to calculate percentage of enzyme in active form.

MUSCLE ATP AND PCr

1. Add 10 µl of blank (water) and 10µl of ATP standards to 1ml of reagent. All samples are to be done in doublets.
2. Vortex and wipe. Read in fluorometer.
3. Add 25µl of HK and incubate for 30min.
4. While ATP incubates add 10µl of PCr standard to 1ml of reagent. Add 25µl of HK, vortex and wipe and read in fluorometer. Then add 20µl of CK, vortex and wipe, and incubate for 60min.
5. For Muscle samples add 10µl of each sample to 1ml of reagent. Vortex and wipe. Read.
6. Add 25µl of HK to samples. Vortex and wipe and incubate for 30min.
7. Read samples in fluorometer.
8. Then add 20µl of CK to all tubes. Vortex and wipe and incubate for 60 min.
9. Read in fluorometer.

Muscle PCr and ATP

Reagent	Stock Conc.	for 25ml	for 50ml	for 100ml	Final Conc.	Solutions
1. Tris	1.0M	1.25ml	2.5ml	5.0ml	50.0mM	6.05g/50ml
2. $MgCl_2$	1.0M	25.0µl	50.0µl	100.0µl	1.0mM	203.3mg/ml
3. D.T.T	0.50M	25.0µl	50.0µl	100.0µl	0.5mM	77.1mg/ml

4. Glucose	100.0mM	25.0µl	50.0µl	100.0µl	100.0uM	18.02mg/ml
5. NADP	50.0mM	25.0µl	50.0µl	100.0µl	50.0uM	38.27mg/ml
6. G-6-PDH	350U/ml	2.50µl	5.0µl	10.0µl	0.02U/ml	
7. Hexokinase	280U/ml	See Procedure			0.14U/ml	
8. ADP	Solid	See Procedure				
9. Creatine Kinase	25U/mg	See procedure				

Note: add reagents 1 to 5. Bring to volume with distilled water. Adjust to pH 8.1. Add reagent 6.

Enzyme Preparation:

1. Add 25µl of HK to 1 ml of reagent, mix by inversion
3. Add 2mg of CK, and 2mg of ADP to 1ml of reagent, and 10µl of BSA to 1ml of reagent.

MUSCLE CREATINE

Procedure for Assay:

1. Add 10µl of blank (water), 10µl of each standard to 1ml of reagent. All standards and blank are to be in doublets.

2. Vortex and wipe. Incubate for 15min.

3. Read in fluorometer.

4. Add 25µl of CK to all tubes, vortex and wipe.

5. Place in the dark for 30 min.

6. Read.

Muscle Creatine

Reagent	Stock Conc.	for 25ml	for 50 ml	for 100ml	Final Conc.	Solutions
1. Imidazole	1M	1.25ml	2.5ml	5ml	50mM	5.23g/50ml
2. $MgCl_2$	1M	125µl	250µl	500µl	5mM	203mg/ml
3. KCl	1M	0.75ml	1.5ml	3ml	30mM	3.7g/50ml
4. PEP	10mM	60µl	120µl	240µl	25uM	1.9mg/ml

5. ATP	Solid	3mg	6mg	12mg	200uM	
6. NADH	15mM	75µl	150µl	300µl	45uM	10.5mg/ml
7. LDH	125OU/ml	5µl	10µl	20µl	0.24U/ml	
8. Pyruvate Kinase	2000U/ml	10µl	20µl	40µl	0.75U/ml	
9. Creatine Kinase	25U/mg	See Below			3.6U/ml	

Note: Add reagents 1 to 6, bring to volume with distilled water and adjust to pH 7.5. Add reagents 7 and 8

Enzyme Preparation: 1. Add 5mg of creatine kinase to 1ml of reagent
2. Add 10µl of 10 % BSA, mix by inversion.

Standards: 1. 12.5mM Cr

CITRATE SYNTHASE ASSAY

Homogenization Media:
5 mM Kpi (monobasic),
1 mM EDTA ,
0.1mM DTT,
*pH solution to 7.4

- Homogenates are 15 mg wet weight muscle to 300 µl homogenate medium. Although reliable results can be achieved with a range of 10-20 mg wet weight of muscle.

Chemicals Required:
0.1 M Tris Buffer, pH 8.0
DTNB (Ellmans Reagent) 4mg/ml, made in Tris Buffer
OAA (Oxalo-acetic-acid) 6.6 mg/ml made in Tris Buffer
Acetyl CoA 30 mM (to 10 mg bottle add 402.8 µl dH_2O

Method:
1. Add 1ml Tris Base (heated to 37 C in water bath) to cuvette.
2. Add 10 µl DTNB
3. Add 2 µl Acetyl CoA
4. Add 10 µl Homogenate and mix. (mixing with cuvette mixing rod is best)
5. Zero spectrophotometer
6. Add 10 µl OAA and mix. This starts the reaction. Read every 30 seconds for 2-4 min at 412 nm. Reaction is very linear. Calculate activity per minute.
(if dual-spectrophotometer is used, blank receives no OAA

C.S. formula = (OD/.0136)(dilution)(200ul/w.wt muscle)
OD = reading per/minute
Dilution = (vol.of buffer/homogenate) = (1ml/10ul)

APPENDIX IV: Sample Consent and Ethics forms

ADDENDUM TO ETHICS APPLICATION FORM

Part I: Brief summary of proposed research for review by laypersons and by members of medical advisory committee.

Most of the energy for exercise comes from fat and carbohydrate. Protein can also serve as an energy source during endurance exercise. This increased protein oxidation results in an increased protein requirement for most athletes. A controversy in the literature relates to the effect of training for it is known that the highest protein requirements are found in the best trained individuals who are training extensively. This is logical if one considers that the rate limiting enzyme for protein oxidation increases with training. However, there are also increases in the ability to oxidise fats and hence spare carbohydrate, which in turn "spares" protein. To date, their have been no training studies to further explore this issue. Furthermore, our lab has found that females oxidise less protein than do similarly trained males (with a lower protein requirement).

Part II: Brief Outline of the Proposed Research and its Objective:

We plan to study the effects of a 10 week endurance training protocol upon the muscle energetics and protein oxidation capacity and the relationship between these adaptations and whole body protein oxidation in both males and females. This is the first training study to examine these variables and most importantly, it considers the potential sex differences in the response. Subjects will be required to come to the laboratory for physiological testing before and after a 10 week endurance training program. There will be 1 session before training and 2 after training (the first at the same relative intensity of exercise (65% VO_{2peak}) and the other at the same absolute intensity (pre-training load for 65 %). They will be infused with a stable isotope (non-radio-active) of an amino acid on each of the testing session to measure amino acid (protein) oxidation and synthesis. Blood and respiratory gas measurements will be made to measure fuel use during exercise and as an indicator of the metabolic stress. Muscle biopsies will be taken during the first and last training session to look at the enzyme adaptations and the metabolic stress of the exercise and fuel use. Urine samples will be collected during each of a rest and exercise day for each testing session (while on a controlled diet) to determine the whole body protein balance and muscle protein degradation.

(1) Sample size:

We have demonstrated sex differences in protein oxidation with only 6 subjects per group using. Power calculations of this data demonstrate that we only needed 4/group to show the sex differences. In previous work we have shown increased aerobic power in males with 7 subjects and power calculations show that most of these would be apparent with only 5 subjects/group. We do not have any reason to believe that the females would respond to

the training stimulus in a different manner (thus increasing the N to 16 for the training effect).

(2) Design:

Subjects will be tested in the morning, after an overnight fast and will abstain from caffeine consumption for 12 hours and exercise for 48 hours prior to testing. Upon arrival, subjects will have weight taken and will have a 20 Ga plastic catheter inserted into an antecubital vein for blood sampling. Following this, they will be prepared for a muscle biopsy of the vastus lateralis (total of one pre-exercise, one at 5 min of exercise, and one post-exercise sample).

(3) Setting:

Human Performance Laboratory, Ivor Wynne Centre, Rm. A106.

(4) Participants/subjects:

N = 8 males and N = 8 females between the ages of 19 - 30 who do not participate in formal physical activity more than 2 X per week and who do not have any medical or orthopedic contraindications to participating in a endurance training programme.

(5) Intervention:

10 weeks of supervised, progressive endurance exercise training.

(6) End Point:

Completion of testing on all subjects after the 10 week training programme will be the endpoint.

(7) Measurements:

The following measurements will be made during the 90 min cycling exercise test:

a) Cardio-respiratory measures (VO_2, VCO_2, respiratory exchange ratio, ventilation, heart rate) throughout the duration of exercise. Breath samples will be analysed for $^{13}/^{12}CO_2$ ratio using an isotope ratio mass spectrometer.

b) Blood samples will be taken (10 mL) at baseline (-75 min), -15 min (4mL), T=0 (REST) and then every 30 min during exercise (T= 30,60,90 min). These will be analysed for: lactate, ammonia, urea, and glucose. Enrichment of leucine and alanine in the plasma will also be determined at these time points by GC/MS.

c) Muscle will be assayed for: glycogen, ATP, phosphocreatine (PCr), creatine (Cr), and several TCA cycle intermediates (fumarate, malate, succinate and citrate) as well as branched chain ketoacid dehydrogenase activity.

d) Urine will be assayed for: urea, 3-methylhistidine, and creatinine.

Section C: Estimate of the Risks and Benefits of the Proposed Research.

1) What are the proposed benefits to the subjects, the scientific community and/or society that would justify asking subjects to participate?

The subjects will increase their aerobic fitness in a controlled and supervised fashion. They will also gain knowledge of many physiological and nutritional variables that are expensive to have measured in the community. With respect to the scientific community, there is an incomplete understanding of the metabolic response of females to exercise training and no studies have examined the effect of a training programme upon protein oxidation (This may have implications for the protein requirements in athletes).

2) What inducement or compensation is offered to subjects?

All participants will be given a dietary assessment, a copy of their maximal workload (VO_{2peak}), and $ 250.00 to cover time and travel costs.

3) Comment on the risks to subjects.

1. Blood sampling: A single needle stick will be used to place each of 2 plastic catheters such that pain will be minimised. The possibility of a small bruise at the site exists. The total amount of blood over the entire study is about 180 mL (12 tablespoons), which should have no negative effects.

2. Cycling/training: These subjects will be healthy university students with no medical problems. There is a risk of about 1/100,000 of major cardiac events during strenuous exercise in non-cardiac patients in all age groups combined (this is less for the group we will be training and we also have a complete "crash cart on site"). All subjects will have continuous heart rate monitoring during the exercise testing. The training and testing may result in muscle and joint discomfort, however, we have not had any significant problems with this in over a decade of training studies aside from the occasional ice pack use. Physical assessment will be available by Dr. Tarnopolsky, and if appropriate, consultation will be arranged.

3. Muscle Biopsy: I have performed over 6,500 of these in patients and healthy control subjects ranging in age from 1 week to 90 y with the following complications:

- 2/6,500 with a local skin infection.

- 6/6,500 with a fibrous lump at the site of biopsy (connective tissue) - all disappeared with massage for < 1 week.
- 4/6,500 with a small patch of cutaneous hypoalgesia just distal to the biopsy incision (size of a quarter) due to cutting a small sensory nerve branch. In all cases complete recovery occurred in < 3 months.
- the muscle usually has a dull ache for 24 - 48 h (markedly reduced with ice and mild analgesics).
- in theory, one could damage a small motor branch of the vastus and partially denervate a distal aspect of the vastus muscle. This should not affect function for the vastus is one of 4 similar muscles and has an assistive role in knee-extension with these 4. I have not seen this in any of the patients/subjects that I have biopsied.

4. Stable isotopes: The stable isotopes by definition are non-radioactive. The leucine that we use has an extra neutron and is called ^{13}C Vs the more abundant ^{12}C. Each human has about 1.1 % of their body leucine as the ^{13}C form naturally and the infusion brings this amount to about 4 - 5% in order for us to detect it. The isotope level is back to baseline within a few hours after terminating the infusion. There have been over 1000 studies using these types of isotopes in the world and we have used them in 10 studies so far. There are no complications from the isotope *per se* and experiments in animals and plants have shown that lifelong exposure to over 80 % ^{13}C atoms had no measurable effect. The possibility of bacterial contamination of the infusate is possible, however, we prepare this under sterile conditions and infuse it after Millipore filtration and after checking the stock solution with plate culture techniques. We have not had a single complication in over 200 infusion trials.

good day thank you very much for your interest in my study. I have included a copy of the consent form used for this study for you to read. once you have read it over, please let me know if you would like to proceed with the study and I will arrange to meet with you and discuss the next steps. thank you very much for your interest.

Consent/Information Form

THE EFFECT OF ENDURANCE TRAINING ON LEUCINE OXIDATION.

I,_____, consent to participate in a study supervised and directed by Dr. M.A. Tarnopolsky.

PURPOSE: Most of the energy for exercise comes from fats and carbohydrates (sugars). It has become clear that the body can also breakdown protein for energy during exercise. This may have negative consequences for a person who is trying to increase the enzymes in

muscle (proteins). Additionally, if the body is protein deficient, there may be negative health consequences such as increased risk of infections and possibly anemia. Endurance exercise results in an increased number of enzymes contained in a part of our cell called the mitochondria. The enzymes responsible for burning protein also increases, however, increased energy efficiency would tend to counter this and predict a lower protein use. The literature concerning the effects of endurance training upon protein oxidation have been contradictory and have used only animal models. Another very interesting observation that we would like to study is the fact that females have a lesser protein oxidation as compared to males. We are therefore very interested in examining protein oxidation in response to endurance training in the human.

OUTLINE: 1. I am aware that I will be required to participate in a 8 week training study where I will come to the Ivor Wynne Centre (McMaster University) for about 1 hour each day from Monday to Friday to cycle on a cycle ergometer. I will have to cycle to exhaustion prior to, and after 4 and 8 weeks of training. I will participate in a total of 3 testing sessions over this period (one at the beginning and 2 at the end (last 2 separated by 7 - 10 days)). Each testing session will require about 3 hours of my time and I will have to record my diet on three occasions that should take about one hour each time. I will not perform vigorous exercise for 48 hours and will fast overnight and abstain from caffeine for 12 hours prior to testing.

2. During each of the 3 testing sessions I will undergo the following protocol:

Upon arrival (0730 - 0900h (7:30 - 9:30 am)) I will have a small plastic catheter placed into a forearm vein for blood sampling. I will then have a small amount of a sterile amino acid solution (protein) injected into my arm, followed by a slow infusion of this solution. After a 70 minutes rest, another plastic catheter will be placed into a hand vein for blood sampling. I will then have my leg cleaned and prepared for a muscle biopsy of the outside of the leg (see below). I will then exercise on a cycle at a moderate pace for 90 minutes at 65 % of my maximal capacity and will breathe into a plastic mouth piece at 0, 30, 60 and 90 minutes for 4 minutes. I will have another muscle biopsy following the exercise (total = 2). This same protocol will be repeated after the 8 weeks of training at the same percent of my maximal capacity and again one week later at the same absolute intensity as in the pre-training test. I will also have to collect urine on the day before and the day of exercise at the first testing session and for each of the 2 post-training sessions.

POTENTIAL RISKS:

I understand the following potential risks:

1. Muscle Biopsy: The muscle biopsy procedure involves administering local anaesthetic (freezing) to the skin using a needle (like that used for stitches), followed by the making of a small incision (4 - 5 mm), and the removal of a small amount of muscle using a small hollow needle (smaller than a pencil). Dr. Tarnopolsky will perform this and has performed

over 6,500 of these in patients and healthy control subjects ranging in age from 1 week to 90 y with the following complications:

- 2 in 6,500 with a local skin infection.
- 6 in 6,500 with a fibrous lump at the site of biopsy (connective tissue) - all disappeared with massage for less than 1 week.
- 4 in 6,500 with a small patch of decreased skin sensation just beyond the biopsy incision (size of a quarter) due to cutting a small sensory nerve branch. In all cases complete recovery occurred in less than 3 months.
- the muscle usually has a dull ache for 24 to 48 hours (markedly reduced with ice and mild analgesics (Tylenol or aspirin or ibuprofen).
- it is possible (this has not been seen to date), that a small nerve going to my outer leg muscle could be damaged and the lower part of the outside leg muscle could shrink. This could take several months to recover but should not affect muscle function at all.

2. I may get a slight bruise at the site of the intra-venous plastic catheter. I will feel a single pinch as the needle is inserted that is identical to getting a blood test.

3. During the cycling tests I will feel exhausted at the end. There should be no complications form this, but unexpected heart complications such as a heart attack may occur in about 1 in 100,000 persons (all ages-this is less for young healthy persons)). I do not have any known heart, breathing or bone/joint problems for which I have been told not to exercise.

4. There is a risk of muscle strain and discomfort with the training programme, however, I will be instructed in proper technique and stretching (warm-up), and will have access to free muscle/joint assessment (by Dr. Tarnopolsky) if I am experiencing discomfort and my training may be altered or discontinued after discussion with him.

I am aware that if I experience any side effects or desire to withdraw from the study at any time, I may do so without any adverse repercussions, even after signing this form.

4. BENEFITS:

I will receive $ 250.00 for completion of the study and will get a copy of my fitness level before and after and can request a copy of the final paper.
The study is expected to take about 3 months to complete and results for participants should be available within 4 - 5 months by calling Dr. Tarnopolsky (see below).

5. TENTATIVE DATES

- Pre-screening, involving a VO_{2peak} test, a 4 day diet record and DEXA body scan will occur the week before the study (June 30-July 5).

- Trial 1 will commence on July 7 and will run until July 22. There will be 2-3 subjects tested on each of those days (mon-fri).
- Post testing will occur from September 15 through October 3. There will be two separate trials at absolute and relative intensities as described earlier.

I have had the study explained to me by Dr. Mark Tarnopolsky, and understand the nature of the investigation and my rights.

_____ _____ _____

Name Signature Date

_____ _____ _____

Witness Signature Date

I, Dr. Mark Tarnopolsky, have explained the nature of the study to the subject and believe that he/she understood it.

_____ _____ _____

Name Signature Date

I am also aware that I will have a copy of this consent/information sheet.

Contact person in case of emergency:

Dr. Mark Tarnopolsky - 905-521-2100 (6367) or 905-525-9140 (24465)
or page me via 905-521-2100 (x6443 - ask for pager 2888).