THE EFFECT OF RESISTANCE TRAINING ON MUSCLE PROTEIN TURNOVER IN THE FED STATE

THE EFFECT OF RESISTANCE TRAINING ON MUSCLE PROTEIN TURNOVER IN THE FED STATE

By

Jennifer Perco, B.Sc. Kin.

A Thesis Submitted to the

School of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree

Master of Science

McMaster University

© Copyright by Jennifer Perco

August 2005

Master of Science (2005)
(Kinesiology)

McMaster University
Hamilton, Ontario

Title: The effect of resistance training on muscle protein turnover in the fed state

Author: Jennifer Perco, B.Sc. Kin. (University of Waterloo)

Supervisor: Dr. Stuart M. Phillips

Number of Pages: x, 165

ABSTRACT

Resistance exercise results in an increase in muscle protein synthesis (MPS); however, in the fasted state net muscle protein balance (synthesis minus breakdown) remains negative. With the ingestion of post-exercise amino acids, or protein, net protein balance becomes positive and protein accretion can occur over time. Previously, we found that resistance training elevated resting, fasted, mixed MPS, but blunted the acute resistance exercise-induced rise in MPS. We aimed to determine how resistance training would affect mixed MPS chronically and acutely, in the fed state. Young men (N = 10, 21 ± 0.47 yrs, BMI = 25.9 ± 1.4 $kg \cdot m^{-2}$; means ± SD) completed an 8-week unilateral leg resistance training protocol which consisted of knee extension exercise performed at ~80% 1 repetition maximum (1 RM) and resulted in one leg being trained (T), while the contralateral leg served as an untrained (UT) control. Strength gains were 3-fold greater in the T versus the UT leg ($P < 0.05$). As well, mean fibre cross-sectional area of Type I and Type II fibres increased ($P < 0.05$) in the T leg only. Following training, in the fed state, subjects underwent primed constant infusions of [ring-d_5 or -$_{13}C_6$]phenylalanine or d_3-α-ketoisocaproic acid in a randomized counterbalance manner to determine rates of MPS using the precursor product equation for incorporation into mixed muscle proteins, sampled via muscle biopsy of the vastus lateralis. MPS was assessed at rest, acutely following a bout of resistance exercise, which was at the same relative intensity for both legs, and approximately 28 h after the resistance exercise bout. Rates of MPS at rest were unchanged following training ($P = 0.97$). Following the resistance exercise bout, rates of MPS were elevated acutely in both the T and UT legs (4 h post-exercise; $P < 0.01$), but

returned to baseline levels by 28 h post-exercise in the T leg only. We conclude that while resistance training did not alter resting MPS, it did affect the time course of MPS following an acute bout of resistance exercise compared to the UT leg. Hence, when taken with our previous results we conclude that resistance training attenuates the MPS response to an acute bout of resistance exercise when loads are matched at the same relative intensity, but the response is not altered by the nutritional status of the individual.

ACKNOWLEDGEMENTS

I would like to thank my Mom, Dad, Grandma, my brother Brad, my fiancée Gavin, and my friends for their constant support, encouragement, and love during the completion of this project. They were always understanding of the pressure and the time constraints involved in completing this thesis and to easy the load they even took over planning the wedding. As well, they truly took an interest in my work and learned a lot more about protein metabolism than they ever thought they would. I need to express special gratitude to my grandmother who allowed me to set up an office in her house to write this manuscript. During this time my grandmother pampered me by cooking for me, as well we were able to spend quality time together watching The Price is Right and A Wedding Story daily. Thank you for everything.

I don't know where to begin thanking my supervisor Dr. Stuart Phillips. He gave me the great opportunity to work in his lab and get excited about research. He allowed me the freedom to try and work things out for myself but he was always there with his advice, guidance, and time when they just wouldn't work out. I really appreciate everything that you have done for me and all that I have gained throughout this process. Thank you.

I would like to express my appreciation to my committee members. To Dr. Martin Gibala, Dr. Mark Tarnopolsky, and Dr. Maureen MacDonald, thank your for your insight, motivation, and most importantly your friendship. I am really privileged to have had the opportunity to work with you.

I would also like to thank the following people who have assisted with this project. Jason Tang and Joe Hartman greatly assisted with the histochemical analysis and I would never have been able to do without them. I had a blast running this study together with Sarah Wilkinson's Ph.D. study, it really lightened the load. I would also like to thank Sarah for her help with the HPLC analysis. I am very grateful to all those people who let me crash on their couches and/or futons, so that I could be up and at school in time for those early morning infusion trials. In addition, I would like to thank the volunteers who spent many of their days training the subjects and greatly assisted during the infusion trials.

I especially need to thank Todd Prior for fixing everything that I broke in the lab without giving me too much of a hard time about it. Todd has become a good friend and he always knew when I needed a caffeine or an ice cream break. Thank you.

Finally, I would like to thank all the friends (you know who you are) that I have made during these two years, you have really made this experience worthwhile. Your friendship has really kept me going through the rough times I encountered with my analysis. We have had many laughs and hopefully there will be many more to come.

TABLE OF CONTENTS

Abstract . . . iii
Acknowledgments . . . v
List of Appendices . . . viii
List of Figures . . . ix
List of Tables . . . x

Chapter I: Literature Review

1.1 Introduction . . . 1
1.2 Protein Breakdown . . . 2
1.2.1 The Effect of Resistance Exercise on Protein Breakdown . . . 3
1.2.2 The Effect of Nutritional Status on Protein Breakdown . . . 4
1.3 Protein Synthesis . . . 5
1.3.1 The Effect of Resistance Exercise on Protein Synthesis . . . 5
1.3.2 The Effect of Nutritional Status on Protein Synthesis . . . 9
1.3.3 Protein Synthesis of Skeletal Muscle Protein Sub-Fractions . . . 11
1.4 Regulation of Protein Synthesis . . . 13
1.4.1 The Effects of Increased Amino Acid Availability and Resistance Exercise on Translation Initiation . . . 16
1.5 Rational for Research . . . 20
1.6 Hypothesis . . . 22

Chapter II: The effect of resistance training on mixed muscle protein synthesis in the fed state

2.1 Introduction . . . 24
2.2 Methods . . . 27
2.2.1 Subjects . . . 27
2.2.2 Study Design . . . 27
2.2.3 Resistance Training Protocol . . . 28
2.2.4 Strength Measurements . . . 29
2.2.5 Experimental Infusion Protocol . . . 30
2.2.5.1 Isotope . . . 33
2.2.5.2 Blood Sampling . . . 34
2.2.5.3 Muscle Biopsies . . . 35
2.2.6 Analysis . . . 36
2.2.6.1 Blood Plasma . . . 36
2.2.6.2 Muscle Analysis . . . 39
2.2.7 Calculations . . . 41

2.2.8 Muscle Fibre Type and Cross-Sectional Area 42
2.2.9 Statistics 43
2.3 Results 46
2.3.1 Strength Measurement 45
2.3.2 Muscle Fibre Cross-Sectional Area 47
2.3.3 Amino Acid Concentrations 48
2.3.4 Glucose and Insulin Concentrations 49
2.3.5 Mixed Muscle Protein Fractional Synthetic Rate 49
2.4 Discussion 52

References 68

LIST OF APPENDICES

Appendix 1. Information and Consent to Participate in Research Form
Subject Screening Questionnaire
Description of Medical Procedures 78

Appendix 2. Subject's Physical Characteristics 91

Appendix 3. 1 RM Strength Measurements and Anova Table
Isokinetic Strength Measurement and Anova Table
Isometric Strength Measurement and Anova Table 93

Appendix 4. Histochemistry Raw Data and Anova Table
Histochemical Images 98

Appendix 5. Daily Caloric Intake and Composition of Feeding During Testing . . . 104

Appendix 6. Plasma Glucose and Insulin Concentration
Raw Data and Anova Table 108

Appendix 7. Plasma Amino Acid Concentration Raw Data and Anova Table 113

Appendix 8. Mixed Muscle Fractional Synthetic Rate Raw Data and Anova Table 120

Appendix 9. Total Ion Chromatograms 130

Appendix 10. Muscle Fibre Analysis/Histochemistry Protocol 136

Appendix 11. Plasma Glucose and Insulin Assays 141

Appendix 12. Preparation of Blood/Plasma for HPLC Analysis
Plasma Amino Acid Analysis by HPLC 147

Appendix 13. Prepartion of Blood/Plasma for Analysis by GCMS
Preparation of Muscle for Analysis by GCMS
Amino Acid Isolation/Purification
Derivatization of Protein Hydrolysate and Amino Acids 161

LIST OF FIGURES

Figure 1.	Schematic representation of protein turnover	1
Figure 2.	The role of various initiation factors in controlling the steps of protein synthesis initiation	15
Figure 3.	The process of translation initiation	15
Figure 4.	The signalling pathways regulated by resistance exercise and amino acid availability	17
Figure 5.	The response of muscle protein turnover following feeding a protein containing meal and the consumption of that same meal following resistance exercise	19
Figure 6.	Time course of the elevation in muscle protein synthesis after an acute bout of resistance exercise	21
Figure 7.	Schematic representation of the testing sessions following training	31
Figure 8.	Knee extension 1 RM strength assessments	45
Figure 9.	Isokinetic leg strength tested concentrically at 0.52 rad·s^{-1}	46
Figure 10.	Isometric strength measurements	46
Figure 11.	Mean fibre Cross-Sectional Area	47
Figure 12.	Mixed muscle FSR	51
Figure 13.	Time course of the elevation in muscle protein synthesis after a single bout of resistance exercise	54

LIST OF TABLES

Table 1. Action of eukaryotic initiation factors in promoting protein synthesis 16

Table 2. Plasma amino acid concentrations 48

Table 3. Plasma glucose and insulin concentrations 49

Table 4. Mean blood isotope enrichment during the infusion trials 50

Table 5. Mean muscle intracellular isotope enrichment during the infusion trials 50

1.1 INTRODUCTION

Approximately 40% of the body's weight is comprised of skeletal muscle and it contains about 50-75% of all protein in the human body. Proteins in the human body are in a continuous state of turnover that is, simultaneously being synthesized and broken down. Protein synthesis and breakdown are two opposing processes that occur at the same time, which may seem counterproductive; however, it does allow for constant repair and remodelling within the tissue to occur. The fluxes of protein synthesis and protein breakdown are termed protein turnover. Within any given day, our skeletal muscle will go through several periods of anabolism and catabolism. Our pattern of feeding and fasting is an example of cycling between states our body goes through regularly. Even though skeletal muscle protein turnover is relatively slow (1-2%·day^{-1}) in comparison to other tissues, it can contribute to approximately 20-30% of whole body protein turnover since skeletal muscle is a far larger reservoir of protein (63).

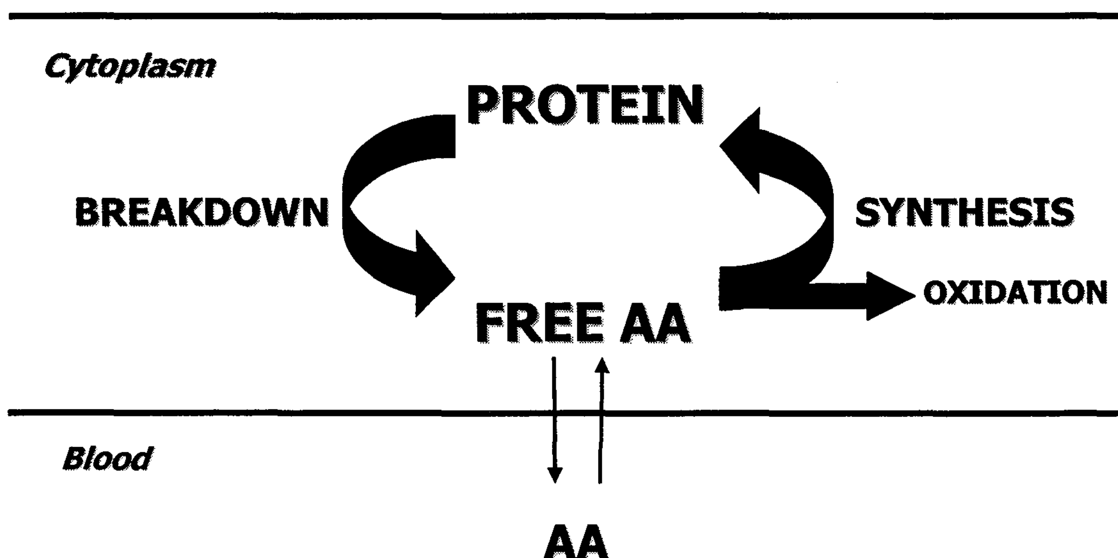


Figure 1: Schematic representation of protein turnover. AA; amino acids.

The algebraic difference between protein synthesis and protein breakdown (i.e., protein synthesis minus protein breakdown), which is termed net protein balance, determines whether protein accretion or attrition occurs. At rest, in the post-absorptive or fasted states, there are low levels of circulating amino acids and insulin, resulting in a net negative protein balance (i.e., protein synthesis < protein breakdown). However, with the feeding of a mixed carbohydrate and protein meal, there is an increase in the level of circulating amino acids and insulin, resulting in a net positive protein balance (i.e., protein synthesis > protein breakdown). Throughout the day the body fluctuates between the post-absorptive to fasted and fed to post-prandial states, with the degree of change in protein turnover between these states being about equal; thus the habitual consumption of a balanced diet containing sufficient protein (especially essential amino acids) does not result in any net gain or loss of bodily proteins. However, skeletal muscle is one of the best examples of a tissue that is able to adapt to different stimuli through changes in protein synthesis and to a lesser degree protein breakdown.

1.2 PROTEIN BREAKDOWN

Skeletal muscle is the body's major reservoir of protein and amino acids. Muscle protein can be hydrolyzed into amino acids likely in an attempt to regulate a constant intracellular amino acid concentration (81) and/or as precursors for gluconeogenesis. There are multiple pathways for protein breakdown, all with distinct physiological functions; the lysosomal system, cytosolic proteases, mitochondrial proteases, and the ubiquitin-proteasome pathway (40). The lysosomal system is composed of acid proteases

(i.e., cathepsins) that primarily breakdown membrane proteins and glycoproteins (37; 40). The cytosolic proteases including calpains, are activated by calcium and appear to play a role in cell injury when cytosolic calcium rises (40). Mitochondria contain their own complete system for breakdown of organelle specific proteins known as mitochondrial proteases (40). However, the bulk of intracellular proteins are degraded by the ubiquitin-proteasome pathway (40). Regrettably, we understand very little about how intramuscular proteolysis is regulated. Most of our information on the regulation of these various protein degradation systems is derived from in vitro or ex vivo systems; hence, extension of these finding to intact physiological systems is dubious at best.

1.2.1 The Effect of Resistance Exercise on Protein Breakdown

The response of muscle protein breakdown following resistance exercise has not been extensively studied and results have differed, which may be attributed to methodological issues. Studies using 3-methylhistidine excretion in the urine as a marker of skeletal muscle myofibrillar protein breakdown have reported increases (20; 20; 58), decreases (49; 62), or no change (59; 61) in myofibrillar proteolysis in response to exercise. Upon examining studies using tracer methodologies, muscle protein breakdown has consistently been shown to increase in response to heavy resistance exercise (9; 56; 57). Phillips *et al.* (56) observed that protein breakdown in response to exercise was elevated by 31% at 3 h post-exercise and remained elevated at 24 h post-exercise; however, protein breakdown returned to resting levels by 48 h post-exercise in untrained participants. It appears to make sense that protein breakdown would be elevated with

resistance exercise, particularly when the lengthening component of the exercise is emphasized, which we know induces damage (30). To date, no relationship between ultrastructural muscle damage and protein breakdown has be observed, at least as measured with a robust method such as stable isotope measured breakdown rate.

1.2.2 The Effect of Nutritional Status on Protein Breakdown

The systemic infusion of insulin has resulted in an improved whole-body and local net protein balance, predominantly by inhibiting protein breakdown (28). However, the inhibition of proteolysis results in a systemic hypoaminoacidemia and thus a reduction in protein synthesis also. Indeed, Biolo *et al.* (9) showed that insulin infused into the femoral artery, which did not elevate systemic insulin concentrations, significantly attenuated the exercise-induced rise in protein breakdown. As well, a drink supplement containing carbohydrate (68) or carbohydrate with amino acids given after exercise also reduced the exercise-induced rise in 3-methylhistidine excretion or myofibrillar protein breakdown (64). Phillips *et al.* (55) found that post-exercise protein breakdown was not different between trained and untrained participants in the fed state. However, the supplement, specifically the insulin released in response to the supplement, did not block the entire exercise-induced rise in protein breakdown in the untrained state. This is could be due to the intensity of the exercise, which was at the same absolute intensity between the trained and untrained states meaning there was a greater stimulus for protein breakdown in the untrained state.

1.3 PROTEIN SYNTHESIS

1.3.1 The Effect of Resistance Exercise on Protein Synthesis

An acute bout of resistance exercise has been shown to increase protein synthesis in both humans (5; 17; 43; 55-57) and animals (24). However, some studies have failed to observe a significant increase in protein synthesis following an acute bout of resistance exercise (38; 68; 75). It has been suggested that these studies that failed to find a significant rise in *mixed* muscle protein synthesis could be due to the fact that the subjects were resistance exercise trained (68) and may have had a biological adaptation to the stress of exercise. For example, the highly trained swimmers studied by Tipton *et al.* (75) showed no rise in deltoid muscle protein synthesis following more than 60 contractions which were performed at more than 65% of 1 repetition maximum (1 RM); it may be, however, that the intensity of the exercise was not sufficient to stimulate protein synthesis. Rennie and Tipton (65) suggested that mixed muscle protein synthesis responds to a repeated stimulus of a constant magnitude (i.e., same absolute workload) like resistance exercise by progressively decreasing the disturbance of homeostasis that stimulus would have caused, like changes to the ADP/ATP ratio, myofibrillar protein disruptions, and possibly connective tissue damage. For example with resistance training there is an increase in the mitochondrial content, and thus oxidative enzyme content (Tang *et al.*, 2005; in review), which would reduce the perturbation in the cell's energy status (ADP/ATP ratio). Maintaining the energy status of the cell would allow protein synthesis to proceed, since protein synthesis is suppressed in conditions of low energy status (16). As well, Gibala *et al.* (29; 30) has shown that resistance exercise results in

less muscle fibre damage in resistance trained individuals (29) compared to untrained individuals (30), especially during the highly fibre damaging eccentric phase (48) of weight lifting. Since less muscle fibre damage is occurring following training a reduced rise in protein synthesis in response to the exercise would be required to repair and regenerate the muscle fibre structure. Therefore, to elicit a response in muscle protein synthesis that is similar in magnitude to that observed before training the magnitude of the stimulus (i.e., resistance exercise) would have to be increased (i.e., greater intensity and/or increased volume) to stress the tissue beyond its existing capacity. Indeed, following 8 weeks of resistance training the excise-induced rise muscle protein synthesis was somewhat attenuated compared to untrained values when measured following an acute bout of resistance exercise at the same absolute intensity (55). In trained subjects, Chesley *et al.* (17) reported a 50% exercise-induced increase in muscle protein synthesis when subjects performed 12 sets of 6-12 repetitions at 80% 1 RM. However, in the studies that did not show a significant exercise-induced rise in muscle protein synthesis the volume and/or intensity of the resistance exercise bout was lower; 6 sets at 65% 1 RM through a whole body routine (75), 6 sets at 80% 1 RM through specific exercises for the muscle group sampled (38), or 8 sets at 85% 1 RM through specific exercises for the muscle group sampled (68). Thus it may be that a heavy bout of resistance exercise is needed to stimulate muscle protein synthesis.

As well, it may be important to examine the time post-exercise when protein synthesis was measured when comparing studies that have observed a change to studies that have failed to observe an exercise-induced rise in protein synthesis. Indeed, studies which

have attempted to delineate the time course of the exercise-induced increase in muscle protein synthesis have reached somewhat different conclusions. Phillips *et al.* (56) showed that protein synthesis was elevated by 112% at 3 h post-exercise and still remained significantly elevated over resting levels by 65% and 34% at 24 and 48 h post-exercise, respectively, in untrained subjects. Conversely, MacDougall *et al.* (43) pooled data from similar studies (17; 43) which demonstrated that protein synthesis was elevated by 50% at 4 h post-exercise, and peaked at 24 h post-exercise with an elevation of 109% over resting levels, but had returned to resting levels by 36 h post-exercise in trained subjects. The shorter time course of elevated protein synthesis reported by MacDougall *et al.* (43) has been hypothesized to be due to the training status of the participants. However, Kim *et al.* (38) failed to find a significant increase in protein synthesis 16 h post-exercise in trained muscle. This may be due to the fact that MacDougall *et al.* (43) studied the protein synthetic response in the biceps brachii, where Kim *et al.* (38) examined the vastus lateralis, so the time course may be slightly altered by the muscle group examined. As well, MacDougall *et al.* (43) used different groups of subjects to study protein synthesis at each time point post-exercise in order to construct the overall time course of muscle protein synthesis. Kim *et al.* (38) used the same subject for each time point which would have resulted in a clearer (i.e., within-subject variability in the response versus between subject variability) time course. Nevertheless, it appears that resistance training has a profound impact on the time course of the exercise-induced rise in muscle protein synthesis.

Resistance exercise training is well known to induce muscle fibre hypertrophy which

is defined as an increase in muscle fibre cross-sectional area (38; 44; 45). The increase in muscle fibre cross-sectional area is the result of the addition of new contractile proteins. It may seem that resting protein turnover should be increased to allow for the constant repair and remodeling of the additional muscle proteins when muscular hypertrophy is present. Indeed a number of studies have observed an increase in resting muscle protein synthesis following training in humans (38; 55; 57; 83) and in rats (22). An increase in resting muscle protein synthesis in both young and elderly humans has been reported in as little as 2 weeks of resistance training (82; 84; 85). These findings may have been confounded by the fact that the post-training measures were made within 24 h following the last bout of resistance exercise. As previously mentioned, protein synthesis can remain elevated for up to 48 h post-exercise and thus it would be difficult to differentiate any carry-over effects from the last exercise bout from a chronic training effect on resting protein synthesis. A number of studies have measured resting protein synthesis at least 72 h following the last training bout to differentiate any carry-over effects from the last training bout (38; 55; 57). They have consistently found elevated resting protein synthesis following training, even through it did not reach statistical significance ($P = 0.19$) in one study (57). Even though resting protein synthesis appears to be increased by resistance training, there appears to be no change in the net muscle protein balance in response to training since proteolysis is also elevated and net balance remains unchanged (55; 57).

1.3.2 The Effect of Nutritional Status on Protein Synthesis

Amino acid infusion and/or oral supplementation has been shown to transiently increase protein synthesis at rest (6; 8; 10; 11; 52). Protein synthesis has been shown to increase to a greater extent following exercise with an increase in amino acid availability (8; 47; 74; 76). This amino acid-exercise symbiosis indicates that the two stimuli are activating protein synthesis either through an additive common mechanism or via separate pathways. Tipton *et al.* (76) determined that only essential amino acids are necessary to stimulate protein synthesis following resistance exercise, since a drink containing both essential and non-essential amino acids did not further stimulate protein synthesis nor promote a more positive protein balance than the drink containing only essential amino acids (76; 79). Another important anabolic response to feeding is the increase in plasma levels of insulin. Some controversy exists about insulin's role in protein synthesis, which may be confounded by the fact that insulin infusion that affects systemic insulin levels produces pronounced systemic hypoaminoacidemia, due to a sharp reduction in proteolysis likely in labile fast turning over tissues such as gut and liver, which results in a lack of substrate (i.e., amino acids) for protein synthesis. At rest, insulin has no effect on protein synthesis, however insulin has been shown to improve the net muscle protein balance by inhibiting protein breakdown (27; 28). Post exercise there is no further stimulation of protein synthesis with insulin (9) although net protein balance improves due to an inhibition of protein breakdown, but the balance is still negative (9; 15). It seems that the action of insulin on protein synthesis is permissive in nature, which means that there is a critical concentration of insulin below which rates of protein

synthesis start to fall; however, it appears that this concentration is remarkably low and is seen only in diabetic states (25; 60). Increasing amino acid availability, by infusion or ingestion, coincident with an increase in insulin levels, which can be achieved from carbohydrate administration, results in increased protein synthesis, at rest (74) and following resistance exercise (64) with an improved and positive net muscle protein balance. It is believed that the net effect of the combination of amino acids and carbohydrates is equal to the sum of the individual effects, although a definitive study has not been done since the previous conclusion is based on the comparison of results from two separate studies (64; 74). The data suggests that to maximize the improvement in net muscle protein balance at rest and following resistance exercise the consumption of amino acids and carbohydrates, to elevate insulin, are required.

An average net positive muscle protein balance needs to occur throughout the resistance training period in order to account for the resultant muscular hypertrophy. It is important to note that the consumption of a drink containing essential amino acids and carbohydrates given either 1 or 3 h following resistance exercise stimulated muscle protein anabolism by increasing protein synthesis to an equivalent extent irrespective of the timing of the drink post exercise (64). This data indicates that the exact timing of a meal post exercise is inconsequential as long as it is within 3 h post exercise. Tipton *et al.* (77) found that consuming a drink containing essential amino acids and carbohydrates prior to exercise resulted in a significantly larger increase in the net muscle protein balance and greater rates of protein synthesis compared to the consumption of the drink post exercise. The increased rate of protein synthesis can be attributed to an increase in

amino acid delivery to the muscle and a resultant increase in uptake of amino acids (77). The greater amount of amino acids delivered to the muscle was attributed to insulin-mediated improvements in blood flow. Indeed, the pre-exercise supplementation group had significantly higher blood flow values both during exercise and 1 h post-exercise which resulted in a greater delivery of amino acids to the muscle and in turn an improved net muscle protein balance (77).

To gain muscle mass with a resistance training program there needs to be a chronic positive net muscle protein balance over the training period. Feeding within 3 h following each training session would be sufficient for protein accretion to occur due to the additive effects of amino acids, obtained through a regular diet, combined with chronic resistance exercise bouts (64; 74; 77). However, overfeeding protein does not increase the size of lean body mass, and excess amino acids are oxidized (73) or their carbon skeletons can be stored as fat. In fact, when amino acids were continuously infused the resultant hyeraminoacidemia transiently increased protein synthesis for the first couple of hours before falling and by 4 h was not significantly different from basal protein synthetic rates despite continued availability of amino acids (11).

1.3.3 Protein Synthesis of Skeletal Muscle Protein Sub-Fractions

The measurement of mixed muscle protein synthesis represents an average synthesis rate of several cellular protein and protein sub-fractions; mitochondrial, sarcoplasmic, and myofibrillar. It is important to examine specific changes in skeletal muscle sub-protein fractions because specific changes in one of these pools could be “masked” when only

mixed muscle protein synthesis is monitored, especially after resistance exercise. Myofibrillar proteins that include myosin, actin, troponin, tropomysin, titin, and nebulin, comprises approximately 70-80%, by weight, of all skeletal muscle proteins (3; 31; 32). Bohé *et al.* (11) found that changes in myofibrillar protein synthesis are closely related to changes in the magnitude of the increase in mixed muscle protein synthesis after an amino acid infusion showing that protein fractions respond very similarly to feeding, which is perhaps not surprising. However, with resistance exercise, a stimulus that likely targets in a more specific fashion the contractile proteins (i.e., myofibrillar) differences might be expected. With an acute exercise program both mixed muscle protein synthesis and myosin heavy chain (MHC) synthesis were increased (32). Recent evidence from Miller *et al.* (46) showed that intense muscle contraction as a result of single leg kicking elicited a rise in myofibrillar and sarcoplasmic protein synthesis that persisted for up to 48 h, but that had returned to baseline by 72 h post-contraction, which is similar to results observed in mixed muscle protein synthesis. However, when subjects become accustomed to resistance exercise, that is become resistance trained, the increase in mixed muscle protein synthesis is attenuated, as well, myofibrillar and MHC protein synthesis contributes to a larger portion of the mixed muscle protein synthetic response (38). Myofibrillar synthesis was unchanged after 3 months of resistance exercise (80) and after 8 weeks of unilateral resistance exercise training (38). The majority of the myofibrillar protein fraction is made up of myosin and myosin composes approximately 25-30% of all muscle protein (4). However, resting MHC synthesis, has been shown to account for approximately 18% of mixed muscle protein synthesis (3), and MHC synthesis rates have

been correlated with changes in muscle mass and strength (4). So as one becomes trained they may be able to synthesize more myofibrillar proteins/MHC needed for the greater force production observed after training, which may result in trained individuals having myofibrillar or MHC synthesis comprise a greater faction of mixed muscle protein synthesis. Even though resting mixed muscle protein synthesis has been shown to increase following training, myofibrillar protein synthesis remains unchanged meaning that the synthesis of non-myofibrillar proteins must have been increased. This seems to be counterintuitive, as the general training principle is that with repeated stress, such as resistance exercise, the body becomes better able to adapt to the stress by 'fine tuning' the response. Recent data from our lab (38) has shown that training does result in less of a perturbation in mixed muscle protein synthesis after a single bout of resistance exercise. Approximately 16 h after exercise mixed muscle protein synthesis increased to a greater proportion in untrained muscle, but post-exercise myofibrillar synthesis was similar between trained and untrained. This shows how trained individuals may be better able to fine tune the synthetic machinery, since myofibrillar synthesis can be selectively increased in response to resistance exercise whereas less of an increase in non-myofibrillar proteins is needed to achieve the necessary myofibrillar protein synthetic response.

1.4 REGULATION OF PROTEIN SYNTHESIS

Protein synthesis is the primarily regulated variable in the protein turnover equation in most physiologic states, as it seems that protein breakdown varies little in

response to the same external stimuli, such as feeding and resistance exercise, in comparison to protein synthesis. Cellular processes, such as transcription and translation can potentially regulate an increase in protein synthesis. However, the protein synthetic response following exercise appears to be too rapid, within 3-4 h, to be due to significant changes in the translational capacity (i.e., increase in RNA content) of the system (63). Indeed, 4 and 24 h following heavy resistance exercise in the human biceps muscle, the exercise-induced rise in protein synthesis was accompanied by an increase in RNA activity, although the total RNA capacity remained unchanged (17). However, measuring total RNA and total RNA activity may mask changes of specific proteins that are necessary in upregulating protein synthesis. Kubica *et al.* (39) did show that there was an increase in a specific translational regulatory protein important in the protein synthetic response prior to an elevation in the relative quantity of mRNA of this protein. It may be that a rapid increase in mRNA translation is followed by a slower elevation in gene transcription in order to mediate the protein synthetic response. The rapid increase in protein synthesis is in response to an upregulation of the systematic translational efficiency; that is, how rapidly existing mRNA are translated into proteins (23; 51). An increase in the translational efficiency of the system is reflective of the number of ribosomes (among other things) available, which would allow for a greater amount of proteins to be synthesized at one time (51).

The process of mRNA translation is composed of three consecutive sequences of events known as peptide chain initiation, elongation, and termination (53). The most tightly regulated of these three phases is peptide chain initiation, which is controlled by a

family of proteins termed eukaryotic initiation factors (eIFs) (53). Without these assisting proteins the process of translation initiation would occur at an extremely slow rate (35). The efficiency of protein translation is primarily controlled by the phosphorylation state of specific eIFs. Changes in the phosphorylation state of these proteins affect their affinity for other proteins by altering the charge and conformation of the protein (53).

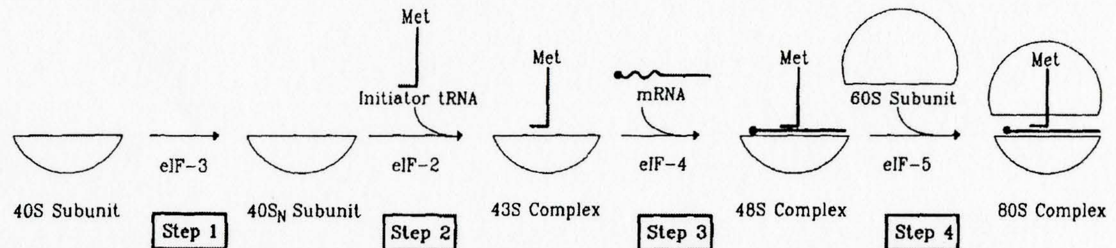


Figure 2: The role of various initiation factors in controlling the steps of protein synthesis initiation (66).

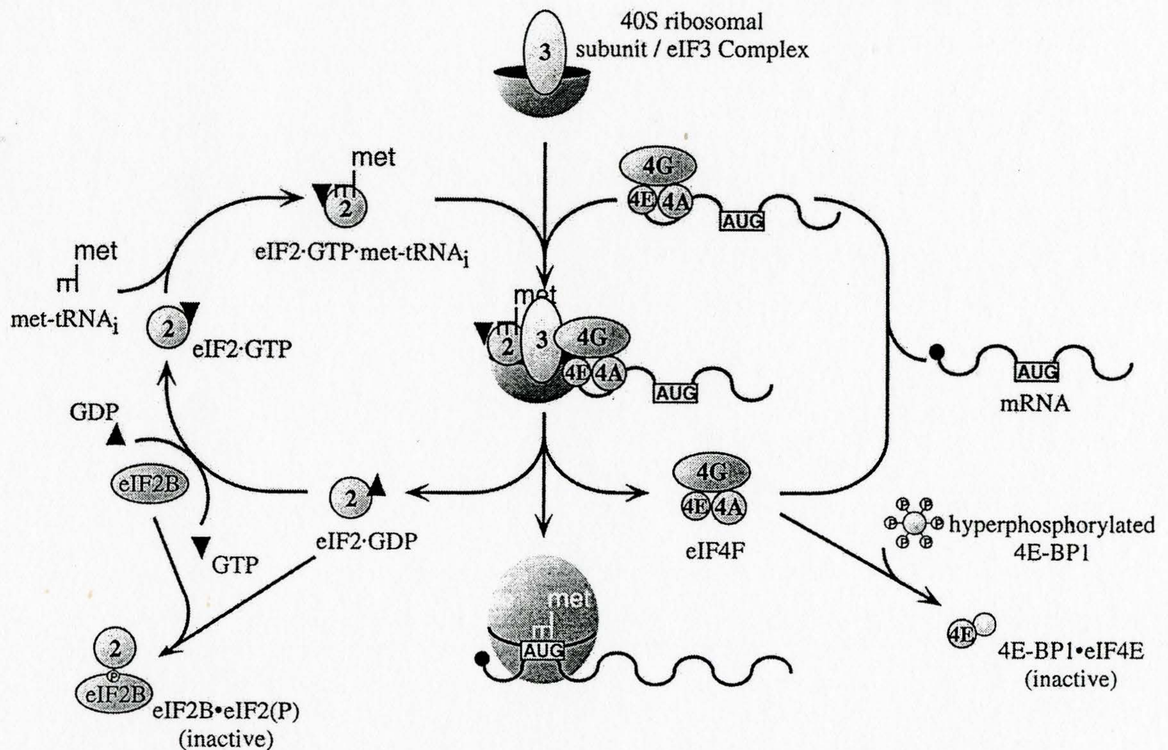


Figure 3: The process of translation initiation. The diagram highlights two key regulatory steps in translation initiation; binding of met-tRNA$_i$ to the 40S subunit (left side) and binding of mRNA to the 43S preinitiation complex (right side) (12).

Table 1: Action of eukaryotic initiation factors in promoting protein synthesis.

Initiation Factor	Action
eIF3	Dissociation of the 80S ribosome into the 40S and 60S subunits
eIF1A	Stimulates the formation of 43S ribosomal subunit
eIF2	Stimulates the binding of Met-tRNA to 40S subunit to form the 43S subunit
eIF2B	Catalyzes the exchange of GDP for GTP on eIF2 thus activating it
eIF4F	Binds mRNA to the 43S complex forming the 48S complex
eIF4A	RNA helicase
eIF4E	Recognizes the 7-methyl cap and binds
eIF4G	Scaffold protein than bridges subunits of eIF4F (i.e., eIF4A and eIF4E)
eIF5	Promotes hydrolysis of eIF2*GTP on the 48S complex allowing it to join with the 60S complex forming an active 80S ribosome

1.4.1 The Effects of Increased Amino Acid Availability and Resistance Exercise on Translation Initiation

Resistance exercise and an increase in amino acid availability independently stimulate protein synthesis, as well the combination of the two results in a greater increase in protein synthesis (11; 56; 64). Resistance exercise provides an efficient stimulus in activating the guanine exchange factor eIF2B (13; 67), but has less of an effect on the formation of the eIF4E·eIF4G complex (13). As well, $p70^{S6k}$ activity is increased in response to resistance exercise, which can stimulate the S6 protein on the ribosome to enhance protein synthesis (13). The increase in $p70^{S6k}$ activity closely parallels the increase in protein synthesis following resistance exercise as $p70^{S6k}$ activity remained elevated 24 h post-exercise (13; 34; 50; 63). Baar and Esser found that the increase in the phosphorylation state and hence the activity of $p70^{S6k}$ was significantly correlated (0.998) with the increase in muscle mass of rats following 6 weeks of resistance training (2). Resistance exercise stimulates protein synthesis initiation through

a PKB pathway, though the increase in this part of the pathway is transient (12; 50; 67); however the stimulation of mTOR closely parallels the increase in proteins synthesis (13).

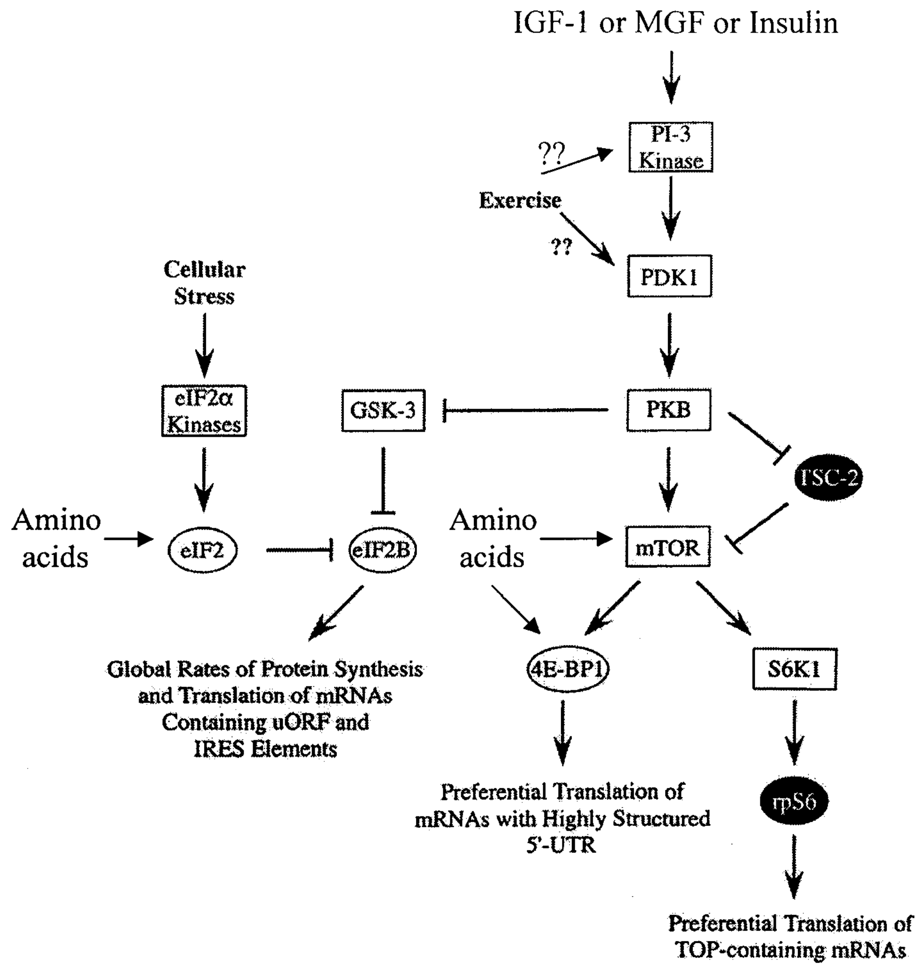


Figure 4: The signalling pathways regulated by resistance exercise and amino acid availability (12).

Feeding resulting in an increased availability of amino acids and insulin stimulates the translational apparatus through a PI3K/PKB dependent pathway, which is important in insulin's permissive effect on protein synthesis, as well amino acids (especially branched chain amino acids) alone can stimulate a mTOR dependent pathway resulting in

the stimulation of p70^{S6k} and the hyperphosphorylation of eIF4E·BP1 (41; 42). However, mTOR stimulation is sufficient but not required in amino acid stimulation of eIF4E to dissociate from its binding protein and associate with eIF4G, thus amino acids can stimulate eIF4E through a mTOR independent pathway (1; 78). The end result, with or without the stimulation mTOR, is increasing amino acid availability activates of the downstream targets of mTOR, 4E·BP1 and p70^{S6k} (18; 19).

The coordination of the signaling pathways involved in protein synthesis initation would result in the greatest increase in the translational efficiency of the system. When resistance exercise is combined with feeding the greater increase in protein synthesis may potentially come from the synergistic enhancement of protein translation initiation. For example, the stimulation of PKB with both resistance exercise and feeding of a carbohydrate and amino acid drink resulted in a prolonged stimulation of PKB, as opposed to a transient rise observed with resistance exercise alone (18). As well, the combination of exercise and intravenously or orally increasing circulating amino acid levels can enhance both targets of mTOR, as well as inhibit GSK-3 in order to stimulate eIF2B. The action of resistance exercise combined with normal feeding can regulate translation initiation by stimulating all potentially limiting factors, thus coordinating the protein synthetic response. The stimulation of the guanine exchange factor eIF2B will allow eIF2 to cycle faster through the building of the 80S ribosome, while the hyperphosphorylation of 4E·BP1 releases eIF4E so that it is free to bind with eIF4G and eIF4A, forming the eIF4F complex to bind mRNA to the ribosome. This action is potentially further enhanced by the phosphorylation of eIF4E to drive the dissociation of

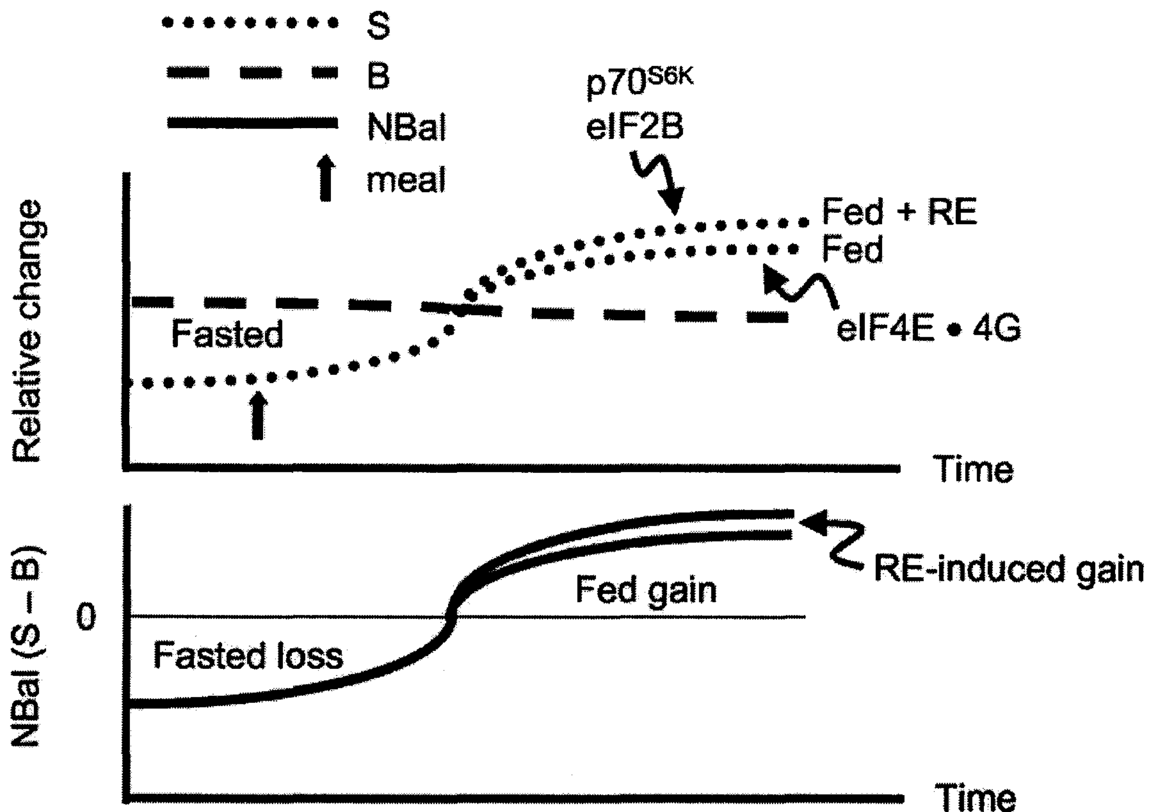


Figure 5: The response of muscle protein turnover following feeding of a protein containing meal and the consumption of that same meal following resistance exercise (63).

eIF4E with its binding protein, while enhancing its ability to bind mRNA and form the eIF4F complex. In addition, the stimulation of p70^{S6k} results in the phosphorylation of ribosomal protein (rp) S6 on the 40S ribosome to enhance the translation of TOP-containing mRNA which code for ribosomal proteins, elongation factors, eIF4G, and poly(A) binding protein (12; 51), which are all important to increasing the capacity of the translational apparatus. However, the full response of protein synthesis and translation initiation to either the combined or single effect of resistance exercise or an increase in amino acid availability is not observed in the absence of a minimal level of circulating insulin.

1.5 RATIONAL FOR RESEARCH

The training status of an individual may impact upon the effect that a single bout of resistance exercise has on protein turnover, since resistance trained individuals have an attenuated response in protein synthesis following resistance exercise (38; 43; 55; 55-57). Phillips *et al.* (56) found that protein synthetic rates after a single bout of resistance exercise peaked at 3 h post exercise and were still elevated 48 h post exercise in *untrained* subjects. In contrast, a study by MacDougall *et al.* (43) found that protein synthetic rates after a single bout of resistance exercise peaked at 24 h post exercise but were back near baseline by 36 h post exercise in *trained* individuals. It is possible that the training status of the individuals in the two studies may account for the differences in the time course of the increases in muscle protein synthesis following resistance exercise. In a recent study (38) from our lab we examined the effect of training status on protein synthesis. The study used an 8-week unilateral resistance training protocol where one limb became trained and the contralateral limb remained as an untrained control. The results from the study showed that resting protein synthesis was elevated in the trained leg compared to the untrained leg and that protein synthesis was elevated 16 h post-exercise in the untrained leg only. However, protein synthesis rates in that study (38), as well as in the study by Phillips *et al.* (56) were measured in fasted conditions. It is important to measure protein synthetic rates in the fed state since amino acids (i.e., protein) are potent stimulators of protein synthesis and result in a positive net protein balance so over time protein accretion can occur. Phillips *et al.* (55) measured protein turnover at rest and following exercise in the fed state before and after 8-weeks of resistance training. They

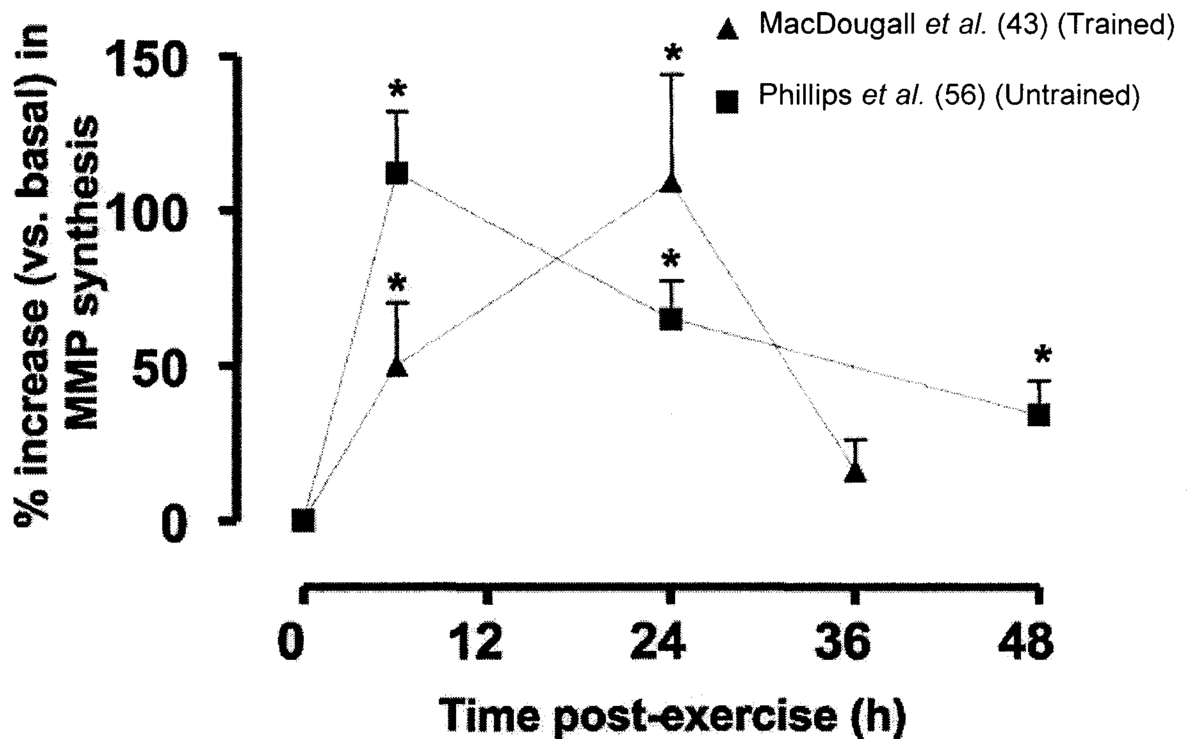


Figure 6: Time course of the elevation in muscle protein synthesis after an acute bout of resistance exercise. * Significantly different from resting synthetic rate (63).

observed a training induced elevation in protein synthesis, but they chose to examine protein turnover following the resistance exercise at the same absolute intensity, which meant a lower relative intensity completed in the post-trained state. Testing subjects at the same absolute intensity following training means that the independent effects of training could be examined since the workload tested at was not changed, but the more relevant issue is in examining the effects of training on post-exercise protein synthesis when tested at the same relative intensity.

1.6 HYPOTHESES

We proposed that eight weeks of progressive unilateral leg resistance-training program involving knee extension exercise would induce muscle fibre hypertrophy in the trained leg. The main purpose of this study was to examine resistance training-induced adaptations in mixed muscle protein synthesis in healthy human males in the fed state. Specifically, we were interested in changes in the magnitude and time course of muscle protein synthetic rates at rest and after an acute bout of resistance exercise performed at the same *relative* intensity following training. We hypothesized that resistance training would result in an elevation in resting mixed muscle protein synthesis, and a different exercise induced time course in mixed muscle protein synthesis, with training resulting in an earlier rise and peak in the protein synthetic response to an acute bout of resistance exercise as seen when comparing the trained and untrained legs.

CHAPTER II

The effect of resistance training on mixed muscle protein synthesis in the fed state

2.1 INTRODUCTION

Proteins in the human body are continually being remodelled, that is; they are simultaneously being synthesized and broken down. The fluxes in the processes of protein synthesis and protein breakdown are termed protein turnover. The algebraic difference between protein synthesis and protein breakdown (i.e., protein synthesis – protein breakdown), which is termed protein net balance, determines whether protein accretion or attrition occurs. At rest, in the post-absorptive or fasted states, there are low levels of circulating amino acids and insulin, resulting in a net negative protein balance (i.e., protein synthesis < protein breakdown). However, with the feeding of a protein-containing meal, there is an increase in the level of circulating amino acids and insulin, resulting in a net positive protein balance (i.e., protein synthesis > protein breakdown). Throughout a normal day the body fluctuates between the post-absorptive to fasted and fed to post-prandial states, depending on meal frequency and likely size, with the degree of change in protein turnover between these states being about equal. Thus the habitual consumption of a "normal" energy balanced diet containing sufficient protein (and essential amino acids) does not result in any net gain or loss of bodily proteins.

Skeletal muscle is one of the best examples of a tissue that is able to adapt to different stimuli through changes in protein synthesis and to a lesser degree protein breakdown. A single bout of resistance exercise has been shown to simultaneously stimulate both muscle protein synthesis and muscle protein breakdown (17; 43; 56; 57). However, resistance exercise is fundamentally anabolic; as such exercise-induced stimulation of muscle protein synthesis is greater, in an absolute and a relative sense, than

the rise observed in muscle protein breakdown. This means that following resistance exercise, when in the fasted state, net protein balance becomes less negative. When amino acids are either infused or ingested (or when protein is ingested) (6; 8; 11) Muscle protein synthesis is increased rapidly, but the increase is transient (11). However, the effect of post-exercise amino acid or protein infusion/ingestion results in an additive or synergistic effect in stimulating protein synthesis compared to amino acid or protein ingestion at rest (8; 74; 77). As well, muscle net protein balance becomes positive and protein accretion can occur over time.

Resistance training often causes muscular hypertrophy (38; 44; 45), which is the consequence of a chronic net positive protein balance resulting from the synthesis of new myofibrillar and non-myofibrillar proteins (i.e., sarcoplasmic and mitochondria). The training state of an individual muscle may also impact on the effect that a single bout of resistance exercise has on protein turnover. For example, resistance trained individuals seem to have an attenuated response of muscle protein synthesis following resistance exercise as compared with their untrained counterparts (38; 55). A similar attenuation of the response of muscle protein synthesis has also been observed in rats following resistance training (23). It may be that not only is the magnitude of response different but the time course of the acute response of muscle protein synthesis to resistance exercise is also different between trained and untrained individuals. In untrained subjects, Phillips *et al.* (56) found that protein synthetic rates after a single bout of resistance exercise peaked at 3 h post-exercise and were still elevated 48 h post-exercise. In contrast, MacDougall *et al.* (43) reported that protein synthetic rates after a single bout of resistance exercise

peaked at 24 h post-exercise but had returned to baseline by 36 h post-exercise in trained individuals. It is possible that the training status of the individuals in the two studies may account for the differences in the time course of the increases in muscle protein synthesis following a single bout of resistance exercise. This attenuated protein synthetic response in trained individuals (38; 55; 57) is in accordance with the general principle of adaptation to a stressor; that is as one becomes accustomed to the stressor, such as resistance exercise, the physiological responses to that stressor are reduced in magnitude and possibly duration.

The purpose of this study was to examine resistance training-induced adaptations in mixed muscle protein synthesis using a progressive unilateral leg resistance-training program in healthy human males in the fed state. Specifically, we were interested in changes in muscle protein synthetic rates at rest and after an acute bout of resistance exercise performed at the same *relative* intensity following training in the fed state. We hypothesized that resistance training would, in comparison to the untrained state, result in a different time course, of activation of muscle protein synthesis with an earlier rise and peak and more rapid decline in response to an acute bout of resistance exercise.

2.2 METHODS

2.2.1 Subjects

Ten healthy males (mean $\pm$ SD; age: 21.0 $\pm$ 1.5 yr, height: 179 $\pm$ 8 cm, weight: 82.7 $\pm$ 14.7 kg, BMI: 25.9 $\pm$ 4.5 $kg \cdot m^{-2}$) who were recreationally active (i.e., participated in no more than one lower body resistance exercise bout per week) were recruited for the study. Each subject was informed of the purpose of the study and the associated risks prior to the commencement of the study. Subjects were then required to complete a health questionnaire; the answers to which established the subject's eligibility to participate in the study. All subjects were informed of the associated risks inherent in the study and signed an approved research consent form. The project was approved by the Research Ethics Board of the Hamilton Health Sciences and McMaster University. All procedures conformed to declaration of Helsinki on the use of human subjects in research.

2.2.2 Study Design

This study used a within-person repeated measure design (two conditions: rest and exercise). The study intervention consisted of a progressive eight-week unilateral leg resistance training protocol involving knee extension exercise. The leg selected for training was randomized in a counter-balance manner, based on 'dominance' as confirmed by strength, to have equal number of subjects train their dominant and non-dominant legs.

2.2.3. Resistance Training Protocol

Prior to the initiation of any testing, subjects had a baseline muscle biopsy taken from the lateral portion of the vastus lateralis of the leg randomly assigned to be trained (T). Following the initial biopsy subjects completed a familiarization session with the dynamomter (Biodex - System 3, Biodex Medical Systems Inc., Shirley, NY) and the dynamic knee extension machine. Prior to the commencement of the first training session all subjects underwent a series of preliminary leg strength tests. The strength tests included a voluntary dynamic one-repetition maximum (1 RM) test to determine the maximum isotonic strength for knee extension of each subject; as well isokinetic and isometric strength tests were performed on the Biodex dynamomter. After all the aforementioned baseline testing was complete, subjects underwent a supervised eight week progressive unilateral leg resistance training program, while their contralateral leg served as an untrained (UT) control. The resistance training program consisted of knee extension exercise only. Prior to each training session, subjects warmed up the leg to be trained by performing 8-12 knee extensions with a low weight (~30% of their 1 RM). The frequency of training began with three sessions per week. Each session consisted of three sets of 10-12 repetitions per set at ~80% 1 RM, with 2 minutes of passive rest between each set. On the third week, the volume of training consisted of three sets with 8-10 repetitions per set and an additional (fourth) set performed until voluntary failure. Subjects performed this volume of training for two weeks. This brought subjects to the mid-point of the training protocol, and prior to the first training session of the fifth week, the 1 RM for both the T and UT legs was reassessed. For the latter part of the training

protocol the frequency of training sessions was reduced to two times per week. However, the volume of training for the fifth and sixth weeks of training was increased to four sets of 8-10 repetitions per set with a fifth set to failure. For the final two weeks of the training protocol the volume of training was further increased to five sets of 6-8 repetitions per set with the sixth set to failure. Throughout the training protocol subjects were given a minimum of one-day rest between training sessions. Following the completion of the training protocol subjects were given two days of rest and then had their dynamic strength, as well as, isokinetic and isometric strength retested.

2.2.4. Strength Measurements

Prior to all strength testing subjects underwent a familiarization with all pieces of strength testing equipment to be used in the study. Specifically, subjects received instruction in the lifting technique to be used while performing knee extensions. In addition, subjects were allowed to sit and make themselves comfortable in the Biodex dynamometer. All familiarization took place at least 1 week prior to testing.

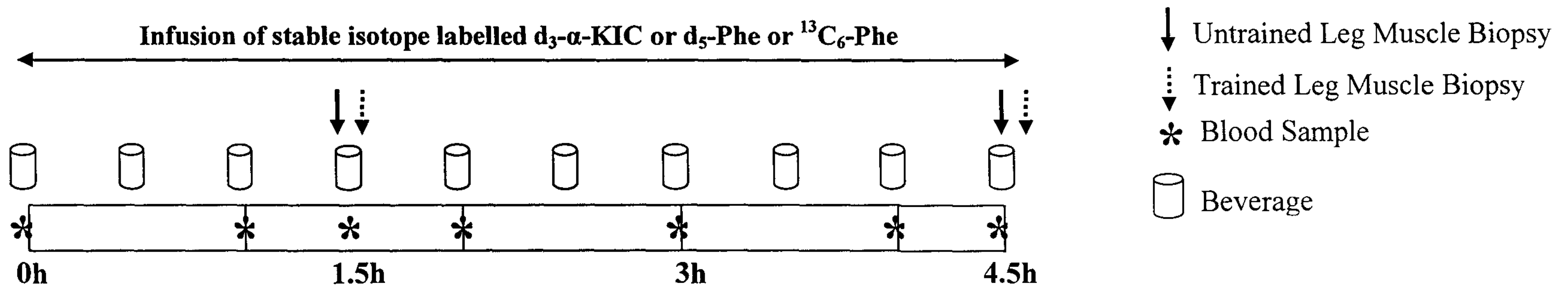
Isotonic knee extension 1 RM strength was tested prior to beginning the training protocol (PRE), after 4 weeks of training (MID), and following the completion of the training protocol (POST). A subject's 1 RM was defined as the maximum weight that could be lifted to full extension and held for one second.

Isometric strength and concentric isokinetic strength (SLOW – angular velocity of 0.52 $rad \cdot s^{-1}$ and FAST – angular velocity of 5.24 $rad \cdot s^{-1}$) were tested before and after the training protocol. During the familiarization session, subjects had all seat settings and leg

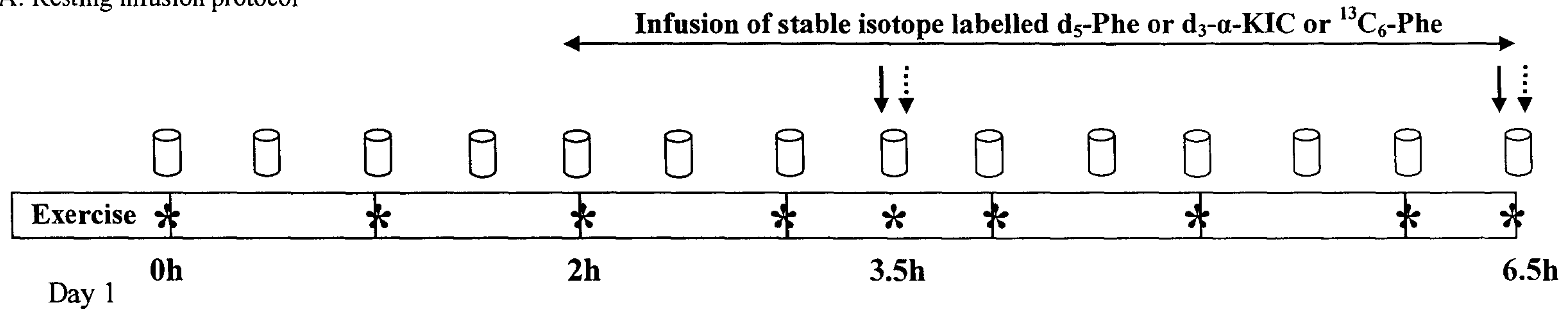
straps of the Biodex dynamometer customized to fit them in a comfortable position for all their testing. The order of testing of each mode was randomly assigned during each testing session and a minimum of 2 min of recovery was given between each exercise mode. For isometric strength testing each subject performed three repetitions of 5 s duration with 90 s rest between each repetition with the knee bent at an angle of ~70° from full extension. The subjects' maximal voluntary force (MVC) was determined as the greatest force generated during one of the repetitions. For concentric isokinetic strength, both fast and slow, subjects carried out ten repetitions of a kicking motion throughout a complete 65° range of motion (from ~90° to ~25°). The highest peak torque value was considered at the maximum value after all repetitions. All strength tests were applied in a randomized order to the T and UT legs separately.

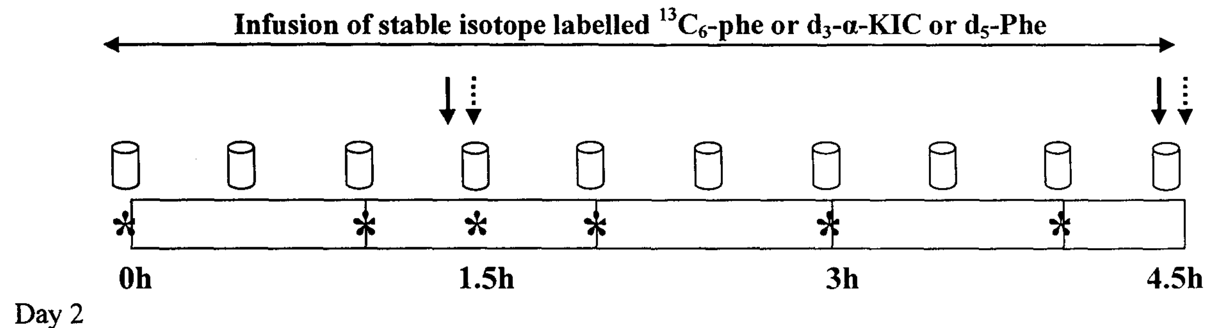
2.2.5. Experimental Infusion Protocol

Subjects were studied under two conditions, resting and following an acute bout of resistance exercise. The experimental infusion protocols are shown schematically in Figure 8. Subjects participated in a resting infusion session, four days following their last training session. Subjects trained two days following the resting infusion protocol according to their last training session (5 sets of 6-8 repetitions, with the sixth set to failure). One week following the resting infusion, subjects then participated in an isotope infusion following an acute bout of resistance exercise (5 sets of 8-10 repetitions at ~80% 1 RM, with the sixth set to failure – same relative intensity for the T and UT leg). Then approximately 24 h following the bout of resistance exercise subjects returned to the lab



A: Resting infusion protocol





B: Post-exercise infusion protocol

Figure 7: Schematic representation of the testing sessions following training.

for another infusion trial. Dietary intake was controlled 48 h prior to each infusion session according to the analysis each subject's 3-day diet record. Subjects were instructed to eat identically prior to the resting and post-exercise infusion sessions, as well subjects had a pre-packed diet given to them between the post-exercise infusion days to ensure that subjects consumed an energy balanced diet.

Subjects arrived in the lab at ~0700-0800 after an overnight fast for the initiation of an infusion testing session. Subjects had a 20-gauge catheter inserted in an antecubital vein from which blood samples were drawn throughout the infusion period (t = 0, 60, 90, 120, 180, 240, and 270 min; see figure 7). The catheter was kept clear by periodic flushing with 1-2 ml of 0.9% saline. Following the baseline blood sample a second catheter was inserted in the opposite arm for the primed, constant infusion of the stable isotope labeled with either d_3-α-ketoisocaproic acid (d_3-α-KIC; sodium salt, 98% enriched), L-[ring-d_5]-phenylalanine (98% enriched), or L-[ring-$^{13}C_6$]-phenylalanine (99% enriched). The priming dose was 3 $\mu mol \cdot kg^{-1}$ for the L-[ring-d_5]phenylalanine and L-[ring-$^{13}C_6$]-phenylalanine tracers, whereas it was 10 $\mu mol \cdot kg^{-1}$ for the d_3-α-KIC tracer. Following the priming dose the continuous infusion was started at a rate of 0.08 $\mu mol \cdot kg^{-1} \cdot min^{-1}$ for the phenylalanine based tracers, and 0.15 $\mu mol \cdot kg^{-1} \cdot min^{-1}$ for d_3-α-KIC. The catheter was placed in such a manner that arm bending would not occlude the infusion. During the infusion, subjects received approximately 35% of their recommended daily caloric intake, as predicted by the Harris-Benedict equation, through a meal supplement (Boost^{TM}: 68% carbohydrates, 17% protein, 15% fat), which they ingested in equally divided aliquots every 30 minutes (total intake over the entire session: 193 g

carbohydrates, 47 g protein, 19 g fat). The supplement aliquots where also enriched with isotope, equivalent to the infusion to achieve a tracer to tracee ratio of ~0.08 or ~0.15, which was designed to be equivalent to the enrichment of the isotope being infused. Muscle biopsies were obtained from both legs within approximately 10 min of each other at t = 90 and 270 min. Muscle samples were immediately frozen in liquid nitrogen and stored at -80°C until further analysis. The testing procedures were identical for both the resting and the 24 hr post-exercise infusion sessions, which were outline above. There were minor differences during the testing session that immediately followed the bout of resistance exercise. Immediately following the exercise subjects had an antecubital catheter inserted for blood sampling (t = 0, 60, 120, 180, 210, 240, 300, 360, and 390 min; see figure 7), however the catheter for the primed, constant infusion was not inserted until t = 120 min and the infusion was then started. Immediately following a background blood sample and every 30 minutes thereafter subjects received equally divided aliquots of the meal supplement, which provided in total 50% of their recommended daily caloric intake (total intake over the entire session: 275 g carbohydrates, 67 g protein, 27 g fat), as predicted by the Harris-Benedict equation.

2.2.5.1 Isotope

Isotopes were purchased from Cambridge Isotope Laboratories (Andover, MA). Phenylalanine was chosen as one of the tracers because it is not synthesized by the body or oxidized in the muscle. KIC was chosen as the other tracer because infusing KIC should generate similar intracellular and arterial leucine enrichments, which are not seen with infusing leucine. This is due to the fact that labeled KIC is transaminated

intracellularly to leucine and thus the labeled leucine is introduced into the body at the same site as the unlabelled leucine (i.e., intracellularly). All isotopes were dissolved in 0.9% saline then filtered and their concentrations were determined. All isotopes were filtered through a 0.2-μm filter during the infusion using a calibrated syringe pump (74900 Series, Cole Palmer Instrument Company, Niles, IL). The infusion rate was calculated by dividing the total volume of infusate (ml) per body weight by the total infusion period (min). The infusion protocol was designed so that steady state was achieved in both the plasma and intramuscular pools.

We chose to use three different tracers as opposed to a single tracer to prevent enrichment 'carryover' from one trial to the next, which can result in increased variability in the measure of MPS (M.J. Rennie, personal communication). While two of the tracers were phenylalanine-based and the other leucine-based (i.e., KIC), the tracers are: a) both essential amino acids; b) both large neutral amino acids and thus share the same transport system in the gut and in muscle; and c) give very similar estimates of MPS when compared across data sets (8; 11). While other phenylalanine tracers are available, for example $^{2}H_{8}$- and ^{15}N-phenylalanine, are inappropriate tracers to use. This is due to variable loss of the deuterated label in the $^{2}H_{8}$ tracer, essentially resulting in a variable infusion rate, and inappropriate sensitivity of analysis using GC-MS techniques to measure MPS using the ^{15}N label since the m+1 to m+0 ratio (i.e., $^{15}N/^{14}N$ ratio) is not sensitive enough to detect incorporation into proteins.

2.2.5.2. Blood Sampling

All blood samples were collected from the designated blood sampling catheter

using a heparinized evacuated tube (Vacutainer^{TM}) for determination of glucose, insulin, and amino acid concentrations, as well as tracer to tracee ratio. The catheter was flushed with 0.9% saline after each sample to prevent clotting. The blood sample was inverted to ensure mixing of the whole blood with the heparin, and from the tube 200 µL of whole blood was added to 500 µL of cold 0.6 M perchloric acid (PCA) to precipitate the blood proteins. The samples were kept cold and centrifuged at 15 000 rpm for 2 min in a refrigerated microfuge (0-4°C). Then 250 µL of 1.25 M potassium bicarbonate ($KHCO_3$) was added to the tube and the reaction was allowed to proceed for 10 min on ice. The samples were then centrifuged at 15 000 rpm for 2 min and the supernatant was removed and stored at -20°C until further analysis. The rest of the whole blood sample was centrifuged at 4 500 rpm for 10 min at 0-4°C to obtain plasma samples. The plasma samples were stored at -20°C until further analysis.

2.2.5.3. Muscle Biopsies

All needle biopsies were obtained from the vastus lateralis under local anesthesia, 2% xylocaine with 8.5% sodium biocarbonate (9:1). After the injection of the anesthetic, an incision was made and a 5 mm Bergstörm biopsy needle modified for manual suction was used to obtain a muscle sample. A sample of ~50-70 mg of mixed muscle tissue was obtained from each biopsy. Samples were immediately dissected free of all fat and connective tissue. A small portion of a biopsy sample prior to training in the T, and post training in the T and UT legs was taken and arranged to be embedded with its fibres perpendicular to the horizontal plane in which it was to be cut and mounted in optimal cutting temperature (OCT, Tissue Tek, Sakura Finetechnical Co. Ltd, Tokyo, Japan)

embedding compound. The sample was then immediately frozen in isopentane cooled by liquid nitrogen and stored at -80°C until further analysis. All other biopsy samples collected were immediately frozen in liquid nitrogen and stored at -80°C until further analysis.

2.2.6. Analysis

2.2.6.1. Blood Plasma

For determination of amino acid enrichment, the PCA extract (as previously described) was transferred into a threaded Pyrex© tube and lyophilized in a Speed-Vac rotary evaporator (Speed Vac® Plus, SC210A-120, Savant Instruments, Farmingdale, NY). To determine the enrichment of the infused isotope in the blood, the *tert*-butyl dimethylsilyl (*t*-BMDS) derivative was prepared. To the dried sample 50 μL of acetonitrile (HPLC grade) and 50 μL *N*-methyl-*N*-(tert-butyldimethylsilyl) trifluoro-acetamide + 1% tert-butyldimethylchlorosilane (MTBSTFA + 1% TBDMCS; Pierce Chemical, Rockford, IL) were added, and then the samples were heated for 1 hr at 90°C. The t-BMDS derivatives of phenylalanine and leucine were analyzed using electron-impact ionization capillary gas chromatography-mass spectrometry (GC-MS; Models 6890 GC and 5973 MS, Hewlett-Packard, Palo Alto, CA) and selected ion monitoring mass-to-charge ratios (*m/z*) for L-[ring-d_5]-phenylalanine of 234 and 239 for *m*+0 and *m*+5, for L-[ring-$^{13}C_6$]-phenylalanine of 234 and 240 for *m*+0 and *m*+6, and for d_3-α-KIC of 200 and 203 for *m*+0 and *m*+3, respectively. For phenylalanine analysis the conditions of the GC column (122-5012 DB-5 350°C; Dimension 15 m x 250 μm x 0.25 μm, J&W

Scientific, Agilent Technologies, Palo Alto, CA) consisted of an initial over ramp temperature of 80°C which was held for 2 min; the first ramp to 230°C at 15°C/min and held for 6 min; the second ramp to 300°C at 90°C/min and held for 2 min; and the post run at 300°C for 2 min. For leucine analysis the conditions of the GC column consisted of an initial over ramp temperature of 80°C which was held for 2 min; the first ramp to 230°C at 10°C/min and held for 2 min; the second ramp to 300°C at 50°C/min and held for 2 min; and the post run at 300°C for 2 min. The split ratio and split flow used were 10:1 and 13.6 ml He/min, respectively.

Amino acid concentrations were determined through High-Performance Liquid Chroatography (HPLC) from plasma samples, which were extracted using PCA, as previously described for whole blood. An amino acid standard was prepared using an Amino Acid Protein Hydrolysate standard (012506H, Pickering Laboratories, Mountain View, CA) which contained 11.4 pmol of asparagine, serine, glutamate, glycine, histidine, threonine, arginine, alanine, proline, tyrosine, valine, methionine, isoleucine, leucine, lysine, and phenylalnine; to make the standard contain all 20 physiological amino acids the following amino acids were added: 26.8 pmol of asparagine (A-8381, Sigma, Oakville, ON), 53.6 pmol of glutamine (G-3126, Sigma, Oakville, ON), 8.9 pmol of taurine (T-0625, Sigma, Oakville, ON), and 8.9 pmol of cycteine (C-77555, Sigma, Oakville, ON). Standard curves were used to calculate the concentration of amino acids in the samples. For the derivatization of the samples and standards, 25 μL of the sample or standard was added to 55 μL AccQ-Fluor™ Borate Buffer (Reagent 1, WAT052880, AccQ- Fluor™ Reagent Kit, Waters, Milford, MA), and then 25μL of reconstituted Accq-

Fluor™ Reagent (1 mL of vial 2B was added to vial 2A and heated until dissolved, for a maximum of 10 min; WAT052880, AccQ- Fluor™ Reagent Kit, Waters, Milford, MA) which are then allowed to sit at room temperature for 1 min and then heated for 10 min at 55°C. The samples/standards are then allowed to sit for at least 10 hrs prior to running in the HPLC. The samples/standards are passed through the column (Waters 2690 Separations Module, W052885 AccQ-Tag™, 3.9 mm x 150 mm, 4 μm particle size, temp 34°C, Waters, Milford, MA) using high pressure (maximum: 4000 psi) and then eluted from the column with a filtered solvent gradient (Eluent A: sodium acetate trihydrate, sodium azide, triethylamine, pH 4.97; Eluent B: same as eluent A, except pH 7.4; 100% acetonitrile; and deionized, distilled water). The eluted samples/standards are detected (Multi λ Fluorescence Dector, Waters 2475; Gain 1.0, Filter 0.5, Waters, Milford, MA) using fluorescents with an excitation wavelength of 250 nm and an emission wavelength of 395 nm.

Plasma glucose was measured by an enzymatic assay adapted for fluorometry. A standard curve (5, 10, 20, 60, 80, 200, 400, and 600 μM) was constructed and fluorescent changes of the samples were then compared to the standard curve to obtain concentrations of the plasma glucose within the sample. The buffer (50 mM Tris, pH 8.1; 1 mM $MgCl_2$; 0.5 mM DTT; 300 μM ATP; 50 μM NADP: pH 8.1 then 0.02 U/mL G-6-P-DH was added) was prepared ahead of time and 1 ml was added to 30 μL of plasma sample or standard. The reaction was allowed to equilibrate for 5 min and then the first reading was taken on the fluorometer (Hitachi model F2500, Hitachi Instruments, Tokyo, Japan). After the first reading, 0.3 U/mL of hexokinase was added to each sample and the

reaction was allowed to proceed for 15 min before the second reading was taken. Glucose samples were analyzed in triplicate.

Insulin concentrations were analyzed by radioimmunoassay (RIA) (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA). Briefly, frozen plasma samples were thawed, vortexed and centrifuged to pellet out any clumped blood lipids. 200 μL of sample and 1 mL of ^{125}I insulin were added to 12x75 mm polypropylene insulin-specific antibody coated tubes and gently vortexed. Samples were analyzed in duplicate. Tubes were incubated for 24 hrs at room temperature (~21°C) and then decanted thoroughly. Radioactivity was analyzed directly using a gamma counter (5000 Minaxi Auto Gamma, Packard, Downers Grove, IL) for a duration of 1 min. Intra-assay variability was 5% or less for duplicate samples.

2.2.6.2. Muscle Analysis

Approximately 20 mg of wet muscle tissues was analyzed for mixed-muscle protein-bound and free intracellular enrichment. Muscle samples were lyophilized to dryness overnight, while being incubated on dry ice. Samples were then manually powdered and 500 μL of 0.6 M PCA was added. After gentle shaking, the samples sat on ice for 10 min and were then centrifuged at 4 500 rpm for 2 min. The supernatant (i.e., intracellular pool free amino acids) was collected and processed in the same manner as the whole blood samples. The remaining pellet from the muscle sample was washed twice with 1 ml of distilled deionized water and then lyophilized to dryness overnight. The dried pellet was placed in 2 ml of 6 N HCl and hydrolyzed for 24 h at 100°C. The acid hydrolysate was passed over ion exchange columns for amino acid

isolation/purification to determine the protein bound enrichments. Ion exchange columns were acid-washed cation exchange column (Dowex Analyticall Grade 50W-X8, 100-200 mesh hydrogen form, BioRad Labs, Hercules, CA). Amino acids were released from the resin using approximately 5 ml of 4 N NH_4OH and the eluate was immediately collected. The samples were dried using a rotary evaporator and the dried pellet was then derivatized in the same manner as the blood samples.

The supernatant from the PCA muscle extraction was lyophilized in a Speed-Vac rotary evaporator and to determine the enrichment of the infused isotope in the intracellular compartment, the *t*-BMDS derivative was prepared, as previously described. The t-BMDS derivatives of phenylalanine and leucine were analyzed using electron-impact ionization capillary GC-MS with selected ion monitoring mass-to-charge ratios (*m/z*), as previously described for blood enrichment. For muscle (intracellular and protein-bound) phenylalanine analysis the conditions of the GC column consisted of an initial over ramp temperature of 120°C which was held for 2 min; the first ramp to 230°C at 15°C/min and held for 6 min; the second ramp to 300°C at 90°C/min and held for 2 min; and the post run at 300°C for 2 min. For muscle leucine analysis the conditions of the GC column consisted of an initial over ramp temperature of 50°C which was held for 2 min; the first ramp to 230°C at 10°C/min and held for 2 min; the second ramp to 300°C at 50°C/min and held for 2 min; and the post run at 300°C for 2 min. The split ratio and split flow used were 6:1 and 11.6 ml He/min, respectively.

A standard curve was used to calculate the protein-bound *m*+5 to *m*+0 and *m*+6 to *m*+0 ratios for L-[ring-d_5]-phenylalanine and L-[ring-$^{13}C_6$]-phenylalanine, respectively.

A series of known standard tracer to tracee ratios (0%, 0.0025%, 0.005%, 0.01%, 0.05%, and 0.1%) were prepared and diluted 10-fold. A number of standard volumes of the standards (25, 50, 100, and 250 μl) were dried and derivaitized to account for any concentration dependency within the samples (54). The standard curve was constructed by plotting the measured *m*+5 to *m*+3 ratio against the known *m*+5 to *m*+0 ratio for L-[ring-d_5]-phenylalanine and by plotting the *m*+6 to *m*+4 ratio against the *m*+6 to *m*+0 ratio for L-[ring-$^{13}C_6$]-phenylalanine, respectively. The abundance (total area) of the unenriched ion (i.e., *m*+0) from each hydrolysate sample determined from a standard curve (25, 50, 100, and 250 μl) which was used to convert the protein-bound *m*+5 to *m*+3 ratio to the *m*+5 to *m*+0 ratio or the *m*+6 to *m*+4 ratio to the *m*+6 to *m*+0 ratio.

2.2.7. Calculations

Fractional synthetic rate (FSR) was calculated from the determination of the rate of tracer incorporation into muscle protein and using the muscle intracellular free phenylalanine or leucine enrichment as a precursor, according to the following equation:

$$\text{FSR} (\%\cdot h^{-1}) = \frac{(Et_1 - Et_0)}{[E_p * (t_1 - t_0)]} * 100$$

Where;

Et_0 is the enrichment of the protein-bound isotope tracer from the first biopsy.

Et_1 is the enrichment of the protein-bound isotope tracer from the second biopsy.

E_p is the mean intracellular tracer enrichment during the time period for determination of protein incorportation.

$(t_1 - t_0)$ is the incorporation time (~3 h).

2.2.8. Muscle Fibre Type and Cross-sectional Area

Frozen tissue samples embedded in OCT were cut into 10 μm serial sections at -20°C in a cryostat microtome (Model HM500OM, Microm International, Waldorf, Germany). Sections were thaw mounted on slides, allowed to dry overnight at 4°C, and subsequently stored at -80°C until histochemical analysis. Myofibrillar adenosine triphosphatase (mATPase) histochemistry was performed using preincubation pH value 4.60 (50 mM potassium acetate and 17.5 mM calcium chloride) for 6.5 min to determine muscle fiber-type composition. Slides were then rinsed with distilled water and incubated in 3 mM ATP using an alkaline solution (75 mM glycine, 40.5 mM calcium chloride, 75 mM NaCl, and 67.5 mM NaOH, adjusted to pH 9.4) for 45 min at 37°C and agitated at regular intervals in a temperature-controlled incubator shaker. After the ATP incubation, a rinse with distilled water was done, and the samples were incubated in 1% $CaCl_2$ for 3 min at room temperature. Slides were again rinsed with distilled water and incubated in 2% $CoCl_2$ for 3 min at room temperature. Another rinse with distilled water and an incubation in 1% ammonium sulfide for 1 min at room temperature followed. Samples were rinsed with distilled water five times before being dehydrated by incubation for 2 min in each ethanol concentration (70, 80, 90, 95, and 100%). Samples were then cleared using xylene. After the slides were dried, coverslips were mounted using Permount (SP15 Fisher, Nepean, ON) and allowed to dry overnight.

Sections were viewed under a light microscope (Olympus BX-60, Olympus America, Melville, NY), then the images were digitized using a SPOT camera (model SP401-115, SPOT Diagnostic Instruments, Sterling Heights, MI) and visualized using

SPOT software (V3.2.4 for Windows, SPOT Diagnostic Instruments, Sterling Heights, MI). The number of images taken at x200 magnification of each sample was between five and seven and was largely dependent on the quality of the serial sections. Each image contained ~30–50 fibers. Images were analyzed by using Image Pro Plus (V4.0 for Windows, Media Cybernetics, Silver Spring, MD). Fibres were classified based on staining intensity as type I (dark) or type II (light) using visual, as well as, optical density bins as previously described (71). When not in use, slides were stored in the dark.

2.2.9. Statistics

The strength measurements (1 RM, isometric strength, and isokinetic strength) were analyzed using a two-way repeated measures analysis of variance (ANOVA), with leg (T and UT) and time (PRE, MID - for 1 RM only, and POST) as within-subject factors. Blood measurements; isotopic enrichment, amino acid concentrations, and blood glucose and insulin concentrations were analyzed using a two-way repeated measures ANOVA, with condition (rest, immediately post-exercise, and 24 h post-exercise) and time (min) as within-subject factors. Intracellular isotopic enrichment was analyzed using a three-way repeated measures ANOVA, with training status (T and UT), condition (rest, immediately post-exercise, and 24 h post-exercise), and time (biopsy 1 and biopsy 2) as within-subject factors. Mixed muscle protein FSR was analyzed using a two-way within-subject ANVOA with training status (T and UT) and condition (rest, immediately post-exercise, and 28 h post-exercise) as within-subject factors. Wherever ANOVA revealed significant differences ($P < 0.05$) a Tukey's post-hoc procedure was used to identify the

difference. Fibre CSA was analyzed using a one-tailed t-test paired for two sample means (pre and post) for each fibre type. Significance was accepted at $P < 0.05$. All data are expressed as means $\pm$ SD.

2.3. RESULTS

2.3.1. Strength Measurements

Prior to training there was no difference between legs in unilateral knee extension 1 RM strength (Figure 8). After 4 weeks of training 1 RM strength significantly increased compared to initial values (UT = 10.1 ± 8 %, P < 0.01; T = 38.0 ± 20 %, P < 0.01; Figure 8) and 1 RM strength in the T leg was significantly higher than the UT leg (P < 0.01; Figure 8). At the end of the eighth week, all subjects continued to increase their unilateral 1 RM strength compared to initial values (UT = 19.7 ± 13 %, P < 0.01; T = 62.3 ± 27 %, P < 0.01; Figure 8). However, by the completion of the training protocol the increase in the T leg was 3-fold greater than of that seen in the UT leg (P < 0.01; Figure 8).

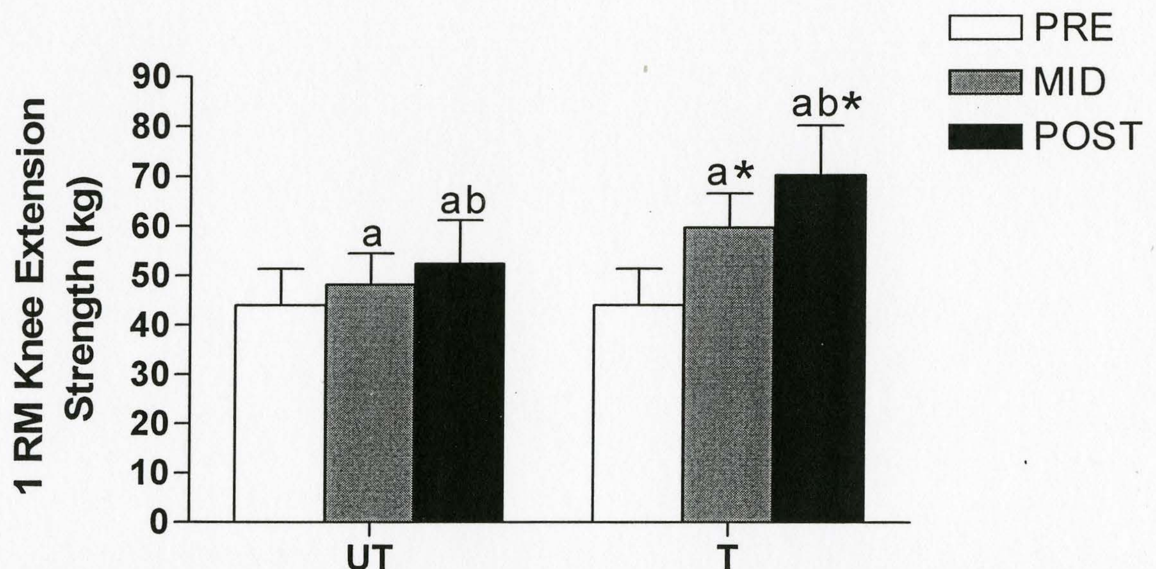


Figure 8. Knee extension 1 RM strength assessments. a Significantly different from Pre testing measures (P < 0.01); b Significantly different from Mid testing (P < 0.01); * Significantly different from UT at the same time (P < 0.01). Values are means ± SD.

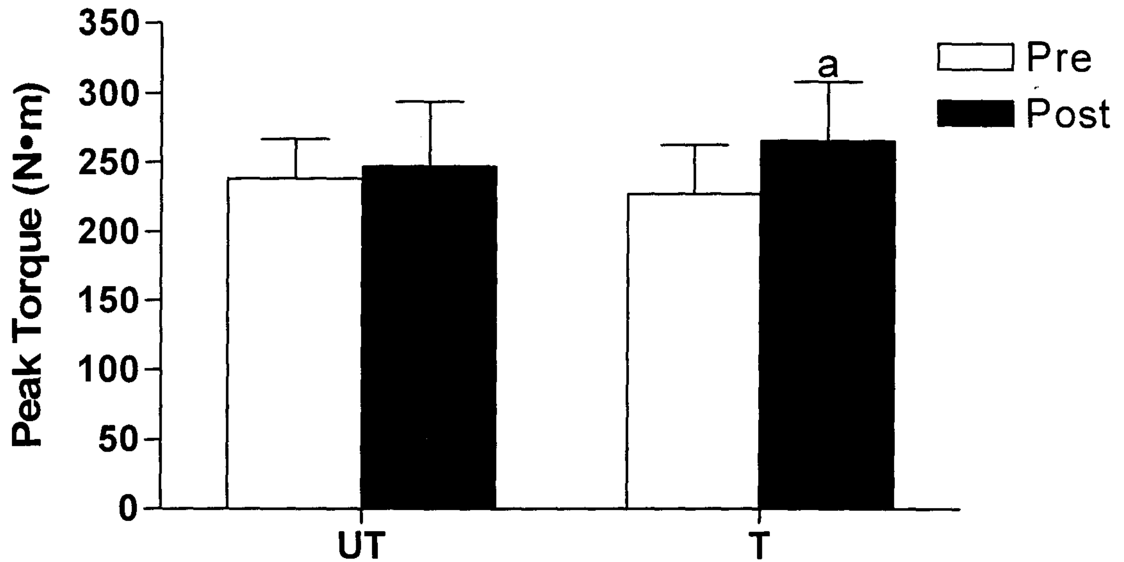


Figure 9. Isokinetic leg strength tested concentrically at 0.52 rad·s^{-1}. a Significantly different from PRE ($P < 0.01$). Values are means ± SD.

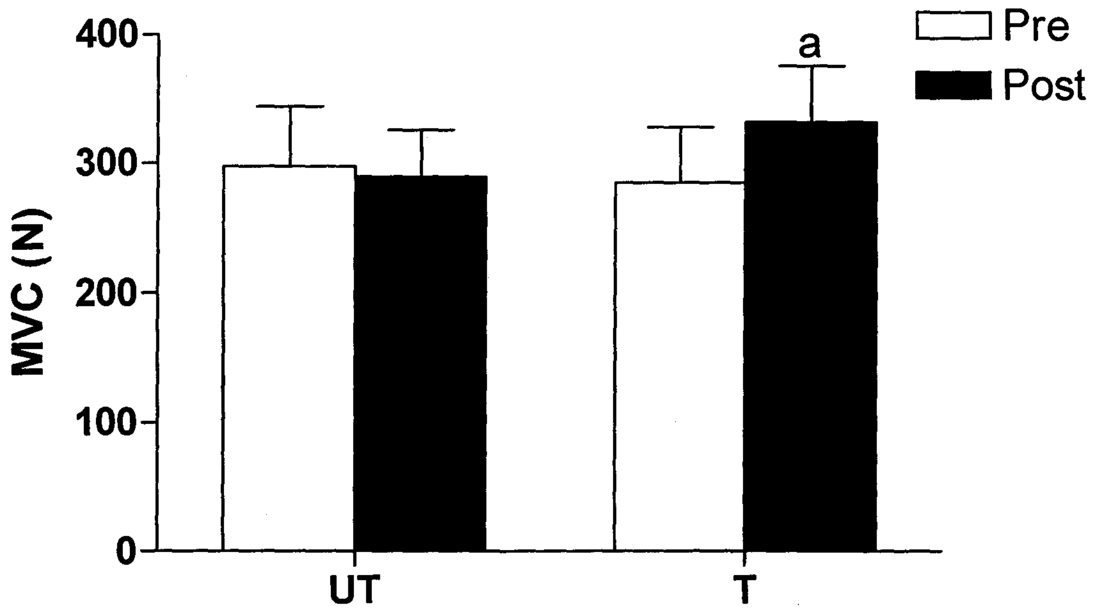


Figure 10. Isometric strength measurements. a Significantly different from PRE ($P < 0.01$). Values are means ± SD.

Prior to training there was no difference between legs in isokinetic (SLOW: P = 0.43; FAST: P = 0.71; Figure 9) or isometric (P = 0.64, Figure 10) leg strength between legs. After training, isokinetic strength was unchanged when tested at 5.24 rad·s^{-1}

(FAST) concentrically in both legs. However, there was a training-induced increase in isokinetic strength when tested at 0.52 rad·s^{-1} (SLOW) concentrically (17.8 ± 16 %, P < 0.01, Figure 9) in the T leg, with no increase in the contralateral UT leg (P = 0.63). As well, after training isometric strength increased in the T leg (17.7 ± 12 %, P < 0.01, Figure 11), with no change in the UT leg (P = 0.86).

2.3.2. Muscle Fibre Cross-Sectional Area

The mean fibre cross-sectional area (CSA) of both type I and type II fibres increased as a result of training (type I: 16 ± 10 %, P < 0.05; type II: 20 ± 19 %, P < 0.05; Figure 11) in the T leg. There was no significant increase in CSA after the training protocol in the UT.

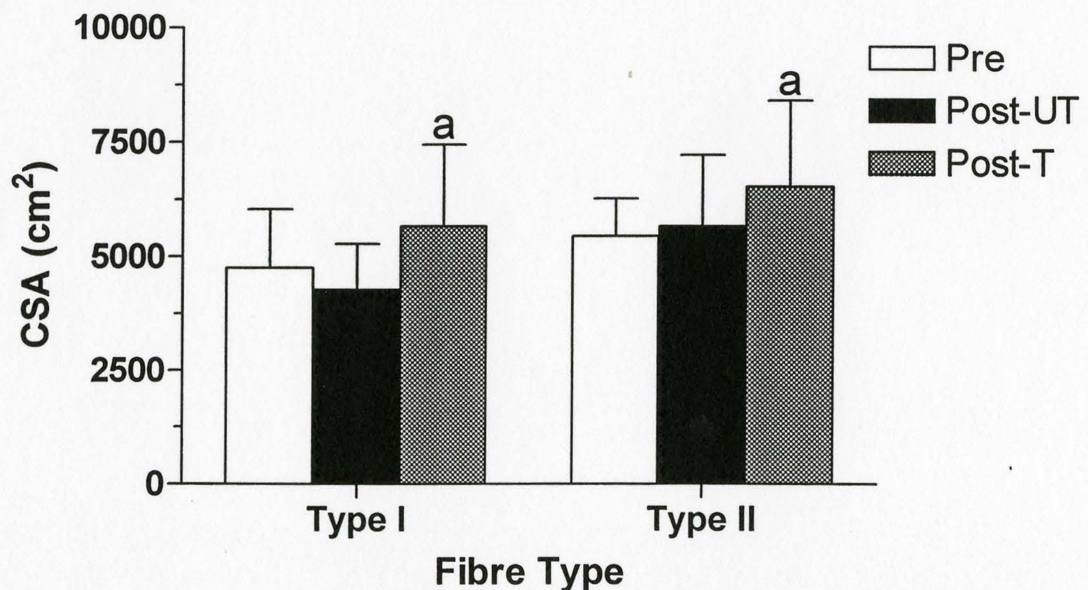


Figure 11. Mean fibre CSA. a Significantly different from PRE (P < 0.05). Values are means ± SD.

2.3.3. Amino Acid Concentrations

Table 2 shows the concentration of plasma total amino acids (TAA), essential amino acids (EAA), and branched-chain amino acids (BCAA) during the three testing sessions. There was a main effect for time in TAA, EAA, and BCAA levels in the plasma ($P < 0.01$). Feeding stimulated an increase in plasma amino acid levels; however the level remained constant at the times when biopsies were taken. There was also a main effect for condition where the amino acid concentrations seen at 24 hrs post-exercise condition were lower than immediately post-exercise ($P < 0.05$) (see Table 2).

Table 2. Plasma amino acid concentrations (μM). Values are means ± SD. Main effect of time where 0 hrs had significantly lower TAA, EAA, BCAA concentrations than all other time points ($P < 0.01$). Main effect for condition where there were lower TAA, EAA, BCAA concentrations 24 hrs post-exercise than immediately post-exercise ($P < 0.05$).

	REST (h)		
	0	1.5	4.5
TAA	4259 ± 1089	5456 ± 664	5170 ± 631
EAA	1573 ± 351	1992 ± 304	1874 ± 277
BCAA	795 ± 182	996 ± 192	927 ± 165

	POST EXERCISE (h)		
	0	3.5	6.5
TAA	4983 ± 402	5541 ± 657	5512 ± 640
EAA	1801 ± 290	2027 ± 338	1866 ± 278
BCAA	894 ± 185	1003 ± 183	921 ± 162

	24 h POST EXERCISE (h)		
	0	1.5	4.5
TAA	4281 ± 525	4952 ± 613	4853 ± 424
EAA	1503 ± 183	1801 ± 278	1779 ± 174
BCAA	732 ± 110	895 ± 156	899 ± 129

2.3.4. Glucose and Insulin Concentrations

Plasma glucose levels were not significantly affected by feeding or by condition. However, there was a main effect of time where insulin concentrations were significantly elevated over resting (t = 0 h) values at all time points ($P < 0.01$; Table 3).

Table 3. Plasma glucose and insulin concentrations. Values are means ± SD. Main effect of time, where 0 h had significantly lower insulin concentrations than all other time points ($P < 0.01$).

	REST (h)			
	0	1.5	3	4.5
Glucose (mM)	4.4 ± 1.2	4.0 ± 0.7	4.7 ± 0.9	4.0 ± 1.1
Insulin (μIU/ml)	6.5 ± 4.2	21.1 ± 13.3	31.3 ± 16.7	25.6 ± 19.1

	POST EXERCISE (h)			
	0	3.5	5	6.5
Glucose (mM)	4.6 ± 0.7	3.8 ± 0.7	4.2 ± 1.3	4.1 ± 0.6
Insulin (μIU/ml)	7.1 ± 3.2	26.1 ± 20.6	29.8 ± 20.8	25.3 ± 18.0

	24 h POST EXERCISE (h)			
	0	1.5	3	4.5
Glucose (mM)	4.6 ± 1.3	4.0 ± 0.5	4.2 ± 0.8	4.2 ± 0.8
Insulin (μIU/ml)	6.5 ± 3.1	27.5 ± 18.8	30.1 ± 20.1	29.5 ± 17.4

2.3.5. Mixed Muscle Protein Fractional Synthetic Rate

All blood enrichments were significantly elevated from resting (t = 0 h) values ($P < 0.01$). Mean blood enrichment was maintained throughout the infusion protocols (Table 4). There was no difference in the blood enrichment at the time points when the muscle biopsies were taken or between conditions.

Mean muscle intracellular enrichment was lower than blood enrichments. The lower values in the intracellular pool may be accounted for by the dilution of the pool from muscle protein breakdown (7). There was a main effect for time where an elevation

Table 4. Mean blood isotope enrichment (tracer/tracee ratio) during the infusion trials. Values are means ± SD.

	TIME (hrs)			
	0	1.5	3	4.5
Rest	0.003 ± 0.004	0.119 ± 0.042	0.138 ± 0.043	0.123 ± 0.022
Post-exercise	0.003 ± 0.004	0.105 ± 0.021	0.129 ± 0.031	0.120 ± 0.024
24 hrs Post-exercise	0.003 ± 0.004	0.114 ± 0.034	0.124 ± 0.027	0.122 ± 0.018

Table 5. Mean muscle intracellular isotope enrichment (tracer/tracee ratio) during the infusion trials. Values are means ± SD. Main effect of time, where Biopsy 1 had a significantly lower tracer/tracee ratio than Biopsy 2 ($P < 0.01$).

	T		UT	
	Biopsy 1	Biopsy 2	Biopsy 1	Biopsy 2
Rest	0.047 ± 0.016	0.064 ± 0.026	0.044 ± 0.016	0.065 ± 0.026
Post-exercise	0.048 ± 0.024	0.060 ± 0.033	0.045 ± 0.023	0.061 ± 0.030
24 hrs Post-exercise	0.050 ± 0.016	0.070 ± 0.023	0.050 ± 0.017	0.071 ± 0.022

in intracellular enrichment over time was observed in both the T and UT legs (46.8 ± 39%; $P < 0.01$, Table 5).

There was no difference in resting mixed muscle FSR between the T and UT leg ($P = 0.97$; Figure 12). An acute bout of resistance exercise elevated FSR above resting values (UT: 108 ± 62 %, $P < 0.01$; T: 162 ± 76 %, $P < 0.01$), however, the increase in the T leg was significantly higher than that seen in the UT leg ~ 4 h post-exercise at the same relative intensity ($P < 0.01$). At about ~28 h after the bout of resistance exercise FSR in

the T leg was back at resting levels, while FSR remained elevated above resting levels in the UT leg (70 $\pm$ 49%, $P < 0.01$).

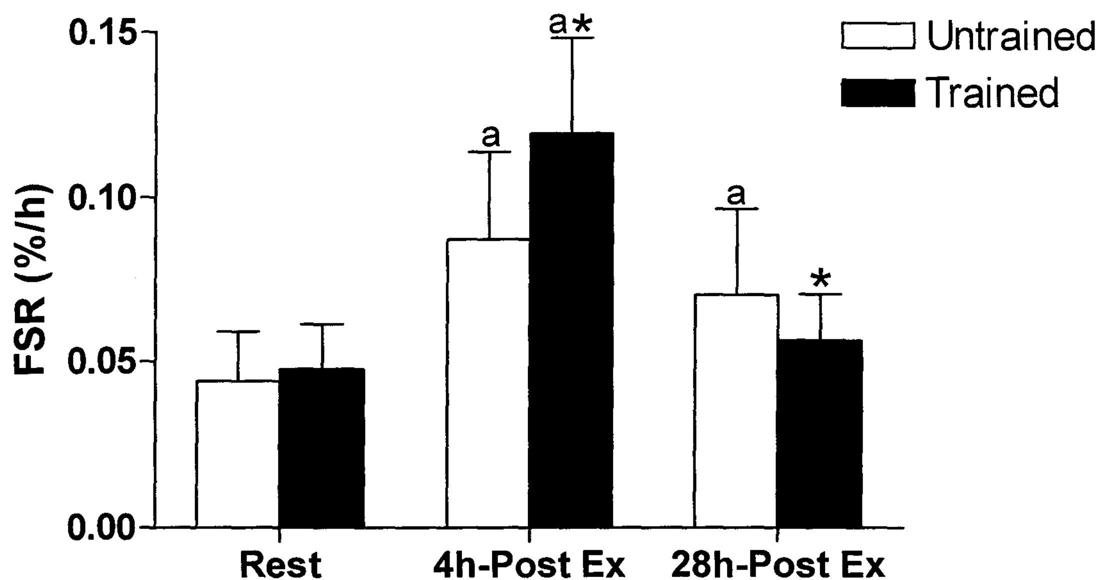


Figure 12. Mixed muscle FSR. a Significantly different from rest ($P < 0.01$). * Significantly different from UT during the same condition ($P < 0.01$). Values are means $\pm$ SD.

2.4. DISCUSSION

The primary findings from the present study were that 8 weeks (20 total training sessions) of resistance training, which resulted in hypertrophy, altered the mixed muscle protein synthetic response to an acute bout of resistance exercise at the same *relative* intensity (i.e., greater work performed by the T leg) in the T and UT legs. Mixed muscle protein synthesis was increased acutely in response to exercise in both the T and UT legs by 162% and 108% immediately post-exercise, respectively. In the UT leg protein synthetic rates remained elevated by 70% at ~28 h post-exercise; however by that time protein synthesis had returned to resting levels in the T leg.

In contrast to previous observations (38; 55; 57) we did not observe an increase in resting mixed muscle protein synthetic rate in the trained compared to the untrained leg. Previously, similar observations have been made in a cross-sectional comparison of trained versus untrained individuals (57); however, these observations were made with subjects in the fasted state. In a longitudinal comparison subjects who trained for 8 weeks were compared pre- versus post-training, in the fed state, and resting mixed muscle protein synthesis was found to be elevated (55). As well, in a similar 8-week unilateral training model resting mixed muscle protein synthesis was elevated in the T leg in the unfed state (38). As with the present study, in our previous work, subjects were fed during the infusions and were rested for at least 72 h prior to the infusion making it unlikely that the effect we previously observed was an acute effect of the last exercise bout. We followed the same procedures in the present study and yet did not see an elevation in resting muscle protein synthesis. Recent evidence from Miller and co-

workers (46) showed that intense muscle contraction elicits a rise in myofibrillar and sarcoplasmic protein synthesis (sub-protein fractions of mixed muscle protein) that persists for up to 48 h, but that returns to baseline at 72 h post-contraction, similar to our previous observations (56). It may be that the intensity and/or volume of the muscular contractions or even the nature of the exercise determined the time course for elevations in muscle protein synthesis. For example, in our previous study (38) the last leg workout 72 h prior to the resting infusion consisted of 6 sets of knee extension and 6 sets of leg press, compared to only 6 sets of knee extension in the current study. Since the results of Miller *et al.* (46) are from strenuous leg kicking it is difficult to truly know how long MPS stays elevated and what effect contraction volume and/or intensity might have on MPS; clearly this needs to be investigated further.

Previous studies attempting to define the time course of the increase in mixed muscle protein synthesis following a bout of resistance exercise have reached somewhat different conclusions (17; 43; 56). These studies are in agreement that protein synthesis rises rapidly in the initial hours post-exercise; however, the decline of the protein synthetic response differed considerably. Phillips *et al.* (56) showed that protein synthesis peaked at 3 h post-exercise but still remained significantly elevated over resting levels at 24 and 48 h post-exercise. Conversely, MacDougall *et al.* (43) demonstrated that protein synthesis was elevated at 4 h post-exercise, but peaked at 24 h and was back down to resting levels by 36 h post-exercise in trained subjects. As previously mentioned the shorter time course of elevated protein synthesis reported by MacDougall *et al.* (43) has been hypothesized to be due to the training status of the participants. In the present study,

muscle protein synthesis was measured at 4 h post-exercise during which protein synthesis peaked in both the UT and T leg and again at 28 h post-exercise when protein synthesis remained significantly elevated over resting levels in the UT leg only. Other studies have reported a similar reduction in muscle protein synthesis induced by resistance training in humans (38; 55; 57) and in rats (22).

The general adaptation principle as it applies to exercise training is that with the repeated stress of resistance exercise the body becomes better able to adapt to the stress by dampening or fine tuning the physiological response to that stress. Indeed, in the untrained state there is a longer protein synthetic response following resistance exercise (Figure 13). From our measures here we are unable to determine why this is the case,

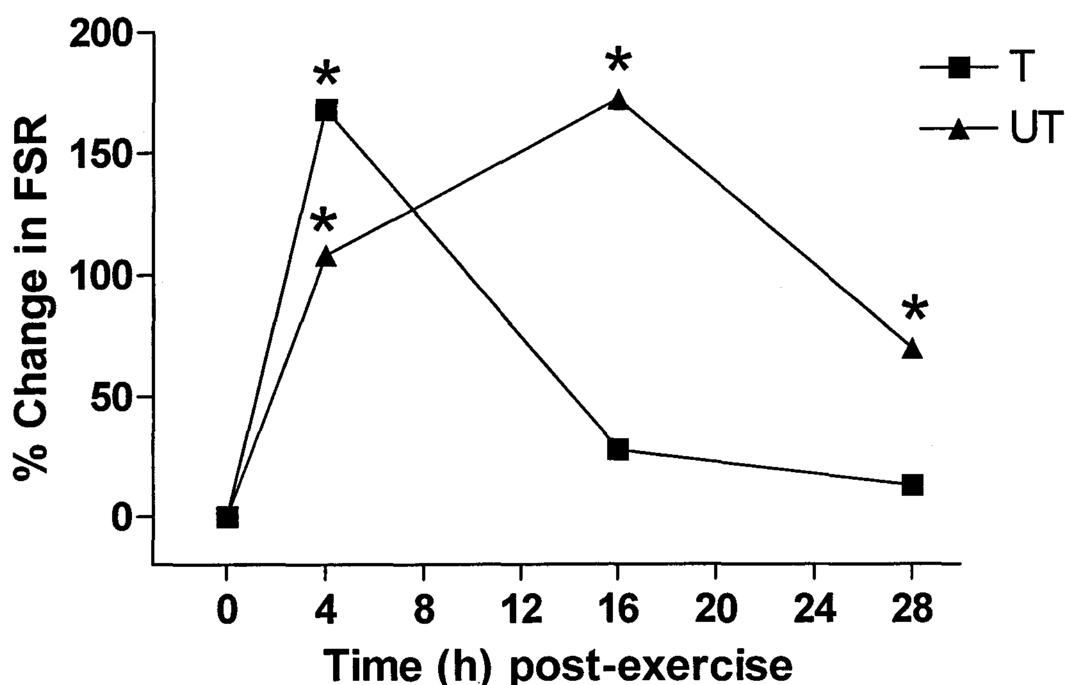


Figure 13. Time course of the elevation in muscle protein synthesis after a single bout of resistance exercise. a Significantly different from rest (P < 0.01). The 16 h time point is from recent data from our lab (38).

however, we speculate that there are several potential possibilities. For example, the bout of exercise in the UT state would result in a greater disturbance of homeostasis in, for example, ADP/ATP ratio, myofibrillar protein disruption, and possibly connective tissue damage. In contrast, resistance training would have likely resulted in adaptations that reduce the degree of perturbation in all of the aforementioned areas caused by a single bout of resistance exercise. For example, an increase in mitochondrial content, and thus oxidative enzyme content with resistance training (Tang *et al.*, 2005; in review) would reduce perturbations in ADP/ATP ratio, which when higher would allow protein synthesis to proceed since protein synthesis is suppressed in conditions of low energy status (16). In addition, it has been shown that damages are reduced with repeated bouts of resistance exercise (72). Hence, this adaptation would reduce myofibrillar disruption or sarcomeres 'popping', which may be a stimulus for MPS and if reduced with training would at least explain the shorter duration of the MPS response, but not necessarily the greater amplitude early.

In the trained state, the exercise-induced increase in mixed muscle protein synthesis rises rapidly indicating the response is likely post-translationally mediated; however, the increase in MPS also returns to near baseline more rapidly in the trained state. The dampened exercise-induced protein synthetic response observed with training would be expected to result in incrementally less muscle protein accretion as training is prolonged. Therefore, theoretically an increased stimulus (greater intensity and/or increased volume) would be necessary in the trained state to achieve a response similar to, or greater, than that observed in the untrained state. Figure 13 illustrates a combination of data from the

present study and that from Kim *et al.* (38) showing the time course of the mixed muscle protein synthetic response following a single bout of resistance exercise at the same relative intensity between the trained and untrained states. The impact of training status on protein synthesis is discernable in this figure and lends some support to our thesis that in the untrained state resistance exercise induces a more prolonged rise in muscle protein FSR. In the study by Kim *et al.* (38), FSR was measured in the fasted state, therefore, these data represent a conservative estimate of protein synthesis since in the fed state the protein synthetic rates at the 16 h post-exercise time point would have been higher due to the synergistic interaction of feeding and resistance exercise (8; 74; 77). Nevertheless, it is unlikely that feeding would drastically alter the time course in either the trained or untrained states.

Chronic resistance exercise leads to muscular hypertrophy which is defined as an increase in muscle fibre cross-sectional area. Hypertrophy only occurs when the muscle is in a net anabolic state, in other words, when a chronic positive net protein balance is present. In the fasted state the net protein balance at rest and following exercise remains negative (56), and it is only with an increase in amino acid availability that the net protein balance becomes positive. At rest amino acid infusions transiently increased protein synthesis (8; 11; 52), with protein synthesis being increased to an even greater extent when the amino acid infusion followed resistance exercise (8). However, Tipton *et al.* (74) determined that only essential amino acids are necessary to stimulate protein synthesis following resistance exercise, since a drink containing both essential and non-essential amino acids did not further stimulate protein synthesis nor promote a more

positive protein balance than essential amino acids alone (74).

Another important component of the anabolic response in the post-prandial state is the plasma levels of insulin. The role of insulin in regulating protein synthesis remains somewhat controversial. At rest, insulin has no effect on protein synthesis; however, insulin does improve muscle net protein balance by virtue of the fact that it inhibits protein breakdown (26-28). In the post exercise period there is no further stimulation of protein synthesis with insulin (9); however, as is seen at rest, elevations in insulin results in an improved (but still negative) protein balance, which occurs due to the inhibition of protein breakdown (9; 15). It seems that the action of insulin on protein synthesis is permissive in nature, which means that there is a critical concentration of insulin below which rates of protein synthesis start to fall (25; 60), but above this level there is no further stimulation of protein synthesis even in the face of resistance exercise or an increase in amino acid availability. When amino acids are given with carbohydrate to stimulate insulin release, there is an increase in protein synthesis at rest (76) and following resistance exercise (64) with an improved net protein balance. In addition, the ingestion of carbohydrate alone elevates insulin and results in a markedly more positive net muscle protein balance that tends toward zero, but does not become positive (14). These findings suggest that to maximize the improvement in protein balance at rest and following exercise a feeding of amino acids and carbohydrates is needed to make sure there is sufficient circulating insulin to allow protein synthesis to proceed and to inhibit protein breakdown. In the present study the subjects were fed using a meal supplement to mimic the effects of a mixed diet (68% carbohydrates, 17% protein, 15% fat) which

would allow for maximal protein anabolism by creating a hyperinsulinemia (Table 3), as well as a hyperaminoacidemia (Table 3).

Since protein balance is the difference between the processes of synthesis and breakdown, maximal protein anabolism can be achieved by stimulating protein synthesis and by inhibiting protein breakdown. In this study however, protein breakdown was not measured. This limits our ability to fully interpret how training affects protein balance, since only one half of the protein balance equation was examined. The response of muscle protein breakdown following resistance exercise has not been extensively studied and results have differed, which maybe attributed to methodology issues. Studies using 3-methylhistidine excretion as a marker of skeletal muscle breakdown have reported increases (20; 58), decreases (49; 62), or no changes (59; 61) in protein breakdown in response to exercise. Examining studies using tracer methodologies, muscle protein breakdown has consistently been shown to increase in response to heavy resistance exercise (9; 56; 57). Phillips *et al.* (56) determined that exercise-induced protein breakdown was elevated by 31% at 3 h post-exercise and was still elevated at 24 h post-exercise, however protein breakdown was down to resting levels by 48 hrs post-exercise in untrained participants. Phillips *et al.* (57) conducted a study on the effects of training on exercise-induced protein breakdown. The results of their investigation found that post-exercise protein breakdown increased to a greater extent in untrained compared to trained individuals (57). It is important to note that the post-exercise increase in protein breakdown in the two aforementioned studies (56; 57) was lower in magnitude than the exercise-induced increase in protein synthesis, resulting in an improved net protein

balance. However, these studies were performed in the post-absorptive state and thus the net protein balance remained negative. While it is certain that we have missed some information in the present study by not studying protein breakdown it should be noted that in contrast to synthesis, breakdown changes far less in response to physiological situations such as exercise and the ingesting of protein, both of which have a substantial impact on protein synthesis. Hence, we would propose that synthesis is the predominant locus of control in determining protein balance and thus, muscle protein gain and likely loss. In addition, changes in protein breakdown have been consistently shown to be closely related to changes in protein synthesis (9; 56; 57), which indicates that protein breakdown would likely track changes in synthesis. Finally, provision of amino acids in the post-exercise period has been shown to suppress the exercise-induced rise in protein breakdown (8); hence, in our trials the change in protein breakdown would not likely play a major role, at least in comparison to protein synthesis, in determining the overall protein balance.

The consumption of a mixed meal containing amino acids and carbohydrates does not only provide substrates for protein synthesis but, as previously mentioned, may inhibit protein breakdown through the anabolic action of insulin. Indeed, Biolo *et al.* (9) showed that insulin infusion blunted the exercise-induced rise in protein breakdown. As well, a drink supplement containing amino acids and carbohydrates given after exercise also prevented the exercise-induced rise in protein breakdown (64). Phillips *et al.* (55) found that post-exercise protein breakdown was not different between trained and untrained in the fed state. However, the feeding, specifically the insulin release, did not inhibit the

entire exercise-induced rise in protein breakdown in the untrained state. This is could be due to the intensity of the exercise, which was at the same absolute intensity between the trained and untrained states. It is possible that exercise performed at a higher intensity (as observed in the untrained state) might result in a greater exercise-induced increase in protein breakdown, and that insulin may not be able to blunt the entire response. However protein breakdown in the trained state was also measured after a lower relative intensity exercise, so the previous explanation does not appear to be correct. The difference in post-exercise protein breakdown in the fasted and fed state could also be attributed to resting protein breakdown levels in the different training states. In the untrained state Phillips *et al.* (55) demonstrated a lower resting rate of protein breakdown in the untrained versus trained state. Despite the difference in resting protein breakdown, training did not affect the resting net protein balance, since protein synthesis was also lower in the untrained state at rest. This differs from the present study since we found no difference in resting protein synthesis with training status.

One possible explanation for the difference in resting protein metabolism could be related to study design. Phillips *et al.* (55) used a longitudinal study to assess the impact of resistance training on protein metabolism, which means that another factor, other than training status, could have changed over the 8-week training period that could have affected resting protein metabolism. In the study by Phillips *et al.* (55) the difference in resting protein metabolism was not affected by insulin levels since they were similar between testing sessions, however circulating amino acids, growth hormone, insulin-like growth factor, and stress hormones were not measured during the different testing

sessions involving training status all which may impact on protein metabolism. In the present study we used a novel unilateral training protocol, such that levels of circulating hormones and amino acids were similar during the simultaneous measurement of protein metabolism in the trained and untrained legs throughout the same infusion trial. However, Kim *et al.* (38) used a similar unilateral experimental protocol but contrary to our observations they observed an elevation in resting protein synthesis in the trained leg. This may be due to the time when protein synthesis was measured following the last training bout. Previous studies that have found a training-induced elevation in resting rates of protein synthesis (38; 55) have measured protein synthetic rates following 3 days of rest, where in the present study we allowed our participants 4 days of rest. It may be that the additional day of rest our participants were allowed resulted in significant detraining, whereby we failed to observe the elevated rate of resting protein synthesis with training. However, there is a lack of knowledge about the detraining process, so this explanation remains speculative. Another possible issue with our unilateral training model that could account for our lack of difference in resting protein synthesis between training states is that our untrained limb may not have been truly 'untrained'. An adaptation that may occur with unilateral training is a cross-training phenomenon, where improvements in strength-production are observed in the untrained limb. The increase in strength in the untrained limb has been attributed to neural adaptation which has been suggested to be due to a diffusion of motor impulses to the untrained limb (33; 36; 69). It is not known what effect this cross-education would have on MPS, however, we propose that any effect would be minor by comparison to the training stimulus. This thesis is

based on the fact that in the UT limb strength gains were almost certainly 100% neural in origin, which is supported by the lack of change in isokinetic and isometric strength in the UT limb, but with significant strength changes in the T limb. In addition, muscle fibres from the UT limb were no different in size than those from the T limb prior to training indicating that the UT limb did not show any hypertrophy.

Twelve weeks of unilateral concentric isokinetic leg extension training produced increases in peak torque production in both the trained and untrained legs (21) yet no changes in EMG amplitude were observed from the trained limb after training. To account for the strength gains independent of any neural adaptation the author's concluded that hypertrophic factors may impact the untrained leg (21). As well, it has been noted that postural positioning needed to properly complete the unilateral exercise may contribute to observed increases in strength in the untrained limb (33). In the present study isotonic 1 RM leg extension strength increased over the trained period in both the trained and untrained legs, however, the increase in strength was much greater in the trained limb and muscle fibre cross-sectional area was only increase in the trained leg. Although neuromuscular activity was not measured in this study, it has generally accepted that the increase strength in the untrained limb can be attributed, for the most part, to neural adaptations. Whether a specific hypertrophic factor, and the nature as well as mechanism of such a factor, may have contributed to the increase in strength remains to be determined. We did observe that isokinetic strength measured at 0.52 $rad \cdot s^{-1}$, as well as isometric strength increased significantly following training in the trained leg, so there was no evidence of a cross-training effect in isokinetic and isometric strength. This

may be due to the fact that isokinetic and isometric strength only increased approximately 18% over pre-trained values, while 1-RM knee extension strength increased approximately 62% after the training period.

One often observed outcome of a resistance training program is the ability to gain muscle mass. The training protocol used in this study resulted in significant increases in CSA of type I and II fibres in the trained leg only. In order to account for the observed muscle fibre hypertrophy, an average net positive muscle protein balance needed to occur over the training period. Feeding soon after each training session would have been sufficient for protein accretion due to the additive effects of amino acids, through a regular diet, combined with the resistance exercise bouts (38; 64; 74; 77). The increase in fibre CSA with hypertrophy is a result of the addition of new contractile proteins into the existing protein lattice in a force-producing arrangement (70). In addition, while controversial, it is possible, to some degree, that hypertrophy is also the result of sarcomerogenesis. The production of new sarcomeres would require an increase in the synthesis of both myofibrillar and non- myofibrillar proteins (i.e., sarcoplasmic and mitochondrial proteins).

The measurement of mixed muscle FSR represents an average synthesis rate of numerous cellular proteins and several protein sub-fractions; mitochondrial, sarcoplasmic, and myofibrillar. With the current training protocol it would be relevant to examine specific changes in the myofibrillar protein fraction because these changes could be "masked" when only mixed muscle protein synthesis is monitored, especially after resistance exercise. Indeed, myofibrillar proteins that include myosin, actin, troponin,

tropomysin, titin, and nebulin, comprise approximately 70-80%, by weight, of skeletal muscle proteins (3; 31; 32). Bohé *et al.* (11) found that changes in myofibrillar protein synthesis were closely related to the magnitude of the increase in mixed muscle protein synthesis after an amino acid infusion. In addition, with an acute exercise program both mixed muscle protein synthesis and myosin heavy chain (MHC) synthesis were increased (32). However, when subjects become accustomed to resistance exercise, that is become resistance trained, the increase in mixed muscle protein synthesis, as previously described, and myofibrillar or MHC protein synthesis were attenuated (80). Myofibrillar synthesis has been found to be unchanged after 3 months of resistance exercise (80) and after 8 weeks of unilateral resistance training (38). The majority of the myofibrillar protein fraction is made up of myosin, which comprises approximately 25-30% of all muscle protein (4). However, resting MHC synthesis, has been shown to account for approximately 18% of mixed muscle protein synthesis (3), but MHC synthesis rates have been correlated with changed in muscle mass and strength (4). So as one becomes trained they may be able to synthesize more MHC needed for greater force production with training, which may result in them having MHC synthesis comprise a greater fraction of mixed muscle protein synthesis. Even though resting mixed muscle protein synthesis has been shown to increase following training, myofibrillar protein synthesis remains unchanged meaning that the synthesis of non-myofibrillar proteins may have been increased. This seems to be counter-intuitive, as the general training principle is that with repeated stress, resistance exercise per se, the body becomes better able to adapt to the stress by fine tuning our responses. Our results show that resting mixed muscle protein

synthesis in not different in trained and untrained, which seems to suggest that resting myofibrillar protein synthesis should be increased in the trained state since the increase in muscle mass observed in our study needs to be constantly turnover or remodeled. Seeing as this study was performed in the fed state, the maximal protein synthetic response was able to occur. Recent data from our lab (38) has shown that training does result in less of a perturbation after a single bout of resistance exercise. Approximately 16 h after exercise mixed muscle protein synthesis increased to a greater extent in the untrained state, but myofibrillar synthesis was similar. This indicates that trained individuals may be better able to fine tune the protein synthetic response, since myofibrillar synthesis can selectively increase in response to resistance exercise where less of an increase in non-myofibrillar protein synthesis is needed to achieve the necessary myofibrillar protein synthetic response. This more 'directed' or 'fine tuned' response of protein synthesis could arise due to changes at any step along the protein synthetic pathway but remains to be determined exactly where in the pathway this is taking place.

The resistance training-induced adaptations appear to be the result of an 'improved metabolic efficiency', which is how rapid existing mRNA is translated into protein, in order for muscle fibres to cope with the subsequent stress of exercise. However, the cellular and molecular mechanisms responsible for these adaptations are just beginning to be understood. In response to both feeding and resistance exercise, protein synthesis seems to be more highly regulated, in comparison protein breakdown, which seems to vary far less than synthesis in response to the same external stimuli. Cellular processes, such as transcription and translation can potentially regulate the increase in protein

synthesis; however, the protein synthetic response appears to be too rapid, within 3-4 h, to induce any significant changes in the translational capacity (i.e., increase in RNA content) of the system. Indeed, 4 and 24 h following heavy resistance exercise in the human biceps muscle, the exercise-induced rise in protein synthesis was accompanied by an increase in RNA activity, but total RNA of the system remained unchanged (17). Measuring total RNA and total RNA activity may not allow detection of changes in specific mRNA abundance or specific proteins necessary in upregulating protein synthesis. However, Kubica *et al.* (39) showed that there was an increase in a specific translational regulatory protein content of the translation initiation pathway, which is important in the protein synthetic response prior to an elevation in the relative quantity of mRNA. The rapid increase in protein synthesis in response to feeding and resistance exercise is largely due to the upregulation of the systematic translational efficiency that is how rapidly existing mRNA are translated into proteins (24; 51). Signal transduction pathways that alter the phosphorylation state of a variety of translational regulatory factors, including eukaryotic initiation factors (eIF) control the change in the translational efficiency of the system. The fact that a combination of resistance exercise and feeding results in a greater protein synthetic response may potentially come from the synergistic enhancement of protein translation initiation by stimulating all potentially limiting factors. This would coordinate the protein synthetic response and resistance training may result in tighter regulation of protein translation initiation (i.e., more rapid onset) but the mechanisms that underlie the muscle protein synthetic response are currently unknown and further investigations are needed.

In summary, 8 weeks of progressive unilateral leg training resulted in muscle fibre hypertrophy and muscular strength gains. In addition, the response of mixed skeletal muscle protein synthesis to resistance exercise was acutely increased in both the trained and untrained legs. However, in the untrained leg protein synthetic rates remained elevated 28 h post-exercise, while rates of protein synthesis returned to resting levels in the trained leg. Resistance training may modify how each muscle protein sub-fraction responds to the exercise stimulus. It may be that part of the reason for the earlier fall in the exercise-induced muscle protein synthetic rate observed with resistance training is due to quantitatively important changes occurring in myofibrillar protein synthesis. These changes in myofibrillar protein synthesis could be 'masked' by smaller changes in sarcoplasmic or mitochondrial proteins, observed when mixed muscle protein responses are measured. The training induced alterations in exercise stimulated protein synthesis could be due, but not mutually exclusive, to changes in mRNA abundance, protein content, and/or the sensitivity of the signalling pathways involved in activating muscle protein synthesis. However, further investigations into the molecular and cellular signalling involved in regulating muscle protein turnover are required.

REFERENCE LIST

1. **Anthony JC, Yoshizawa F, Anthony TG, Vary TC, Jefferson LS and Kimball SR**. Leucine stimulates translation initiation in skeletal muscle of postabsorptive rats via a rapamycin-sensitive pathway. *J Nutr* 130: 2413-2419, 2000.

2. **Baar K and Esser K**. Phosphorylation of p70(S6k) correlates with increased skeletal muscle mass following resistance exercise. *Am J Physiol* 276: C120-C127, 1999.

3. **Balagopal P, Ljungqvist O and Nair KS**. Skeletal muscle myosin heavy-chain synthesis rate in healthy humans. *Am J Physiol* 272: E45-E50, 1997.

4. **Balagopal P, Rooyackers OE, Adey DB, Ades PA and Nair KS**. Effects of aging on in vivo synthesis of skeletal muscle myosin heavy-chain and sarcoplasmic protein in humans. *Am J Physiol* 273: E790-E800, 1997.

5. **Biolo G, Declan Fleming RY and Wolfe RR**. Physiologic hyperinsulinemia stimulates protein synthesis and enhances transport of selected amino acids in human skeletal muscle. *J Clin Invest* 95: 811-819, 1995.

6. **Biolo G, Fleming RY, Maggi SP and Wolfe RR**. Transmembrane transport and intracellular kinetics of amino acids in human skeletal muscle. *Am J Physiol* 268: E75-E84, 1995.

7. **Biolo G, Maggi SP, Williams BD, Tipton KD and Wolfe RR**. Increased rates of muscle protein turnover and amino acid transport after resistance exercise in humans. *Am J Physiol* 268: E514-E520, 1995.

8. **Biolo G, Tipton KD, Klein S and Wolfe RR**. An abundant supply of amino acids enhances the metabolic effect of exercise on muscle protein. *Am J Physiol* 273: E122-E129, 1997.

9. **Biolo G, Williams BD, Fleming RY and Wolfe RR**. Insulin action on muscle protein kinetics and amino acid transport during recovery after resistance exercise. *Diabetes* 48: 949-957, 1999.

10. **Bohe J, Low A, Wolfe RR and Rennie MJ**. Human muscle protein synthesis is modulated by extracellular, not intramuscular amino acid availability: a dose-response study. *J Physiol* 552: 315-324, 2003.

11. **Bohe J, Low JF, Wolfe RR and Rennie MJ**. Latency and duration of stimulation of human muscle protein synthesis during continuous infusion of amino acids. *J Physiol* 532: 575-579, 2001.

12. **Bolster DR, Kimball SR and Jefferson LS**. Translational control mechanisms modulate skeletal muscle gene expression during hypertrophy. *Exerc Sport Sci Rev* 31: 111-116, 2003.

13. **Bolster DR, Kubica N, Crozier SJ, Williamson DL, Farrell PA, Kimball SR and Jefferson LS**. Immediate response of mammalian target of rapamycin (mTOR)-mediated signalling following acute resistance exercise in rat skeletal muscle. *J Physiol* 553: 213-220, 2003.

14. **Borsheim E, Aarsland A and Wolfe RR**. Effect of an amino acid, protein, and carbohydrate mixture on net muscle protein balance after resistance exercise. *Int J Sport Nutr Exerc Metab* 14: 255-271, 2004.

15. **Borsheim E, Cree MG, Tipton KD, Elliott TA, Aarsland A and Wolfe RR**. Effect of carbohydrate intake on net muscle protein synthesis during recovery from resistance exercise. *J Appl Physiol* 96: 674-678, 2004.

16. **Bylund-Fellenius AC, Ojamaa KM, Flaim KE, Li JB, Wassner SJ and Jefferson LS**. Protein synthesis versus energy state in contracting muscles of perfused rat hindlimb. *Am J Physiol* 246: E297-E305, 1984.

17. **Chesley A, MacDougall JD, Tarnopolsky MA, Atkinson SA and Smith K**. Changes in human muscle protein synthesis after resistance exercise. *J Appl Physiol* 73: 1383-1388, 1992.

18. **Cuthbertson DJR, Babraj J, Smith K, Fedele M, Esser K and Rennie MJ**. Temporal relationship between human protein synthesis, protein kinase B and $p70^{S6}$ kinase phosphorylation after shortening or stretching exercise. *J Physiol* 555P: C60, 2003.

19. **Cuthbertson DJR, Smith K, Babraj J, Waddell T, Watt PW, Meier-Augustein W, Rennie MJ, Hinsch M and Esser K**. Myofibrillar protein synthesis and the activity of p70 S6 kinase in human quadriceps muscle after contractile activity with muscle shortening or stretching. *J Physiol* **539P**: S160, 2002.

20. **Dohm GL, Kasperek GJ, Tapscott EB and Beecher GR**. Effect of exercise on synthesis and degradation of muscle protein. *Biochem J* 188: 255-262, 1980.

21. **Evetovich TK, Housh TJ, Housh DJ, Johnson GO, Smith DB and Ebersole KT**. The effect of concentric isokinetic strength training of the quadriceps femoris on electromyography and muscle strength in the trained and untrained limb. *J Strength Cond Res* 15: 439-445, 2001.

22. **Farrell PA, Fedele MJ, Hernandez J, Fluckey JD, Miller JL, III, Lang CH, Vary TC, Kimball SR and Jefferson LS**. Hypertrophy of skeletal muscle in diabetic rats in response to chronic resistance exercise. *J Appl Physiol* 87: 1075-1082, 1999.

23. **Farrell PA, Fedele MJ, Vary TC, Kimball SR, Lang CH and Jefferson LS**. Regulation of protein synthesis after acute resistance exercise in diabetic rats. *Am J Physiol* 276: E721-E727, 1999.

24. **Farrell PA, Hernandez JM, Fedele MJ, Vary TC, Kimball SR and Jefferson LS**. Eukaryotic initiation factors and protein synthesis after resistance exercise in rats. *J Appl Physiol* 88: 1036-1042, 2000.

25. **Fedele MJ, Hernandez JM, Lang CH, Vary TC, Kimball SR, Jefferson LS and Farrell PA**. Severe diabetes prohibits elevations in muscle protein synthesis after acute resistance exercise in rats. *J Appl Physiol* 88: 102-108, 2000.

26. **Fluckey JD, Vary TC, Jefferson LS, Evans WJ and Farrell PA**. Insulin stimulation of protein synthesis in rat skeletal muscle following resistance exercise is maintained with advancing age. *J Gerontol A Biol Sci Med Sci* 51: B323-B330, 1996.

27. **Fluckey JD, Vary TC, Jefferson LS and Farrell PA**. Augmented insulin action on rates of protein synthesis after resistance exercise in rats. *Am J Physiol* 270:

E313-E319, 1996.

28. **Gelfand RA and Barrett EJ**. Effect of physiologic hyperinsulinemia on skeletal muscle protein synthesis and breakdown in man. *J Clin Invest* 80: 1-6, 1987.

29. **Gibala MJ, Interisano SA, Tarnopolsky MA, Roy BD, MacDonald JR, Yarasheski KE and MacDougall JD**. Myofibrillar disruption following acute concentric and eccentric resistance exercise in strength-trained men. *Can J Physiol Pharmacol* 78: 656-661, 2000.

30. **Gibala MJ, MacDougall JD, Tarnopolsky MA, Stauber WT and Elorriaga A**. Changes in human skeletal muscle ultrastructure and force production after acute resistance exercise. *J Appl Physiol* 78: 702-708, 1995.

31. **Hasten DL, Morris GS, Ramanadham S and Yarasheski KE**. Isolation of human skeletal muscle myosin heavy chain and actin for measurement of fractional synthesis rates. *Am J Physiol* 275: E1092-E1099, 1998.

32. **Hasten DL, Pak-Loduca J, Obert KA and Yarasheski KE**. Resistance exercise acutely increases MHC and mixed muscle protein synthesis rates in 78-84 and 23-32 yr olds. *Am J Physiol Endocrinol Metab* 278: E620-E626, 2000.

33. **HELLEBRANDT FA**. Cross education; ipsilateral and contralateral effects of unimanual training. *J Appl Physiol* 4: 136-144, 1951.

34. **Hernandez JM, Fedele MJ and Farrell PA**. Time course evaluation of protein synthesis and glucose uptake after acute resistance exercise in rats. *J Appl Physiol* 88: 1142-1149, 2000.

35. **Iiboshi Y, Papst PJ, Kawasome H, Hosoi H, Abraham RT, Houghton PJ and Terada N**. Amino acid-dependent control of p70(s6k). Involvement of tRNA aminoacylation in the regulation. *J Biol Chem* 274: 1092-1099, 1999.

36. **Kannus P, Alosa D, Cook L, Johnson RJ, Renstrom P, Pope M, Beynnon B, Yasuda K, Nichols C and Kaplan M**. Effect of one-legged exercise on the strength, power and endurance of the contralateral leg. A randomized, controlled study using isometric and concentric isokinetic training. *Eur J Appl Physiol Occup*

Physiol 64: 117-126, 1992.

37. **Kettelhut IC, Wing SS and Goldberg AL**. Endocrine regulation of protein breakdown in skeletal muscle. *Diabetes Metab Rev* 4: 751-772, 1988.

38. **Kim PL, Staron RS and Phillips SM**. Fasted-state skeletal muscle protein synthesis after resistance exercise is altered with training. *J Physiol* 2005. *In press.*

39. **Kubica N, Kimball SR, Jefferson LS and Farrell PA**. Alterations in the expression of mRNAs and proteins that code for species relevant to eIF2B activity after an acute bout of resistance exercise. *J Appl Physiol* 96: 679-687, 2004.

40. **Lecker SH, Solomon V, Mitch WE and Goldberg AL**. Muscle protein breakdown and the critical role of the ubiquitin-proteasome pathway in normal and disease states. *J Nutr* 129: 227S-237S, 1999.

41. **Liu Z, Jahn LA, Wei L, Long W and Barrett EJ**. Amino acids stimulate translation initiation and protein synthesis through an Akt-independent pathway in human skeletal muscle. *J Clin Endocrinol Metab* 87: 5553-5558, 2002.

42. **Long W, Saffer L, Wei L and Barrett EJ**. Amino acids regulate skeletal muscle PHAS-I and p70 S6-kinase phosphorylation independently of insulin. *Am J Physiol Endocrinol Metab* 279: E301-E306, 2000.

43. **MacDougall JD, Gibala MJ, Tarnopolsky MA, MacDonald JR, Interisano SA and Yarasheski KE**. The time course for elevated muscle protein synthesis following heavy resistance exercise. *Can J Appl Physiol* 20: 480-486, 1995.

44. **McCall GE, Byrnes WC, Dickinson A, Pattany PM and Fleck SJ**. Muscle fiber hypertrophy, hyperplasia, and capillary density in college men after resistance training. *J Appl Physiol* 81: 2004-2012, 1996.

45. **McCall GE, Byrnes WC, Dickinson AL and Fleck SJ**. Sample size required for the accurate determination of fiber area and capillarity of human skeletal muscle. *Can J Appl Physiol* 23: 594-599, 1998.

46. **Miller BF, Olesen JL, Hansen M, Dossing S, Crameri RM, Welling RJ, Langberg H, Flyvbjerg A, Kjaer M, Babraj JA, Smith K and Rennie MJ**. Coordinated collagen and muscle protein synthesis in human patella tendon and quadriceps muscle after exercise. *J Physiol* 2005.

47. **Miller SL, Tipton KD, Chinkes DL, Wolf SE and Wolfe RR**. Independent and combined effects of amino acids and glucose after resistance exercise. *Med Sci Sports Exerc* 35: 449-455, 2003.

48. **Moore DR, Burgomaster KA, Schofield LM, Gibala MJ, Sale DG and Phillips SM**. Neuromuscular adaptations in human muscle following low intensity resistance training with vascular occlusion. *Eur J Appl Physiol* 92: 399-406, 2004.

49. **Mussini E, Colombo L, De Ponte G, Calzi M and Marcucci F**. Effect of swimming on protein degradation: 3-methylhistidine and creatinine excretion. *Biochem Med* 34: 373-375, 1985.

50. **Nader GA and Esser KA**. Intracellular signaling specificity in skeletal muscle in response to different modes of exercise. *J Appl Physiol* 90: 1936-1942, 2001.

51. **Nader GA, Hornberger TA and Esser KA**. Translational control: implications for skeletal muscle hypertrophy. *Clin Orthop* S178-S187, 2002.

52. **Paddon-Jones D, Sheffield-Moore M, Zhang XJ, Volpi E, Wolf SE, Aarsland A, Ferrando AA and Wolfe RR**. Amino acid ingestion improves muscle protein synthesis in the young and elderly. *Am J Physiol Endocrinol Metab* 286: E321-E328, 2004.

53. **Pain VM**. Initiation of protein synthesis in eukaryotic cells. *Eur J Biochem* 236: 747-771, 1996.

54. **Patterson BW, Zhao G and Klein S**. Improved accuracy and precision of gas chromatography/mass spectrometry measurements for metabolic tracers. *Metabolism* 47: 706-712, 1998.

55. **Phillips SM, Parise G, Roy BD, Tipton KD, Wolfe RR and Tamopolsky MA**. Resistance-training-induced adaptations in skeletal muscle protein turnover in the

fed state. *Can J Physiol Pharmacol* 80: 1045-1053, 2002.

56. **Phillips SM, Tipton KD, Aarsland A, Wolf SE and Wolfe RR.** Mixed muscle protein synthesis and breakdown after resistance exercise in humans. *Am J Physiol* 273: E99-107, 1997.

57. **Phillips SM, Tipton KD, Ferrando AA and Wolfe RR.** Resistance training reduces the acute exercise-induced increase in muscle protein turnover. *Am J Physiol* 276: E118-E124, 1999.

58. **Plante PD and Houston ME.** Effects of concentric and eccentric exercise on protein catabolism in man. *Int J Sports Med* 5: 174-178, 1984.

59. **Plante RI and Houston ME.** Exercise and protein catabolism in women. *Ann Nutr Metab* 28: 123-129, 1984.

60. **Preedy VR and Garlick PJ.** The response of muscle protein synthesis to nutrient intake in postabsorptive rats: the role of insulin and amino acids. *Biosci Rep* 6: 177-183, 1986.

61. **Price GM, Halliday D, Pacy PJ, Quevedo MR and Millward DJ.** Nitrogen homeostasis in man: influence of protein intake on the amplitude of diurnal cycling of body nitrogen. *Clin Sci (Lond)* 86: 91-102, 1994.

62. **Radha E and Bessman SP.** Effect of exercise on protein degradation: 3-methylhistidine and creatinine excretion. *Biochem Med* 29: 96-100, 1983.

63. **Rasmussen BB and Phillips SM.** Contractile and nutritional regulation of human muscle growth. *Exerc Sport Sci Rev* 31: 127-131, 2003.

64. **Rasmussen BB, Tipton KD, Miller SL, Wolf SE and Wolfe RR.** An oral essential amino acid-carbohydrate supplement enhances muscle protein anabolism after resistance exercise. *J Appl Physiol* 88: 386-392, 2000.

65. **Rennie MJ and Tipton KD.** Protein and amino acid metabolism during and after exercise and the effects of nutrition. *Annu Rev Nutr* 20: 457-483, 2000.

66. **Rhoads RE**. Regulation of eukaryotic protein synthesis by initiation factors. *J Biol Chem* 268: 3017-3020, 1993.

67. **Rommel C, Bodine SC, Clarke BA, Rossman R, Nunez L, Stitt TN, Yancopoulos GD and Glass DJ**. Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. *Nat Cell Biol* 3: 1009-1013, 2001.

68. **Roy BD, Tarnopolsky MA, MacDougall JD, Fowles J and Yarasheski KE**. Effect of glucose supplement timing on protein metabolism after resistance training. *J Appl Physiol* 82: 1882-1888, 1997.

69. **Sale DG**. Neural adaptation to resistance training. *Med Sci Sports Exerc* 20: S135-S145, 1988.

70. **Staron RS, Karapondo DL, Kraemer WJ, Fry AC, Gordon SE, Falkel JE, Hagerman FC and Hikida RS**. Skeletal muscle adaptations during early phase of heavy-resistance training in men and women. *J Appl Physiol* 76: 1247-1255, 1994.

71. **Stewart BG, Tarnopolsky MA, Hicks AL, McCartney N, Mahoney DJ, Staron RS and Phillips SM**. Treadmill training-induced adaptations in muscle phenotype in persons with incomplete spinal cord injury. *Muscle Nerve* 30: 61-68, 2004.

72. **Stupka N, Tarnopolsky MA, Yardley NJ and Phillips SM**. Cellular adaptation to repeated eccentric exercise-induced muscle damage. *J Appl Physiol* 91: 1669-1678, 2001.

73. **Tarnopolsky MA, Atkinson SA, MacDougall JD, Chesley A, Phillips S and Schwarcz HP**. Evaluation of protein requirements for trained strength athletes. *J Appl Physiol* 73: 1986-1995, 1992.

74. **Tipton KD, Ferrando AA, Phillips SM, Doyle D, Jr. and Wolfe RR**. Postexercise net protein synthesis in human muscle from orally administered amino acids. *Am J Physiol* 276: E628-E634, 1999.

75. **Tipton KD, Ferrando AA, Williams BD and Wolfe RR**. Muscle protein

metabolism in female swimmers after a combination of resistance and endurance exercise. *J Appl Physiol* 81: 2034-2038, 1996.

76. **Tipton KD, Gurkin BE, Matin S and Wolfe RR**. Nonessential amino acids are not necessary to stimulate net muscle protein synthesis in healthy volunteers. *J Nutr Biochem* 10: 89-95, 1999.

77. **Tipton KD, Rasmussen BB, Miller SL, Wolf SE, Owens-Stovall SK, Petrini BE and Wolfe RR**. Timing of amino acid-carbohydrate ingestion alters anabolic response of muscle to resistance exercise. *Am J Physiol Endocrinol Metab* 281: E197-E206, 2001.

78. **Vary TC, Jefferson LS and Kimball SR**. Amino acid-induced stimulation of translation initiation in rat skeletal muscle. *Am J Physiol* 277: E1077-E1086, 1999.

79. **Volpi E, Kobayashi H, Sheffield-Moore M, Mittendorfer B and Wolfe RR**. Essential amino acids are primarily responsible for the amino acid stimulation of muscle protein anabolism in healthy elderly adults. *Am J Clin Nutr* 78: 250-258, 2003.

80. **Welle S, Thornton C and Statt M**. Myofibrillar protein synthesis in young and old human subjects after three months of resistance training. *Am J Physiol* 268: E422-E427, 1995.

81. **Wolfe RR**. Control of muscle protein breakdown: effects of activity and nutritional states. *Int J Sport Nutr Exerc Metab* 11 Suppl: S164-S169, 2001.

82. **Yarasheski KE, Campbell JA, Smith K, Rennie MJ, Holloszy JO and Bier DM**. Effect of growth hormone and resistance exercise on muscle growth in young men. *Am J Physiol* 262: E261-E267, 1992.

83. **Yarasheski KE, Pak-Loduca J, Hasten DL, Obert KA, Brown MB and Sinacore DR**. Resistance exercise training increases mixed muscle protein synthesis rate in frail women and men >/=76 yr old. *Am J Physiol* 277: E118-E125, 1999.

84. **Yarasheski KE, Zachwieja JJ and Bier DM**. Acute effects of resistance exercise on muscle protein synthesis rate in young and elderly men and women. *Am J Physiol* 265: E210-E214, 1993.

85. **Yarasheski KE, Zachwieja JJ, Campbell JA and Bier DM**. Effect of growth hormone and resistance exercise on muscle growth and strength in older men. *Am J Physiol* 268: E268-E276, 1995.

APPENDIX 1

INFORMATION & CONSENT TO PARTICIPATE IN RESEARCH FORM

SUBJECT SCREENING QUESTIONNAIRE

DESCRIPTION OF MEDICAL PROCEDURES

EXERCISE METABOLISM RESEARCH GROUP
DEPARTMENT OF KINESIOLOGY, McMASTER UNIVERSITY

INFORMATION AND CONSENT TO PARTICIPATE IN RESEARCH

THE IMPACT OF RESISTANCE TRAINING ON PROTEIN TURNOVER

This research study is sponsored by Natural Sciences and Engineering Research Council of Canada.

You are being asked to participate in a research study being conducted by the investigators listed below. Prior to participating in this study you are asked to read this form which outlines the purpose and testing procedures and a separate form that describes the medical procedures (Description of Medical Procedures) used in this study. In addition, you must answer some questions regarding your health which is included in the attached forms (Subject Screening Questionnaire). Unless otherwise stated all testing and experimental procedures will be conducted in the Exercise Metabolism Research Laboratory, Room A103, Ivor Wynne Centre.

INVESTIGATORS	DEPARTMENT	CONTACT
Dr. Stuart M. Phillips	Kinesiology, IWC AB116	x24465 or x27037
Dr. Mark A. Tarnopolsky	Medicine, HSC 4U4	x76593
Jennifer Perco, B.Sc.	Kinesiology, IWC A103	x27037
Sarah Wilkinson, B.Kin.	Kinesiology, IWC A103	x27037

PURPOSE

A net gain in muscle mass (i.e. muscle hypertrophy) occurs as a consequence of resistance training or weightlifting, which is a result of a chronic net positive protein balance (protein synthesis > protein breakdown). The training status of an individual may impact upon the effect that a single bout of resistance exercise has on protein turnover.

The balance between protein synthesis and protein breakdown determines protein gain or loss. Proteins in the human body are being continually turned over or remodelled. At rest, and with feeding there is no net gain or loss in bodily proteins. One of the most potent stimulators of muscle protein turnover is resistance exercise or weightlifting. A single bout of resistance exercise stimulates both protein synthesis and, to a lesser degree, breakdown. With resistance training there is a net gain in muscle mass (i.e. muscle hypertrophy), which is a result of a chronic net positive protein balance (protein synthesis > protein breakdown). The training status of an individual may also impact upon the

effect that a single bout of resistance exercise has on protein turnover, since resistance trained individuals have an attenuated response in protein synthesis following resistance exercise. Previous studies have examined the time course of protein synthesis following a single bout of resistance exercise but the results from these studies differed. It is possible that the training status of the individuals in the two studies (i.e. one group of subjects were resistance trained while the other group of subjects were untrained) may account for the difference in time course of the increases in muscle protein synthesis. However, the mechanisms that underlie this attenuated muscle protein synthetic response are unknown. Our lack of information in this area has important consequences since imbalances in muscle protein turnover exist in a number of disease conditions (i.e. AIDS, cancer, sepsis), as well as, numerous physiological states (i.e. spinal cord injury, microgravity, aging). Understanding the mechanistic underpinnings of muscle protein turnover from our resistance training resulting in muscle hypertrophy will undoubtedly provide useful information in treating and even reversing the muscle wasting associated with these conditions.

Changes in protein synthesis are controlled by cellular mechanisms such as gene transcription, initiation of protein synthesis, and proteolysis. A large proportion of the change in muscle protein turnover that occurs after resistance exercise is due to translational control, since the synthetic response is too rapid, peaking at about 3 hours post-exercise, to be due to any substantial change in gene expression. It has been suggested that this is largely due to an increase in translational efficiency for a given amount of mRNA transcript. One working hypothesis is that the increase in translational efficiency results from increased activation of signalling pathways that are activating translational initiation factors.

DESCRIPTION OF TESTING PROCEDURES

Prior to the commencement of the study you will be required to complete a health questionnaire. You will have a familiarization session with the equipment and procedures involved in the study. After the familiarization session you will undergo a series of preliminary tests. The isometric strength measurements will be performed on a Biodex machine (isokinetic dynamometer) to determine maximum isometric strength both during eccentric and concentric contractions. As well, you will undergo a preliminary voluntary dynamic one-repetition maximum (1RM) to determine maximum dynamic strength. Additionally, you will receive a resting muscle biopsy in the leg that is going to be training to determine the baseline muscle fibre size of the quadriceps muscle prior to the commencement of the training protocol.

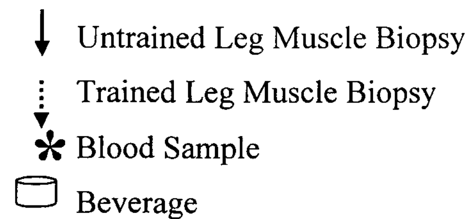
After all the aforementioned baseline testing is complete, you will undergo a progressive 8-week unilateral leg (one leg only) resistance-training program, while your contralateral leg will serve as a non-resistance trained control. The leg to be trained will be randomly

assigned from your dominant and non-dominant leg. The unilateral leg resistance program will consist of knee extension exercise. The volume of training will consist of 2-3 training sessions per week with each session consisting of 3-6 sets of 6-12 repetitions per set at 80% of your one repetition maximum (1RM). At the mid-point of the training protocol we will assess your 1RM again. Throughout the training protocol you will be given at least one day of rest between training days. Following the completion of the training protocol you will be tested for your dynamic strength (via 1RM) and isometric strength (via the Biodex machine) post training.

You will then participate in two infusion sessions, resting and following an acute bout of resistance exercise. You will first participate in a resting infusion session, four days after your last training session. You will arrive in the lab at ~0800 h after an overnight fast. You will have a venous catheter inserted in an antecubital vein from which blood samples will be drawn at specified times (3 samples x ~10mL/sample). Following the baseline blood sample a second catheter will be inserted in your opposite arm for infusion of the stable (i.e. non-radioactive) isotope-labeled D_3-α-ketoisocaproic acid. Following the initiation of the infusion, you will receive small portions of a protein drink, which you will ingest every 30 minutes for the duration of the infusion (4 hours). As well, blood samples will be taken every 2 hours for the duration of the infusion. You will rest supine on a bed for 2 hours before a local anesthetic is injected into your outer portion of both thighs and then a small incision will be made in each thigh in which muscle biopsies will taken from. After resting for another 2 hours, an additional muscle biopsy will be taken from each leg by separate incisions, again following injections of small amounts of local anesthetic. A schematic of this protocol is shown in Figure 1. You will train two days following the resting infusion at 6 sets of 6-8 repetitions at 80% 1RM. One week following the resting infusion, you will then participate in an isotope infusion following an acute bout of resistance exercise (5 sets of 8-10 repetitions at 80% 1RM – same relative intensity for the trained and the untrained leg). You will arrive in the lab at ~0630 h on the testing day after an overnight fast and perform the exercise bout. Two catheters will be inserted as previously described for blood sampling and isotope infusion. The isotope infusion will start 2 hours following the completion of the exercise bout. Biopsy samples from both legs will be taken, as previously described, at 4 and 8 hours following the completion of the exercise bout. As well, you will be given a small portion of drink, which you will ingest every 30 minutes for the duration of the testing session. Following the 8-hour biopsy you will be free to the leave the lab for the evening but are required to return to the lab at ~0800 h the next morning for continuation of the testing protocol. Upon arrival in the lab you will have the blood and isotope catheters inserted. Two hours following the initiation of the infusion a biopsy will be taken from each leg as previously described. The final biopsies will be taken 6 hours following the start of the infusion. A schematic of this protocol is shown in Figure 2. Each biopsy will be cleaned and sutured. Upon completing each testing protocol (i.e. rest, 1st day of acute exercise bout testing, 2nd day of acute exercise bout testing), catheters will be removed. You will also be provided with adequate band-aids and alcohol wipes to clean and care for the biopsy incisions. As well, you will receive the attending physician's pager and telephone

number.

The entire study will require that you complete a progressive 8-week resistance training protocol, and then on two separate testing sessions, have a total of 12 blood samples drawn (total ~120 mL). This amount of blood will not have any noticeable effect on your well being or normal day-to-day function. You will also have at total on 13 muscle biopsies, 7 from the trained leg and 6 from the untrained leg. Past research experience with subjects indicates that the biopsies and weightlifting protocols are generally well tolerated and that the main complaint is diffuse leg soreness following the resistance exercise protocol.



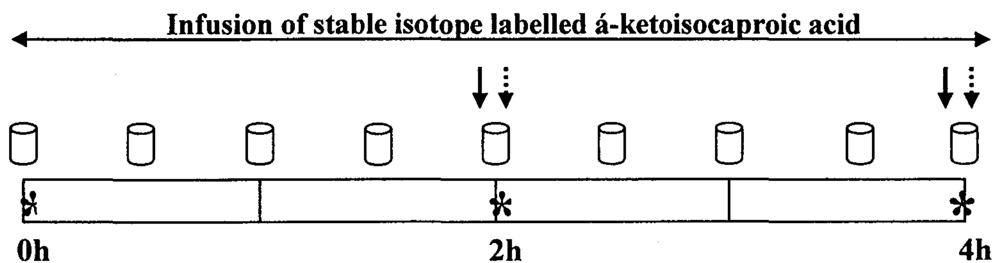


Figure 1: Schematic representation of resting infusion protocol

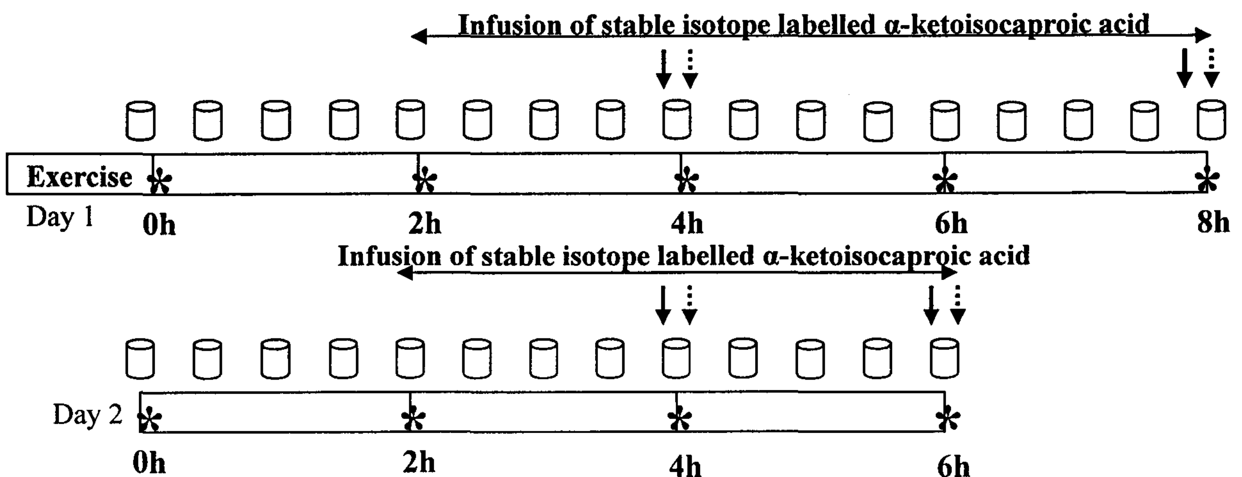


Figure 2: Schematic representation of post exercise infusion protocol

PONTENTIAL RISKS AND DISCOMFORTS

Please refer to the attached form entitled "Description of Medical Procedures" for a complete description of the medical procedures to be performed during the study and the potential risks associated with these procedures.

BENEFITS AND REMUNERATION

In participating in this study you realize that there are no direct benefits to you. You will receive an honorarium of $550 upon the completion of the study to compensate you for you time commitment.

CONFIDENTIALITY

The blood and biopsy samples will be used for this research project only. All data collected during this study will remain confidential and stored in offices and on computers to which only the investigators haves access. You should be aware that the results of this study will be made available to the scientific community, through publication in a scientific journal, although neither your name nor any reference to you will be used in compiling or publishing these results. Additionally, if you are interested you will have access to your own data, as well as the group data, when it becomes available.

PARTICIPATION WITHDRAWL

You can choose whether to participate in this study or not. You should be aware that your participation in this study will no way affect your academic performance in any course offered within the Department of Kinesiology. You may exercise the option of removing yourself or your data from the study at any time if you wish. You may also refuse to answer any questions posed to you during the study and still remain as subject in the study. The investigators reserve the right to withdraw you from the study if they believe that circumstances have arisen that warrant doing so.

RIGHTS OF RESEARCH PARTICIPANTS

You will receive a completed (i.e. signed) copy of this ethics form. You may withdraw your consent to participate in this study at any time, and you may also discontinue participation at any time without penalty. In signing this consent form or in participating in this study you are not waiving any legal claims or remedies. Finally please realize that this study has been reviewed by the Hamilton Health Sciences Research Ethics Board. If you have any further questions regarding your rights as a research participant please feel free to contact:

REB Secretariat, CNH-111
McMaster University
1280 Main St. W.
Hamilton, ON
L8S 4L9

Tel: (905) 525-9140 x24765
Fax: (905) 540-8019
e-mail: grntoff@mcmaster.ca
http://www.mcmaster.ca/ors/

OR

You may also contact the Hamilton Health Science Patient Relations Specialist at 905-521-2100 ext. 75240

INFORMATION

You will be able to contact Dr. Stuart. Phillips at 905-525-9140 (x24465 or x27037) or 905-524-1262 regarding any questions about the study.

I HAVE READ AND UNDERSTAND THE ABOVE EXPLANATION OF THE PURPOSE AND PROCEDURES OF THE PROJECT. I HAVE ALSO READ AND UNDERSTOOD THE ATTACHED FORM ENTITLED "DESCRIPTION OF MEDICAL PROCEDURES" AND COMPLETED THE ATTACHED FORM ENTITLED "SUBJECT SCREENING QUESTIONNAIRE" AND AGREE TO PARTICIPATE AS A SUBJECT. I HAVE ALSO RECEIVED A COPY OF THE INFORMATION AND CONSENT FORM. MY QUESTIONS HAVE BEEN ANSWERED TO MY SATISFACTION AND I AGREE TO PARTICIPATE IN THIS STUDY. I HAVE RECEIVED A SIGNED COPY.

______________________________ ______________________

SIGNATURE DATE

____

PRINTED NAME OF WITNESS

______________________________ ______________________

WITNESS DATE

____

PRINTED NAME OF WITNESS

INVESTIGATOR

In my judgment the participant in voluntarily and knowingly giving informed consent and possesses the legal capacity to give informed consent and participate in this research study.

______________________________ ________________________

SIGNATURE OF INVESTIGATOR DATE

EXERCISE METABOLISM RESEARCH GROUP
DEPARTMENT OF KINESIOLOGY, MCMASTER UNIVERSITY

SUBJECT SCREENING QUESTIONNAIRE

Your responses to this questionnaire are confidential and you are asked to complete it for your own health and safety. If you answer "YES" to any of the following questions, please give additional details in the space provided and discuss the matter with one of the investigators. You may refuse to answer any of the following questions.

Name:______________________ Date: ______________________

1. Have you ever been told that you have a 'heart problem'?

   YES NO

2. Have you ever been told that you have a breathing problem, such as asthma?

   YES NO

3. Have you ever been told that you sometimes experience seizures?

   YES NO

4. Have you ever had any major joint instability or ongoing chronic pain such as in the knee or back?

   YES NO

5. Have you ever been told that you have 'kidney problems'?

   YES NO

6. Have you had any allergies to any medications (including 'over-the-counter' medicines such as aspirin or Tylenol™)?

YES NO

7. Have you had any allergies to food (fish or nuts) or environmental factors (dust, pollen, or mold)?

YES NO

8. Have you had any stomach problems, such as ulcers?

YES NO

9. When you experience a cut do you take a long time to stop bleeding?

YES NO

10. When you receive a blow to your muscle, do you develop bruises easily?

YES NO

11. Are you currently taking any medication (including aspirin) or have you taken any medication in the last two days?

YES NO

12. Is there any medical condition with which you have been diagnosed and are under the care of a physician (e.g. diabetes, high blood pressure)?

YES NO

13. Have you previously participated in a study under the supervision of Dr.'s Stuart Phillips, Mark Tarnopolsky, or Martin Gibala that involved having muscle biopsies taken?

YES NO

EXERCISE METABOLISM RESEARCH GROUP
DEPARTMENT OF KINESIOLOGY, MCMASTER UNIVERSITY

DESCRIPTION OF MEDICAL PROCEDURES

The study in which you are invited to participate involves three procedures which require medical involvement: muscle biopsy sampling, stable isotope-labeled amino acid infusion, and venous blood sampling. Prior to any involvement, you are asked to read this form, which outlines the potential medical risks inherent to these procedures. In addition, you must also complete the "Subject Screening Questionnaire" which is designed to identify any medical reason, which might preclude your participation as a subject.

NOTE: If you have participated in another research study conducted by Dr. Stuart Phillips, Dr. Martin Gibala, or Dr. Mark Tarnopolsky that has involved muscle biopsies then you will not be allowed to participate in this study.

Muscle Biopsy

This procedure involves the removal of a small piece of muscle tissue using a sterile hollow needle. A medical doctor will clean an area over your quadriceps muscle (vastus lateralis) and inject a small amount of local anesthetic ("freezing") into and under the skin. He will then make a small incision (~4-5 mm) in the skin in order to create an opening through which to put the biopsy needle into your thigh. There is a small amount of bleeding from the incision, but this is minimal. He will then quickly cut off a very small piece of muscle (~50-100 mg; about the size of the eraser on the end of a pencil) and remove the needle from your leg. During the time that the sample is being taken (~5 sec), you may feel the sensation of deep pressure in your thigh and on some occasions this is moderately painful. However, the discomfort very quickly passes and you are quite capable of performing exercise and daily activities.

Following the biopsies, the incisions will be closed with sterile suture (stitch), and wrapped with a tensor bandage. You should refrain from excessive muscle use for the remainder of the day. Once the anesthetic wears off, your leg may feel tight and often there is the sensation of a deep bruise or "Charlie Horse". Analgesics (pain killers) such as Tylenol or Ibuprofen (Motrin) are acceptable if you experience significant pain associated with the biopsy. It is also beneficial to periodically apply an ice pack to the biopsy site the following day, as this will help to reduce any swelling and any residual soreness. The following day your leg may feel uncomfortable when going down stairs. The tightness in the muscle

usually disappears within 2 days and subjects routinely begin exercising at normal capacity within a day. In order to allow the incisions to heal properly and minimize any risk of infection, you should avoid prolonged submersion in water for 2-3 days. Daily showers are acceptable, but baths, swimming, saunas, etc. should be avoided for at least 4 days following the biopsy procedure.

Potential Risks. The biopsy technique is routinely used in physiological research, and complications are rare provided that proper precautions are taken. However, there is a risk of internal bleeding at the site of the biopsy, which can result in bruising and temporary discoloration of the skin. On occasion a small lump may form under the site of the incision, but this normally disappears within 2-3 months. As with any incision there is also a slight risk of infection, however this risk is virtually eliminated through proper cleansing of the area and daily changing of wound coverings. If the incision does not heal within a few days or you are in any way concerned about inflammation or infection, please contact us immediately. In very rare occasions there can be damage to a superficial sensory nerve, which will result in temporary numbness in the area. There is also an extremely remote chance (1 in 1,000,000) that you will be allergic to the local anesthetic.

In past experience with healthy young subjects, approximately 1 in 3000 have experienced a local skin infection; 1 in 500 have experienced a small lump at the site of the biopsy (in all cases this disappeared within ~2-3 wk using local massage); 1 in 1,500 have experienced a temporary loss of sensation in the skin at the site of incision (an area of numbness about the size of a quarter which lasted up to 3 months), and 1 in 30 have experienced bruising around the site of incision which lasted for ~4-5 days. While there is also a theoretical risk of damage to a small motor nerve (that is used to allow your muscle to move) branch of the medial vastus lateralis, this has never been seen in over 9,500 biopsies performed at McMaster University in the IWC. Hence, the risk of damaging a small motor nerve branch is impossible to estimate.

Catheterization and Venous Blood Sampling

A small plastic catheter will be inserted into a forearm vein by a physician or a medically trained and certified member of the laboratory group. The catheter will be inserted with the assistance of a small needle, which is subsequently removed. The discomfort of this procedure is transient and is very similar to having an injection by a needle, or when donating blood. Once the needle is removed there should be no sensation from the catheter. During the course of the experiment, blood will be drawn periodically from the catheter. In any this experiment the total blood taken is ~120 ml, which is approximately 1/4 of the blood removed during a donation to a blood bank. It is not enough of a blood loss to affect your physical performance in any way. After each blood sample

has been taken, the catheter is "flushed" with a sterile saline solution in order to prevent blood from clotting in the catheter. This is a salt solution that is very similar in composition to your own blood and it will not affect you. Following removal of the catheter, pressure will be placed on the site in order to minimize bleeding and facilitate healing.

Potential Risks. The insertion of catheters for blood sampling is a common medical practice and involves few risks if proper precautions are taken. The catheters are inserted under completely sterile conditions, however there is a theoretical risk of infection. There is a chance of internal bleeding if adequate pressure is not maintained upon removal of the catheter. This may cause some minor discomfort and could result in bruising/skin discoloration, which could last up to a few weeks. In very rare occasions (less than 1 in 1 000 000), trauma to the vessel wall could result in the formation of a small blood clot, which could travel through the bloodstream and become lodged in a smaller vessel. However, we have never experienced such a complication after several thousand catheter placements.

Stable isotope-labeled amino acid infusion

You will receive, through a small catheter placed in your arm, an infusion (slow measured amount) of an amino acid (a small component of protein) solution. The amino acid will be dissolved in saline (a salt solution similar to your blood). The amino acid will be labeled with a stable isotope of carbon, hydrogen, or nitrogen. An isotope is slightly heavier form of these elements. Since the isotope is stable (i.e., non-radioactive) it poses no health risk to you due to radioactive exposure. Also, a certain fraction of all of the carbon, hydrogen, and nitrogen within your body is already in the same form as that of the stable isotope. Hence, the infusion of the stable isotope-labeled amino acid will simply result in a slight increase in the amount of stable isotope within your body, we refer to this as "enriching" the amount of stable isotope within your body. This enrichment will not remain high, however, and will be back to pre-infusion levels within 24 hours. All of the infused solutions are prepared under sterile conditions and are filtered through a very selective filter prior to entering your body. All solutions that enter your body do not contain, except for the amino acid, anything that will affect your health.

Potential Risks. Despite all precautions there is a theoretical risk (less than 1 in 1,000,000) that you could have a rapid drop in blood pressure due to some small bacterial contamination of the infusate. This has never occurred in our experience.

APPENDIX 2

SUBJECTS' PHYSICAL CHARACTERISTICS

Subject Data

	Weight (kg)	Height (cm)	Height (m)	BMI (kg/m2)	Age (yrs)	Dominant Leg	Trained Leg
S1	89.5	172	1.72	30.3	23	right	right
S2	85.2	177	1.77	27.2	22	right	left
S3	63.2	169.5	1.695	22.0	19	right	right
S4	83.5	176	1.76	27.0	23	left	right
S5	117	181	1.81	35.7	20	right	left
S6	82.6	180	1.8	25.5	19	left	right
S7	70	187	1.87	20.0	21	right	right
S8	70.9	170	1.7	24.5	22	left	left
S9	87.3	194	1.94	23.2	21	left	left
S10	78.1	182	1.82	23.6	20	right	left
average	82.73	178.85	1.79	25.89	21.00		
std	14.73	7.72	0.08	4.50	1.49		
std error	4.658	2.440	0.024	1.424	0.471		

APPENDIX 3

1RM STRENGTH MEASUREMENTS AND ANOVA TABLE

ISOKINETIC STRENGTH MEASUREMENTS AND ANOVA TABLE

ISOMETRIC STRENGTH MEASUREMENTS AND ANOVA TABLE

Isotonic Strength Measurements (1RM – knee extension)

	Trained (kg)			Untrained (kg)		
	Pre	Mid	Post	Pre	Mid	Post
S1	55	65	70	55	55	55
S2	40	70	90	40	50	60
S3	35	50	56	35	40	41
S4	45	55	71	45	45	51
S5	50	66	75	50	56	60
S6	50	56	75	50	50	65
S7	45	60	70	45	50	50
S8	45	65	75	45	50	55
S9	45	60	65	45	50	51
S10	30	50	56	30	35	36
average	44.0	59.7	70.3	44.0	48.1	52.4
std	7.38	6.88	9.96	7.38	6.45	8.80
stderror	2.33	2.18	3.15	2.33	2.04	2.78

Summary of all Effects; design: (strength.sta)
1-TRAINED, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	1450.417	9	13.97222	103.8072	0.00000306
2	2	1515.117	18	34.11666	44.40987	0.00000011
12	2	412.2167	18	4.327778	95.24904	0.00000000

Tukey HSD test; variable Var.1 (strength.sta)
Probabilities for Post Hoc Tests
INTERACTION: 1 x 2

	T - pre	**T - mid**	**T - post**	**UT - pre**	**UT - mid**	**UT - post**
	44.00000	**48.10000**	**52.40000**	**44.00000**	**59.70000**	**70.30000**
T – pre		0.003989	0.000158	1	0.000158	0.0001577
T – mid	0.003989		0.002572	0.003989	0.000158	0.0001577
T – post	0.000158	0.002572		0.000158	0.000159	0.0001577
UT – pre	1	0.003989	0.000158		0.000158	0.0001577
UT – mid	0.000158	0.000158	0.000159	0.000158		0.0001577
UT - post	0.000158	0.000158	0.000158	0.000158	0.000158	

Isokinetic Strength Measurements

Concentric 5.2 rad/s

	Trained (N·m)		Untrained (N·m)	
	Pre	**Post**	**Pre**	**Post**
S1	132	173	161	179
S2	164	153	184	180
S3	115	163	104	118
S4	175	169	158	152
S5	161	187.9	179	161.8
S6	162	160	181	138
S7	170	150	173	155
S8	158	140	152	152
S9	123	166	107	131
S10	149	168	161	176
average	150.9	163.0	156.0	154.3
std	20.60	13.32	28.68	20.91
stderror	6.52	4.21	9.07	6.61

NO EFFECT

Summary of all Effects; design: (slow torque.sta)

1-CONDI, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	32.5802498	9	299.2024841	0.10889	0.748962
2	1	268.842255	9	448.631134	0.59925	0.458716
12	1	476.790253	9	115.4902496	4.128403	0.072717

Isokinetic Strength Measurements

Concentric 0.52 rad/s

	Trained (N·m)		Untrained (N·m)	
	Pre	**Post**	**Pre**	**Post**
S1	248	279	256	221
S2	235	298	231	242
S3	172	181	180	150
S4	228	313	259	268
S5	273	292.5	266	309
S6	250	277	268	302
S7	258	273	233	215
S8	244	269	260	265
S9	183	199	216	228
S10	181	278	216	272
average	227.2	266.0	238.5	247.2
std	35.71	42.35	28.55	46.85
stderror	11.29	13.39	9.03	14.82

Summary of all Effects; design: (strength.sta)
1-TRAIN, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	138.7563	9	553.5341	0.250673	0.628616
2	1	5628.756	9	693.9785	8.110851	0.019153
12	1	2257.506	9	253.1729	8.916855	0.015297

Tukey HSD test; variable Var.1 (strength.sta)
Probabilities for Post Hoc Tests
INTERACTION: 1 x 2

	T-pre	**t-post**	**ut-pre**	**ut-post**
	227.2000	**265.9500**	**238.5000**	**247.2000**
T –pre		0.002031	0.430968	0.079891
T- post	0.002031		0.016814	0.103787
UT – pre	0.430968	0.016814		0.629136
UT - post	0.079891	0.103787	0.629136	

Isometric Strength Measurements

	Trained (N)		Untrained (N)	
	Pre	Post	Pre	Post
S1	320	327	301	325
S2	357	400	344	291
S3	229	243	234	212
S4	265	326	252	300
S5	320	351	328	312.3
S6	315	382	323	342
S7	284	324	306	285
S8	268	351	372	296
S9	268	304	279	272
S10	219	315	235	256
average	284.5	332.3	297.4	289.1
std	43.46	43.42	46.82	36.73
Std error	13.74	13.73	14.81	11.62

Summary of all Effects; design: (slow work.sta)
1-TRAIN, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	2287.656	9	425.1563	5.380743	0.045512
2	1	3902.6	9	554.4336	7.038896	0.026342
12	1	7854.006	9	569.3396	13.79494	0.004814

Tukey HSD test; variable Var.1 (slow work.sta)
Probabilities for Post Hoc Tests
INTERACTION: 1 x 2

	T - pre 284.5000	T - post 332.2800	UT - pre 297.4000	UT - post 289.1300
T –pre		0.006991	0.636935	0.971156
T- post	0.006991		0.040192	0.012862
UT – pre	0.636935	0.040192		0.863892
UT - post	0.971156	0.012862	0.863892	

APPENDIX 4

HISTOCHEMISTRY RAW DATA AND ANOVA TABLE

HISTOCHEMICAL IMAGES

Mean Muscle Fibre Cross Sectional Area Raw Data

	PRE		Total Fibres	POST UT		Total Fibres	POST T		Total Fibres
	Type I	Type II		Type I	Type II		Type I	Type II	
S1	5354	6787	99	#DIV/0!	#DIV/0!	0	5266	6969	171
S2	3717	4464	425	2732	4563	162	5484	6390	224
S3	3822	5586	195	4068	5097	231	3738	5203	285
S4	5514	4281	229	5306	5555	97	6221	5183	187
S5	4018	5232	262	#DIV/0!	#DIV/0!	0	2451	3954	323
S6	5400	5893	131	3102	3089	305	8252	7279	104
S7	7046	6632	204	5028	6806	227	7582	10336	70
S8	3632	5871	320	4576	7423	137	4358	6269	131
S9	4866	6328	97	4963	7075	120	4459	7102	15
S10	4082	5318	137	5034	7636	240	3958	4963	358
average	4745	5639	210	4351	5906	190	5177	6365	187
std dev	1136	886		#DIV/0!	#DIV/0!		1836	1798	
std error	359	280		#DIV/0!	#DIV/0!		580	569	

Mean Fibre Cross-Sectional Area (μm^2)

Type I	PRE	POST T
	3717	5484
	3822	3738
	5514	6221
	5400	8252
	7046	7582
	3632	4358
	4082	3958
Mean	4745	5656
SD	1285	1782
SE	486	673

t-Test: Paired Two Sample for Means — n=7

	Variable 1	Variable 2
Mean	4744.8453	5656.150133
Variance	1650269	3173883.465
Observations	7	7
Pearson Correlation	0.8072739	
Hypothesized Mean Difference	0	
df	6	
t Stat	-2.26909	
P(T<=t) one-tail	0.0318737	
t Critical one-tail	1.9431809	
P(T<=t) two-tail	0.0637474	
t Critical two-tail	2.4469136	

Type I	PRE	POST UT
	3717	2732
	3822	4068
	5514	5306
	5400	3102
	7046	5028
	3632	4576
	4082	5034
Mean	4745	4264
SD	1285	1008
SE	486	381

t-Test: Paired Two Sample for Means — n=7

	Variable 1	Variable 2
Mean	4744.8453	4263.570541
Variance	1650269	1015924.833
Observations	7	7
Pearson Correlation	0.3471394	
Hypothesized Mean Difference	0	
df	6	
t Stat	0.9578457	
P(T<=t) one-tail	0.1875643	
t Critical one-tail	1.9431809	
P(T<=t) two-tail	0.3751285	
t Critical two-tail	2.4469136	

Type

t-Test: Paired Two Sample for Means — n=7

II	PRE	POST T
	4464	6390
	5586	5203
	4281	5183
	5893	7279
	6632	10336
	5871	6269
	5318	4963
Mean	5435	6518
SD	831	1878
SE	314	710

	Variable 1	*Variable 2*
Mean	5434.8356	6517.561055
Variance	690860.65	3527793.471
Observations	7	7
Pearson Correlation	0.6897476	
Hypothesized Mean Difference	0	
df	6	
t Stat	-1.993438	
P(T<=t) one-tail	0.0466353	
t Critical one-tail	1.9431809	
P(T<=t) two-tail	0.0932706	
t Critical two-tail	2.4469136	

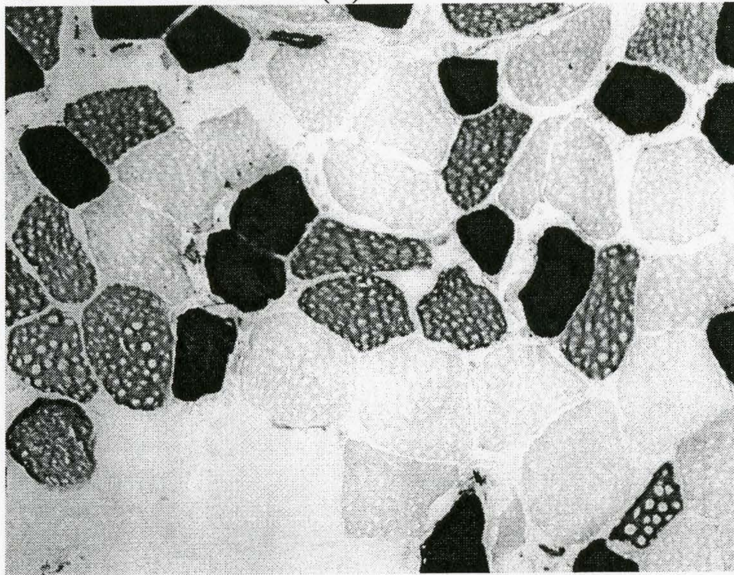
t-Test: Paired Two Sample for Means

n=7

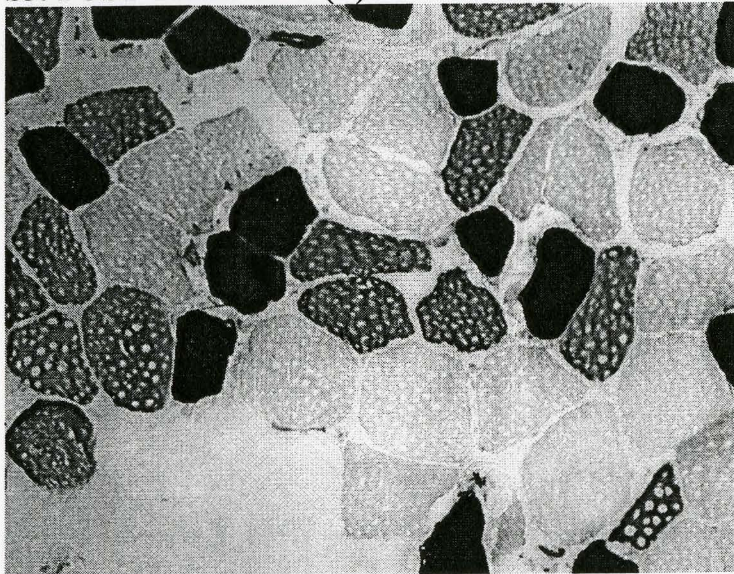
Type II	PRE	POST UT
	4464	4563
	5586	5097
	4281	5555
	5893	3089
	6632	6806
	5871	7423
	5318	7075
Mean	5435	5658
SD	831	1558
SE	314	589

	Variable 1	*Variable 2*
Mean	5434.8356	5658.400691
Variance	690860.65	2427463.612
Observations	7	7
Pearson Correlation	0.2444537	
Hypothesized Mean Difference	0	
df	6	
t Stat	-0.375209	
P(T<=t) one-tail	0.3602054	
t Critical one-tail	1.9431809	
P(T<=t) two-tail	0.7204108	
t Critical two-tail	2.4469136	

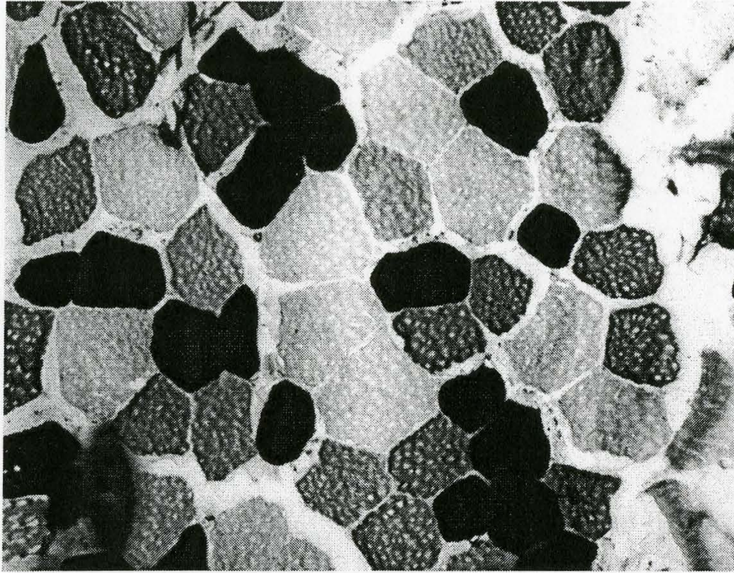
S3 – PRE-TRAINING (T)



S3: POST TRAINING (T)



S3: POST TRAINING (UT)



APPENDIX 5

DAILY CALORIC INTAKE AND COMPOSITION OF FEEDING DURING TESTING SESSIONS

Harris-Benedict Equation

BMR = 66 + [13.7 * weight (kg)] + [5 * height (cm)] – [6.8 * age (yrs)]
= X
= X * 1.7 (moderate activity factor)

Rest and 24 hrs post-exercise

- subjects to receive ~35% of their daily caloric intake

Immediately post-exercise

- subjects to receive ~50% of their daily caloric intake

BOOST

- per can (237 ml)

- 240 kCal
- 41 g CHO
- 10 g PRO
- 4 g Fat

Caloric Intake During Infusion Sessions

REST - ~35% of Predicted Daily Caloric Intake

	Predicted Total Caloric Intake	Caloric Intake During Session	g CHO	g PRO	g FAT	Cans of Boost
S1	3393	1200	205	50	20	5
S2	3346	1200	205	50	20	5
S3	2805	960	164	40	16	4
S4	3287	1200	205	50	20	5
S5	4133	1440	246	60	24	6
S6	3346	1200	205	50	20	5
S7	3038	960	164	40	16	4
S8	2954	960	164	40	16	4
S9	3552	1200	205	50	20	5
S10	3247	960	164	40	16	4
average	3310.1	1128.0	192.7	47.0	18.8	4.7
std dev	366.0	162.0	27.7	6.7	2.7	0.7

POST-EXERCISE- ~50% of Predicted Daily Caloric Intake

	Predicted Total Caloric Intake	Caloric Intake During Session	g CHO	g PRO	g FAT	Cans of Boost
S1	3393	1680	287	70	28	7
S2	3346	1680	287	70	28	7
S3	2805	1440	246	60	24	6
S4	3287	1680	287	70	28	7
S5	4133	1920	328	80	32	8
S6	3346	1680	287	70	28	7
S7	3038	1440	246	60	24	6
S8	2954	1440	246	60	24	6
S9	3552	1680	287	70	28	7
S10	3247	1440	246	60	24	6
average	3310.1	1608.0	274.7	67.0	26.8	6.7
std dev	366.0	162.0	27.7	6.7	2.7	0.7

24 hr POST -EXERCISE- ~35% of Predicted Daily Caloric Intake

	Predicted Total Caloric Intake	Caloric Intake During Session	g CHO	g PRO	g FAT	Cans of Boost
S1	3393	1200	205	50	20	5
S2	3346	1200	205	50	20	5
S3	2805	960	164	40	16	4
S4	3287	1200	205	50	20	5
S5	4133	1440	246	60	24	6
S6	3346	1200	205	50	20	5
S7	3038	960	164	40	16	4
S8	2954	960	164	40	16	4
S9	3552	1200	205	50	20	5
S10	3247	960	164	40	16	4
average	3310.1	1128.0	192.7	47.0	18.8	4.7
std dev	366.0	162.0	27.7	6.7	2.7	0.7

Summary of all Effects; design: (fsr.sta)

1-CONDITIO, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	2	0.386	18	1.142	0.338	0.717
2	3	1.998	27	0.86	2.322	0.098
12	6	0.289	54	0.651	0.444	0.846

	REST				POST EXERCISE					24 hrs POST EXERCISE			
	0	1.5	3	4.5	0	2	3.5	5	6.5	0	1.5	3	4.5
S1	7	16	30	27	12	24	25	30	33	12	20	40	29
S2	3.2	24.8	33.8	17.8	5.7	21.7	14.8	8.8	22.2	3.9	31.2	25.3	26.2
S3	3	16	23.9	15.2	12.1	19.2	14.6	25.1	13.5	5.3	11.4	4.9	13.2
S4	6.4	45.6	62.8	63.1	7.8	55.1	70.1	64	48.5	6.9	29.4	44.9	39.6
S5	9.6	24	49.7	29	7.1	35.6	41	62.4	28.5	10.5	72.8	65.4	65.7
S6	6.5	22.7	29.1	18.8	5.4	22.4	19.9	22.9	27.1	7.4	21.8	20.8	29.6
S7	4.5	6.3	28.4	10.9	4	10.2	11.7	14.7	4.7	3.6	14.6	19	8.7
S8	3	6	7.4	9.2	4.1	7.7	7	6	3.8	3.6	9	11.8	16.2
S9	4.4	9.8	9.1	9.6	6.1	18	8.7	19.3	12.9	4.8	21.9	14.3	19.1
S10	16.8	39.5	38.2	55.8		51.5	48.4	45.7	59	10.4	42.6	54.8	47.5
average	6.5	21.1	31.3	25.6	7.1	26.5	26.1	29.8	25.3	6.8	27.5	30.1	29.5
std	4.2	13.3	16.7	19.1	3.0	16.1	20.6	20.8	18.0	3.1	18.8	20.1	17.4
se	1.334	4.2062	5.2889	6.0437	0.9938	5.0758	6.5237	6.5834	5.704	0.978	5.952	6.3469	5.5087

Summary of all Effects; design: (insulin.sta)

1-CONDITIO, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F
1	2	12113	18	21364	0.567
2	3	343105	27	20011	17.145
12	6	7237.4	54	7422.2	0.9751

Tukey HSD test; variable Var.1 (insulin.sta)
Probabilities for Post Hoc Tests
MAIN EFFECT: TIME

	{1}	{2}	{3}	{4}
	68.39167	248.6667	302.9333	281.1333
.... 1 {1}		0.0003	0.0002	0.0002
.... 2 {2}	3E-04		0.4597	0.8107
.... 3 {3}	2E-04	0.4597		0.9322
.... 4 {4}	2E-04	0.8107	0.9322	

APPENDIX 7

PLASMA AMINO ACID CONCENTRATION RAW DATA AND ANOVA TABLE

Total Amino acid concentration (µM)

SUBJECT	REST			POST EXERCISE			24 hrs POST EXERCISE		
	0	1.5	4.5	0	3.5	6.5	0	1.5	4.5
S1	4692.7	5615.4	5539.4	4256.4	5410.2	5885.1	4877.4	5022.1	5235.7
S2	5630.1	6509.2	5619.2	5010.7	5493.0	5475.6	4813.0	5168.2	5158.0
S3	3858.3	4964.4	4592.6	4991.8	5776.6	5194.6	4197.9	4398.3	5005.1
S4	3118.0	5459.4	5213.4	5125.7	6140.2	5914.8	4564.4	5234.2	4901.8
S5	5752.7	5651.0	5295.1	4375.7	6416.9	6370.2	4041.2	5399.3	4543.3
S6	4224.1	5770.3	6579.7	5396.2	6186.1	5732.0	4154.7	4988.7	4983.8
S7	5127.0	4832.8	4574.3	5035.2	4248.0	4215.2	4049.2	4110.4	4761.4
S8	2646.0		4847.8	5407.6	5221.0	5396.9	4063.5	6188.4	4822.3
S9	3035.1	4302.2	4865.0	4846.7	4847.8	4838.3	3176.6	4274.6	3831.8
S10	4507.9	5995.2	4571.9	5387.9	5668.9	6099.1	4878.7	4739.0	5284.4
average	4259.19	5455.55	5169.83	4983.38	5540.85	5512.16	4281.65	4952.32	4852.78
std	1089.23	663.66	631.22	401.97	657.32	639.61	524.64	612.88	423.78
se	344.4439	221.2196	199.6098	127.1135	207.8614	202.2622	165.9053	193.8091	134.0124

Summary of all Effects; design: (aa.sta)
1-CONDITIO, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	2	3202417	18	394952	8	0.00309
2	2	5606539	18	264663	21	0.00002
12	4	296098	36	284057	1	0.39896

Tukey HSD test; variable Var.1 (aa.sta)
Probabilities for Post Hoc Tests
MAIN EFFECT: **TIME**

	{1} 4508.076	{2} 5316.238	{3} 5178.258
Rest		0.000168	0.000360
Post-ex	0.000168		0.562924
24hr post-ex	0.000360	0.562924	

Tukey HSD test; variable Var.1 (aa.sta)
Probabilities for Post Hoc Tests
MAIN EFFECT: **CONDITIO**

	{1} 4961.525	{2} 5345.465	{3} 4695.582
Rest		0.07199	0.25566
Post-ex	0.07199		0.00238
24hr post-ex	0.25566	0.00238	

Essential Amino acid concentration (µM)

SUBJECT	REST			POST EXERCISE			24 hrs POST EXERCISE		
	0	1.5	4.5	0	3.5	6.5	0	1.5	4.5
S1	1612.7	1920.1	1904.7	1332.8	1941.8	2057.1	1566.0	1704.0	1919.0
S2	2077.8	2436.7	2079.4	1834.6	2080.3	1870.3	1679.0	1869.9	1906.8
S3	1402.4	1747.5	1547.3	1606.0	1936.6	1641.7	1337.6	1440.2	1651.8
S4	1176.0	2340.4	2329.2	1968.3	2622.2	2295.2	1764.1	2185.6	2056.0
S5	2207.8	2216.0	2080.9	2337.7	2529.3	1722.4	1592.0	2137.5	1788.8
S6	1359.0	1889.0	2151.1	1669.4	1996.8	1806.3	1418.6	1646.7	1690.2
S7	1646.6	1634.7	1519.0	1563.6	1462.6	1347.9	1407.9	1454.3	1681.0
S8	1549.4		1708.5	1807.4	1891.3	1930.2	1397.3	2153.9	1645.6
S9	1116.8	1607.0	1778.3	1763.6	1773.7	1773.2	1181.5	1653.4	1498.1
S10	1586.3	2133.8	1645.5	2131.6	2034.9	2214.7	1681.4	1769.2	1949.0
average	1573.49	1991.71	1874.41	1801.49	2026.95	1865.91	1502.54	1801.47	1778.64
std	350.58	304.33	277.16	290.38	337.80	278.49	182.85	278.27	173.86
se	110.8644	101.442	87.64563	91.8247	106.8233	88.06579	57.82096	87.99623	54.97802

Summary of all Effects; design: (new.sta)
1-CONDITIO, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	2	314722	18	40443	8	0.00367
2	2	772584	18	58774	13	0.00030
12	4	52243	36	32042	2	0.18780

Tukey HSD test; variable Var.1 (new.sta)
Probabilities for Post Hoc Tests
MAIN EFFECT: **CONDITION**

	{1} 1813.203	{2} 1898.119	{3} 1694.217
Rest		0.25706	0.08294
Post-ex	0.25706		0.00281
24hr post-ex	0.08294	0.00281	

Tukey HSD test; variable Var.1 (new.sta)
Probabilities for Post Hoc Tests
MAIN EFFECT: **TIME**

	{1} 1625.842	{2} 1940.044	{3} 1839.655
Rest		0.00037	0.00833
Post-ex	0.00037		0.26975
24hr post-ex	0.00833	0.26975	

Branched Chain Amino acid concentration (μM)

	REST			POST EXERCISE			24 hrs POST EXERCISE		
	0	1.5	4.5	0	3.5	6.5	0	1.5	4.5
S1	848	994	1018	699	1000	1088	824	894	1056
S2	1091.2	1330.7	1094.9	919.3	1075.7	958.7	835.9	960.1	1017
S3	768.6	948.2	785.2	775.4	958.5	817.8	642.1	705.8	821
S4	612.1	1171.4	1209.0	924.7	1337.3	1160.9	885.0	1128.2	1097
S5	1053.7	1061.2	1014.4	1262.5	1229.6	850.8	783.0	1125.9	919
S6	672	890	1020	777	918	872	716	806	832
S7	867.4	755.4	729.0	733.4	737.1	641.9	653.7	691.3	818
S8	734.2		751.8	825.3	845.7	851.2	622.2	974.3	747
S9	505.3	731.1	801.4	859.0	855.2	843.0	555.6	772.6	730
S10	793.6	1078.3	843.6	1162.0	1077.5	1128.1	807.4	887.0	956
average	794.56	995.57	926.74	893.83	1003.40	921.19	732.47	894.52	899.31
std	182.45	192.15	165.01	184.53	182.74	162.13	109.80	155.80	129.15
se	57.6945	64.0506	52.1793	58.353	57.787	51.2715	34.722	49.26736	40.84173

Summary of all Effects; design: (aa.sta)
1-CONDITIO, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	2	73308	18	13532	5	0.01439
2	2	195156	18	18741	10	0.00099
12	4	15957	36	8953	2	0

Tukey HSD test; variable Var.1 (aa.sta)
Probabilities for Post Hoc Tests
MAIN EFFECT: **CONDITIO**

	{1}	{2}	{3}
	905.6251	939.4722	842.1027
1 .... {1}		0.51053	0.114905
2 .... {2}	0.510531		0.012082
3 .... {3}	0.114905	0.012082	

Tukey HSD test; variable Var.1 (aa.sta)
Probabilities for Post Hoc Tests
MAIN EFFECT: **TIME**

	{1}	{2}	{3}
	806.9559	964.4971	915.7469
.... 1 {1}		0.00095	0.01707
.... 2 {2}	0.00095		0.37231
.... 3 {3}	0.01707	0.37231	

APPENDIX 8

MIXED MUSCLE FRACTIONAL SYNTHESIS RATE RAW DATA AND ANOVA TABLE

Mean blood enrichment.

SUBJECT	REST			
	0	1.5	3	4.5
S1	0.009044	0.223862	0.233628	0.172197
S2	0.009407	0.121230	0.165154	0.137355
S3	0.000000	0.089498	0.097200	0.102387
S4	0.000000	0.114404	0.127631	0.135896
S5	0.000000	0.110013	0.133639	0.125078
S6	0.009054	0.157008	0.183468	0.123757
S7	0.000000	0.106305	0.106384	0.119633
S8	0.000000	0.088349	0.118764	0.105736
S9	0.000000	0.091981	0.102635	0.096326
S10	0.000000	0.090851	0.114277	0.113730
average	0.002750	0.119350	0.138278	0.123209
std	0.004430	0.042216	0.043363	0.021931
se	0.001401	0.01335	0.013712	0.006935

SUBJECT	POST EXERCISE				
	0	2	3.5	5	6.5
S1	0.000000	0.000000	0.101711	0.130672	0.122614
S2	0.000000	0.000000	0.091838	0.113340	0.126217
S3	0.007930	0.008986	0.108333	0.126993	0.085907
S4	0.000000	0.000000	0.080271	0.112407	0.104624
S5	0.007865	0.000000	0.139018	0.202689	0.151991
S6	0.000000	0.000000	0.083077	0.093415	0.096920
S7	0.008182	0.008666	0.128272	0.110146	0.160083
S8	0.000000	0.000000	0.080876	0.104115	0.097132
S9	0.000000	0.000000	0.111595	0.137971	0.131606
S10	0.009935	0.010317	0.128180	0.154056	0.119166
average	0.003391	0.002797	0.105317	0.128581	0.119626
std	0.004415	0.004522	0.021468	0.031455	0.024184
se	0.001396	0.00143	0.006789	0.009947	0.007648

SUBJECT	24 hr POST EXERCISE			
	0	1.5	3	4.5
S1	0.000000	0.108803	0.111430	0.127429
S2	0.000000	0.114423	0.107982	0.103047
S3	0.000000	0.094793	0.118770	0.114545
S4	0.008261	0.196653	0.180537	0.159371
S5	0.000000	0.110371	0.116106	0.113057
S6	0.000000	0.096091		0.122084
S7	0.000000	0.098681	0.106093	0.132969
S8	0.009764	0.125301	0.148834	0.138714
S9	0.008035	0.130384		0.113739
S10	0.000000	0.067573	0.099747	0.099597
average	0.002606	0.114307	0.123687	0.122455
std	0.004219	0.033906	0.027322	0.017915
se	0.001334	0.010722	0.00966	0.005665

Summary of all Effects; design: (fibre typei t2.sta)

1-CONDITIO, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	2	0.000504	18	0.002162	0.233281	0.794288
2	3	0.107557	27	0.000273	394.4911	2.13E-22
12	6	0.000196	54	0.000328	0.597274	0.731152

Tukey HSD test; variable Var.1 (fibre typei t2.sta)

Probabilities for Post Hoc Tests

MAIN EFFECT: TIME

		{1}	{2}	{3}	{4}
		.0027178	.1129914	.1301820	.1217636
....	1 {1}		0.000167	0.000167	0.000167
....	2				
{2}		0.000167		0.002276	0.192788
....	3				
{3}		0.000167	0.002276		0.22248
....	4				
{4}		0.000167	0.192788	0.22248	

Tukey HSD test; variable Var.1 (fibre typei t2.sta)
Probabilities for Post Hoc Tests
INTERACTION: 1 x 2

			{1} .0027505	{2} .1193500	{3} .1382780	{4} .1232094	{5} .0027969	{6} .1053168	{7} .1285806	{8} .1196260	{9} .0026060	{10} .1143073	{11} .1236873	{12} .1224554
1	1	{1}		0.00012	0.00012	0.00012	1	0.00012	0.00012	0.00012	1	0.0001	0.00012	0.00012
1	2	{2}	0.00012		0.46528	1	0.00012	0.84543	0.99131	1	0.00012	1	0.99999	1
1	3	{3}	0.00012	0.46528		0.77767	0.00012	0.00788	0.98707	0.48788	0.00012	0.1502	0.81044	0.72153
1	4	{4}	0.00012	1	0.77767		0.00012	0.55125	0.99994	1	0.00012	0.9935	1	1
2	1	{5}	1	0.00012	0.00012	0.00012		0.00012	0.00012	0.00012	1	0.0001	0.00012	0.00012
2	2	{6}	0.00012	0.84543	0.00788	0.55125	0.00012		0.1809	0.82857	0.00012	0.993	0.51122	0.61468
2	3	{7}	0.00012	0.99131	0.98707	0.99994	0.00012	0.1809		0.99322	0.00012	0.8308	0.99998	0.99979
2	4	{8}	0.00012	1	0.48788	1	0.00012	0.82857	0.99322		0.00012	0.9999	1	1
3	1	{9}	1	0.00012	0.00012	0.00012	1	0.00012	0.00012	0.00012		0.0001	0.00012	0.00012
3	2	{10}	0.00012	0.99997	0.15017	0.99354	0.00012	0.99299	0.83082	0.99995	0.00012		0.99009	0.99697
3	3	{11}	0.00012	0.99999	0.81044	1	0.00012	0.51122	0.99998	1	0.00012	0.9901		1
3	4	{12}	0.00012	1	0.72153	1	0.00012	0.61468	0.99979	1	0.00012	0.997	1	

IC enrichment

SUBJECT	Condition	TIME			
	REST	T - B1	T - B2	UT - B1	UT - B2
S1	D3-KIC	0.041477	0.039852	0.031821	0.032402
S2	D3-KIC	0.033053	0.032051	0.028611	0.031990
S3	D5-Phe	0.059252	0.081503	0.036865	0.068595
S4	13C6-Phe	0.036043	0.063951	0.049546	0.078387
S5	13C6-Phe	0.063465	0.075291	0.048941	0.090712
S6	D3-KIC	0.022994	0.022994	0.020890	0.034331
S7	D5-Phe	0.056476	0.064597	0.053759	0.060417
S8	13C6-Phe	0.073366	0.087306	0.071808	0.099509
S9	D5-Phe	0.030450	0.079295	0.040044	0.078095
S10	13C6-Phe	0.053722	0.092676	0.061707	0.074363
average		0.047030	0.063951	0.044399	0.064880
std		0.016488	0.024311	0.015711	0.024533
se		0.005213864	0.007687655	0.004968123	0.007758135

SUBJECT	Condition	TIME			
	POST-EX	T - B1	T - B2	UT - B1	UT - B2
S1	13C6-Phe	0.065534	0.075916	0.065360	0.077377
S2	13C6-Phe	0.067287	0.079189	0.047326	0.081583
S3	D3-KIC	0.022460	0.023139	0.019017	0.025045
S4	D5-Phe	0.050606	0.073301	0.039040	0.082176
S5	D3-KIC	0.022375	0.025275	0.025310	0.026687
S6	D5-Phe	0.046511	0.093470	0.066887	0.089990
S7	D3-KIC	0.025339	0.027394	0.024107	0.031494
S8	D5-Phe	0.055762	0.070873	0.052136	0.059532
S9	13C6-Phe	0.094893	0.108885	0.085410	0.103582
S10	D3-KIC	0.027911	0.020730	0.023626	0.028025
average		0.047868	0.059817	0.044822	0.060549
std		0.023975	0.032653	0.022544	0.030225
se		0.007581693	0.010325924	0.007129109	0.00955811

SUBJECT	Condition 24 hr POST-EX	TIME			
		T - B1	T - B2	UT - B1	UT - B2
S1	D5-Phe	0.073314	0.084006	0.042880	0.088597
S2	D5-Phe	0.043962	0.119121	0.030497	0.082372
S3	13C6-Phe	0.051136	0.066897	0.045190	0.066144
S4	D3-KIC	0.040233	0.038186	0.030776	0.036650
S5	D5-Phe	0.044988	0.074769	0.067002	0.076200
S6	13C6-Phe	0.077642	0.080629	0.072482	0.081941
S7	13C6-Phe	0.058269	0.081476	0.067382	0.103582
S8	D3-KIC	0.035341	0.052109	0.035370	0.038663
S9	D3-KIC	0.028924	0.047620	0.035123	0.049840
S10	D5-Phe	0.040781	0.058927	0.063802	0.081327
average		0.049459	0.070374	0.049050	0.070532
std		0.015900	0.023191	0.016790	0.022233
		0.005028164	0.007333566	0.005309517	0.007030599

Summary of all Effects; design: (ic enrichment.sta)
1-TRAIN, 2-CONDITON, 3-PREPOST

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	4.58483E-05	9	0.000299479	0.153093308	0.704699
2	2	0.000461413	18	0.002608299	0.176901996	0.839301
3	1	0.011832451	9	0.000153723	76.97267914	1.05E-05
12	2	0.000108402	18	0.000156441	0.692923784	0.512967
13	1	0.000319718	9	0.0001602	1.995745182	0.191376
23	2	0.000299943	18	0.000353564	0.848343253	0.444543
123	2	0.000148828	18	0.000162721	0.914623737	0.418501

Tukey HSD test; variable Var.1 (ic enrichment.sta)
Probabilities for Post Hoc Tests
MAIN EFFECT: PREPOST

	{1} .0471047	{2} .0669646
Biopsy 1		0.000214279
Biopsy 2	0.000214279	

Bound enrichment

SUBJECT	Condition	TIME			
	R	T - B1	T - B2	UT - B1	UT - B2
S1	D3-KIC	0.006841	0.006909	0.007071	0.007103
S2	D3-KIC	0.006758	0.006803	0.006759	0.006805
S3	D5-Phe	0.000278	0.000377	0.000336	0.000389
S4	13C6-Phe	0.000181	0.000602	0.000309	0.007005
S5	13C6-Phe	0.000092	0.000219	0.000078	0.000223
S6	D3-KIC	0.006692	0.006728	0.006764	0.006804
S7	D5-Phe	0.000368	0.000424	0.000365	0.000463
S8	13C6-Phe	0.000082	0.000238	0.000086	0.000204
S9	D5-Phe	0.000168	0.000214	0.000233	0.000286
S10	13C6-Phe	0.000038	0.000149	0.000067	0.000167
average		0.002150	0.002266	0.002207	0.002945
std		0.003185	0.003141	0.003217	0.003431
se		0.001007257	0.000993148	0.00101734	0.001085059

SUBJECT	Condition	TIME			
	Ex1	T - B1	T - B2	UT - B1	UT - B2
S1	13C6-Phe	0.000095	0.000358	0.000088	0.000221
S2	13C6-Phe	0.000078	0.000358	0.000075	0.000308
S3	D3-KIC	0.006956	0.007049	0.006945	0.007005
S4	D5-Phe	0.000116	0.000312	0.000144	0.000308
S5	D3-KIC	0.006884	0.006981	0.006731	0.006794
S6	D5-Phe	0.000309	0.000518	0.000309	0.000511
S7	D3-KIC	0.006758	0.006845	0.006674	0.006721
S8	D5-Phe	0.000096	0.000383	0.000093	0.000330
S9	13C6-Phe	0.000143	0.000299	0.000207	0.000330
S10	D3-KIC	0.006684	0.006799	0.006774	0.006858
average		0.002812	0.002990	0.002804	0.002939
std		0.003451	0.003382	0.003424	0.003363
se		0.001091413	0.001069561	0.001082857	0.001063484

SUBJECT	Condition	TIME			
	Ex2	T - B1	T - B2	UT - B1	UT - B2
S1	D5-Phe	0.000180	0.000299	0.000069	0.000173
S2	D5-Phe	0.000102	0.000323	0.000120	0.000334
S3	13C6-Phe	0.000064	0.000168	0.000063	0.000170
S4	D3-KIC	0.006774	0.006827	0.006725	0.006774
S5	D5-Phe	0.000275	0.000381	0.000308	0.000513
S6	13C6-Phe	0.000190	0.000293	0.000138	0.000262
S7	13C6-Phe	0.000114	0.000202	0.000104	0.000216
S8	D3-KIC	0.006840	0.006908	0.006756	0.006848
S9	D3-KIC	0.006763	0.006817	0.006825	0.006918
S10	D5-Phe	0.000110	0.000218	0.000110	0.000292
average		0.002141	0.002243	0.002122	0.002250
std		0.003210	0.003180	0.003208	0.003174
		0.001015101	0.001005569	0.001014323	0.001003592

Summary of all Effects; design: (ic enrichment.sta)
1-TRAIN, 2-CONDITIO, 3-BIOPSY

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	3.6722E-07	9	3.4817E-07	1.054715037	0.331233054
2	2	5.1412E-06	18	6.28135E-05	0.081848517	0.921752453
3	1	1.62968E-06	9	3.9561E-07	4.119413853	0.072977588
12	2	4.97336E-07	18	3.51018E-07	1.416840911	0.268261462
13	1	3.03573E-07	9	3.27237E-07	0.927683711	0.360628814
23	2	2.87382E-07	18	4.11823E-07	0.697828889	0.510636568
123	2	3.33884E-07	18	3.30067E-07	1.011562824	0.383411974

FSR

SUBJECT	REST		POST-EXERCISE		24 hrs POST-EXERCISE	
	UT	T	UT	T	UT	T
S1	0.03102347	0.05214145	0.063445	0.12879584	0.05074843	0.04950076
S2	0.04546936	0.0399493	0.11642981	0.12208039	0.11633045	0.08442471
S3	0.03490074	0.04798052	0.08968962	0.13806321	0.06364085	0.05849409
S4	0.06199951	0.05581254	0.08471591	0.10120675	0.052935	0.04890052
S5	0.07344237	0.06587771	0.08129818	0.13652728	0.11694624	0.07288635
S6	0.04627381	0.04679122	0.08458862	0.0972232	0.05436138	0.0461109
S7	0.02493955	0.02694363	0.0607401	0.11684595	0.04172111	0.04195434
S8	0.04372052	0.06582398	0.13690781	0.14689821	0.07561404	0.04865874
S9	0.02953714	0.0269106	0.04764863	0.0567651	0.06938247	0.04437524
S10	0.0479417	0.04780831	0.10582264	0.15217553	0.07922681	0.06756647
average	0.04392482	0.04760392	0.08712863	0.11965815	0.07209068	0.05628721
std	0.0150458	0.01361523	0.02698715	0.02851765	0.0261838	0.0141776
std error	0.0047579	0.00430551	0.00853409	0.00901807	0.00828004	0.00448335

Summary of all Effects; design: (fsr.sta)

1-CONDITIO, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	0.00069395	9	0.0002282	3.0409472	0.11515563
2	2	0.01732519	18	0.00045596	37.9973946	3.4635E-07
12	2	0.00295666	18	0.00011345	26.0620804	4.8378E-06

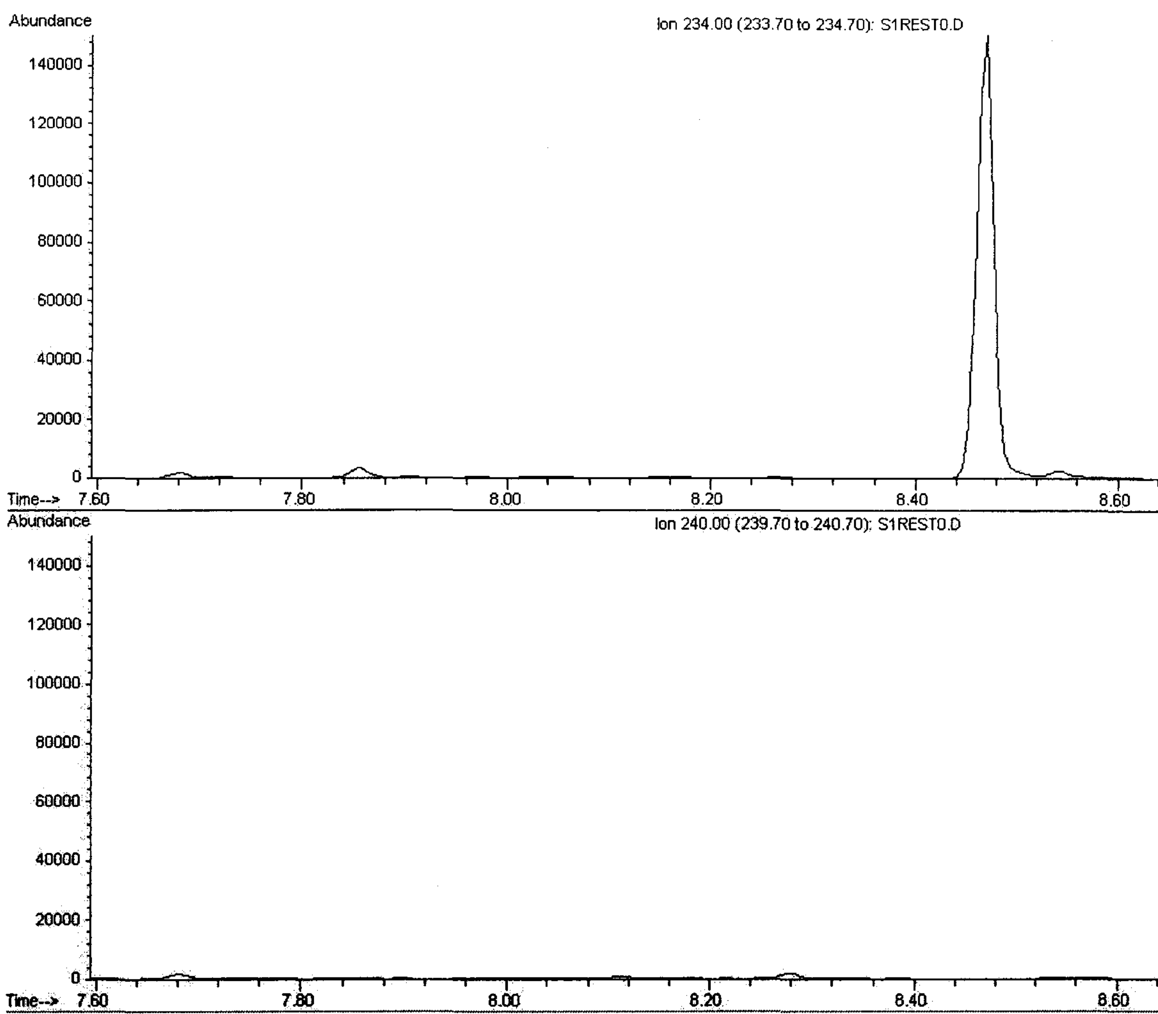
Tukey HSD test; variable Var.1 (fsr.sta)
Probabilities for Post Hoc Tests
INTERACTION: 1 x 2

	UT - R .0439248	UT - Ex1 .0871286	UT - Ex2 .0720907	T - R .0476039	T - Ex1 .1196581	T - Ex2 .0562872
UT - R		0.00015783	0.00029784	0.96885401	0.00015771	0.14922792
UT - Ex1	0.00015783		0.05220336	0.00015813	0.0001747	0.0001967
UT - Ex2	0.00029784	0.05220336		0.00094134	0.00015771	0.03793627
T - R	0.96885401	0.00015813	0.00094134		0.00015771	0.47673374
T - Ex1	0.00015771	0.0001747	0.00015771	0.00015771		0.00015771
T - Ex2	0.14922792	0.0001967	0.03793627	0.47673374	0.00015771	

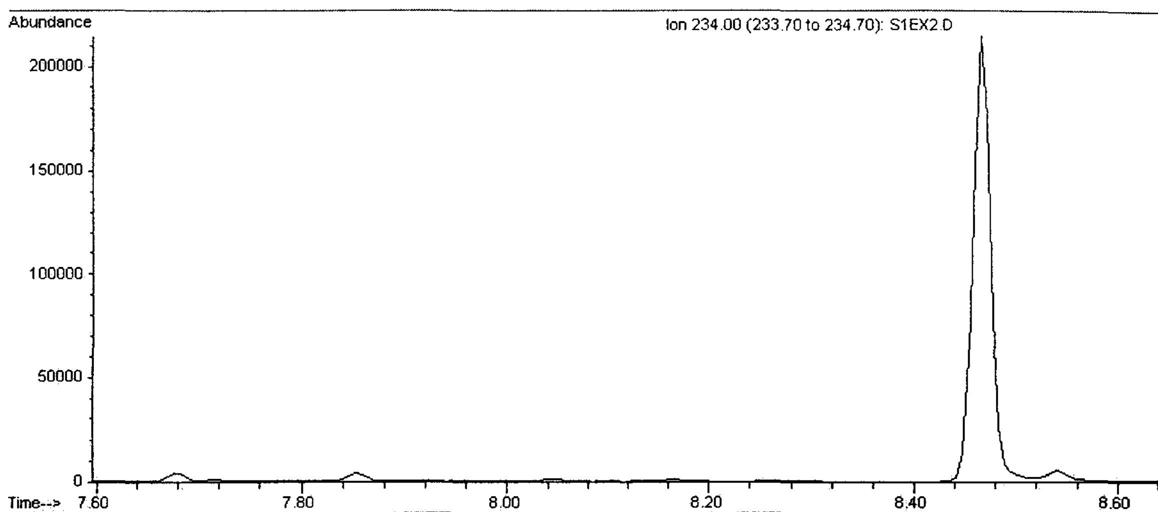
APPENDIX 9

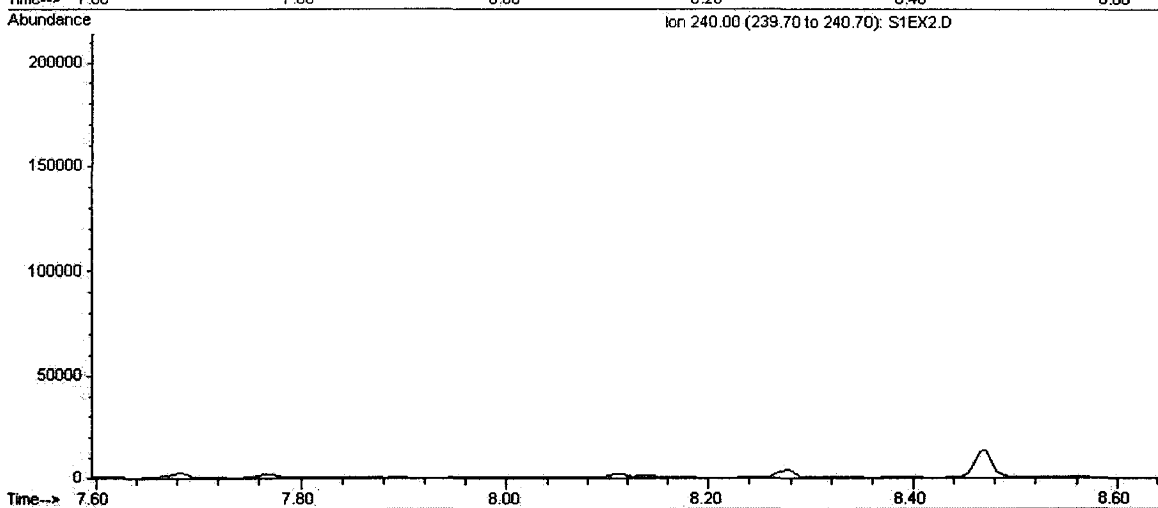
Total Ion Chromatograms

Plasma Enrichment: S1 REST 0 hrs – D_5 Phe

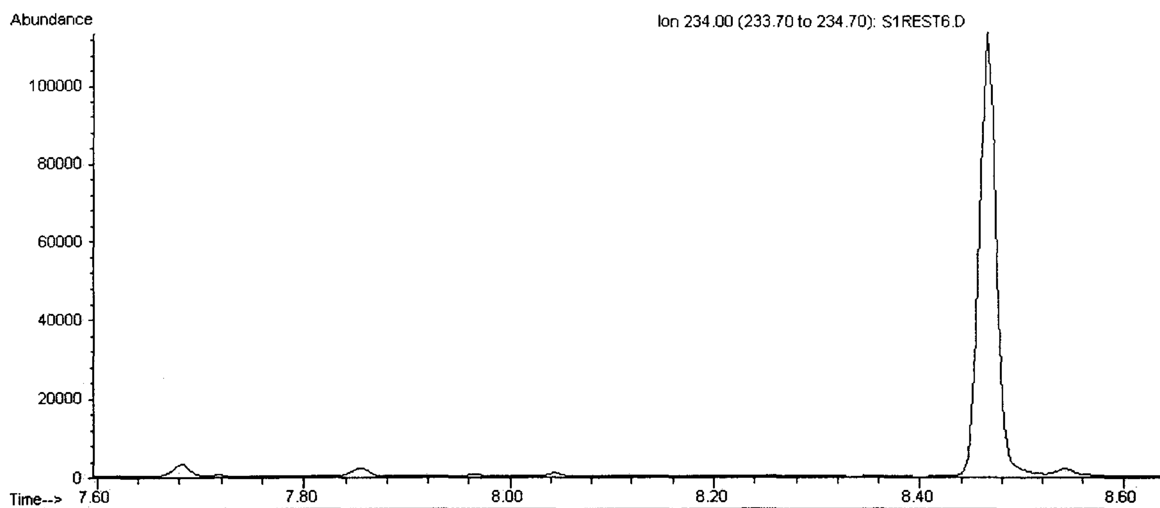


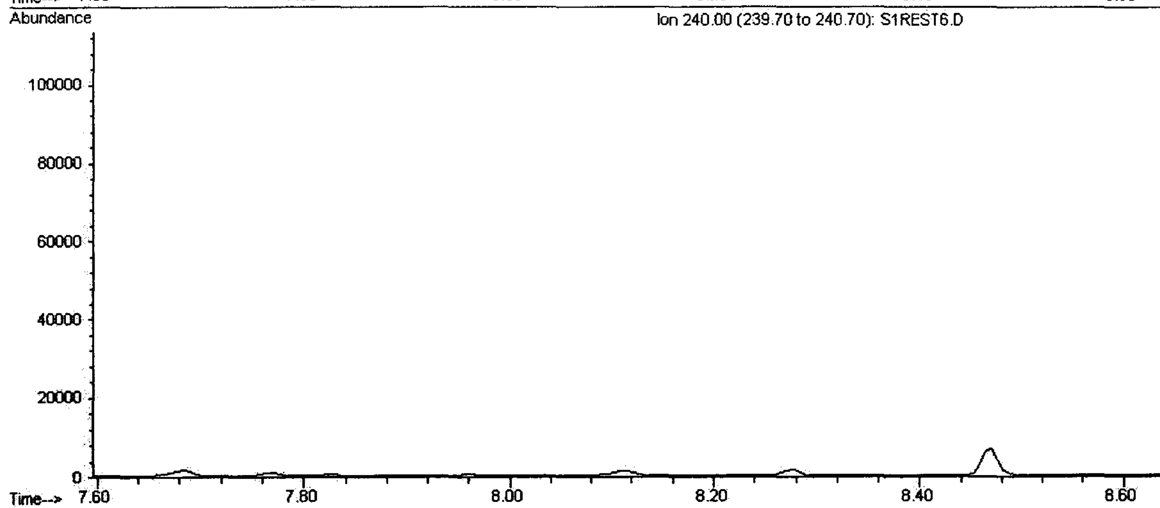
Plasma Enrichment: S1 REST 1.5 hrs – D_5 Phe



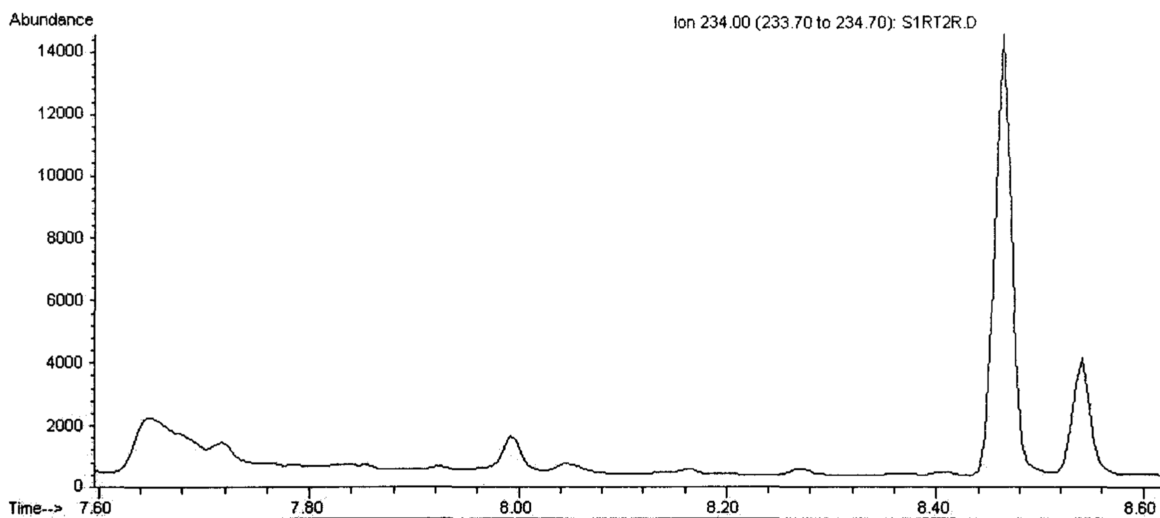


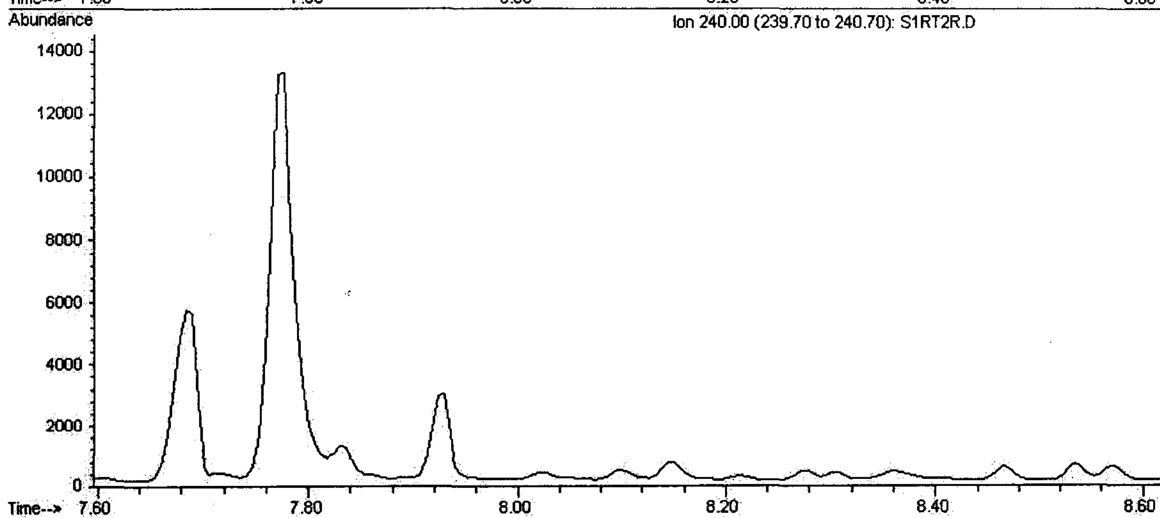
Plasma Enrichment: S1 REST 4.5 hrs – D_5 Phe



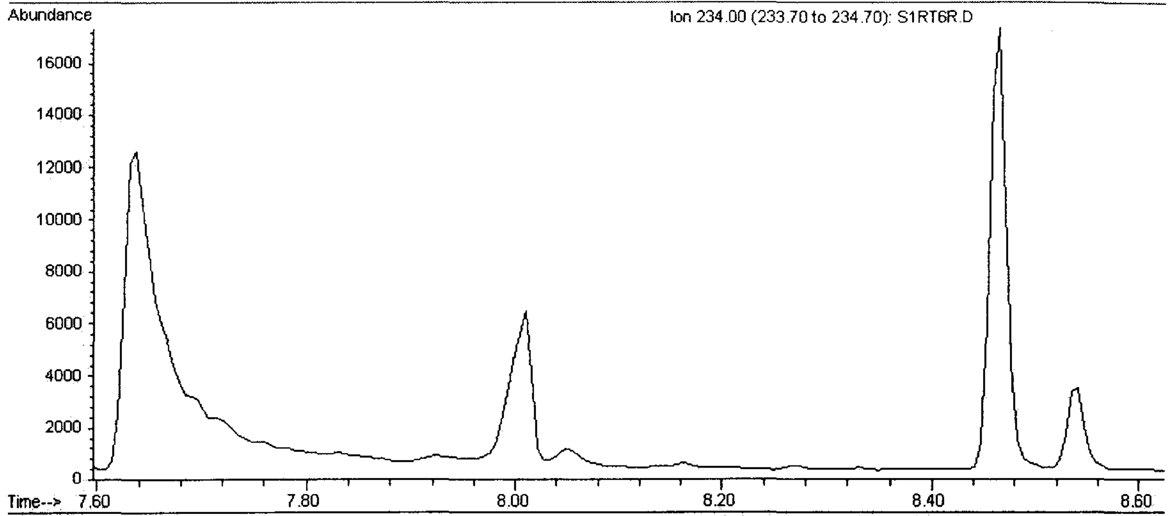


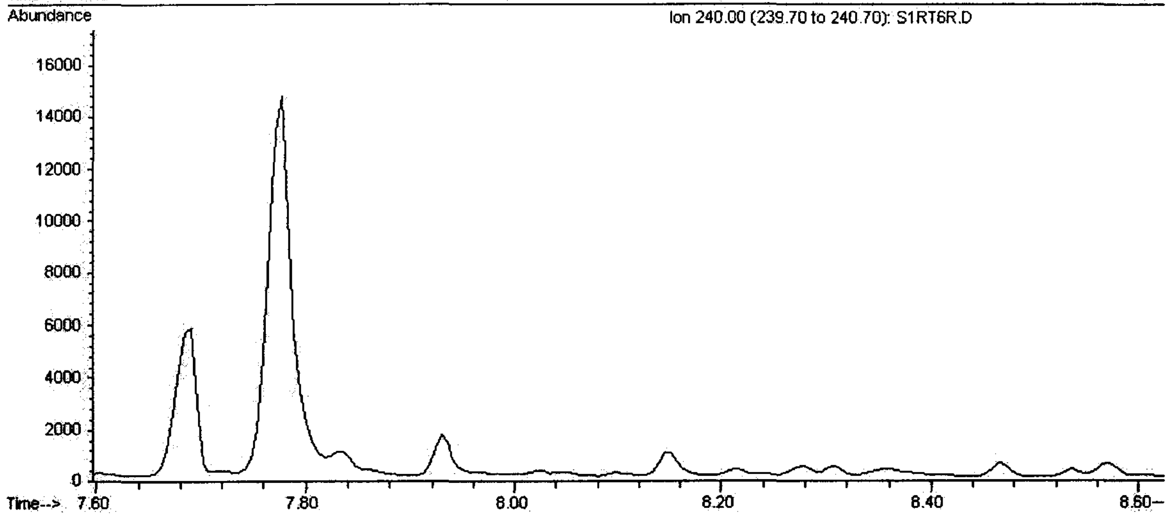
Intramuscular Enrichment : S1 REST 1.5 hrs UT – D_5 Phe





Intramuscular Enrichment S1 REST 4.5 hrs UT – D_5 Phe





APPENDIX 10

MUSCLE FIBRE ANALYSIS/HISTOCHEMISTRY PROTOCOL

ATPASE HISTOCHEMICAL ANALYSIS

Reference: Dubowitz, V. Muscle Biopsy: A practical approach 2ed. London: Bailliere Tindall, 1985.

Adapted by: Snow, R.J. School of Health Sciences, Deakin University, Australia

PART A: CUTTING MUSCLE

1. Store OCT mounted muscle at -80°C.
2. Prior to cutting muscle, place mounted muscle in cryostat for at least 15 min to reach -20°C.
3. Trim the OCT covered portion of the sample at 20µm/cut. Once muscle sample is exposed, reduce thickness of cut to 10µm for ATPase stain.
4. Cut 2-3 samples per slide.
5. Cover slides with paper towel and allow slides to dry overnight at 4°C.
6. Once dried, wrap slides in aluminum foil and store at -80°C until further analysis.

PART B: PREPARATION OF SOLUTIONS

Alkaline Stock Solution, pH 9.4

	Reagent	Manufacturer	Qty
1	Glycine	BioShop Biotechnology Grade - GLN 001	2.8163g
2	$CaCl_2 \cdot 2H_2O$	BDH 10070/EM Science 10070-34	3.00g
3	NaCl	BioShop Reagent Grade - SOD 002	2.1938g
4	NaOH	BDH Analytical Reagent ACS 816	1.3500g
5	MilliQ H_2O		500mL

1. Dissolve reagents in MilliQ H_20 and bring to volume.
2. Calibrate pH meter prior to adjusting pH to 9.4 with conc. HCl/5M KOH.
3. Store stock solution in fridge (4°C).

Acid Preincubation Stock Solution, pH 4.6

	Reagent	Manufacturer	Qty
1	Potassium Acetate	EM PX 1330-1	2.45g
2	$CaCl_2 \cdot 2H_2O$	BDH 10070/ EM Science 10070-34	1.30g
3	MilliQ H_2O		500mL

1. Dissolve reagents in MilliQ H_2O and bring to volume.

2. Calibrate pH meter prior to adjusting pH to 4.6 with glacial acetic acid.
3. Store stock solution in fridge (4°C).

5M NaOH (MW: 40.00g/mol) – Dissolve 20.00 g in 100 mL

5M KOH (MW: 56.11g/mol) – Dissolve 28.055 g in 100 mL

Alkaline Preincubation Solution

1. Remove alkaline stock solution from fridge and allow stock solution to reach room temperature.
2. Adjust pH of an appropriate volume (50mL) of alkaline stock solution to 10.50 using 5M NaOH (This should be done with continuous mixing and a pH meter sensitive to 0.001 pH units).

Acid Preincubation Solution

1. Remove acid stock solution from fridge and allow stock solution to get to reach room temperature.
2. Adjust pH of an appropriate volume (50mL) of acid stock solution to 4.30, 4.54, 4.60 with glacial acetic acid (This should be done with continuous mixing and a pH meter sensitive to 0.001 pH units).

ATP Preincubation Solution, pH 9.4 (PREPARE FRESH DAILY)

1. Add 170 mg of ATP (SIGMA A2383) to 100mL volumetric flask and bring up to volume using ALKALINE STOCK SOLUTION.
2. Adjust pH to 9.4.
3. Keep in fridge (4°C) until ready for use.

1% Calcium Chloride Stock Solution

1. Dissolve 10 g of $CaCl_2 \cdot H_2O$ in 1000mL volumetric flask using MilliQ H_2O and bring up to volume.
2. Store at room temperature.

2% Cobalt Chloride

1. Dissolve 5 g of $CoCl_2.6H_2O$ in 250mL volumetric flask using MilliQ H_2O and bring up to volume.
2. Cover in aluminum foil and store at room temperature.

1% Ammonium Sulfide (PREPARE FRESH DAILY)

1. Add 5 mL of 20% ammonium sulfide solution to 100mL volumetric flask.
2. Bring to volume.
3. Store in fume hood until ready for use.

PART C: FIBRE TYPE STAINING PROCEDURE

1. Incubate the sections in acid preincubation solutions adjusted to a pH of 4.30, 4.54 and 4.60; and alkaline preincubation solution adjusted to pH of 10.50 at the following time periods:

pH	Incubation time (min)
10.50	25
4.54	7.5
4.60	7.0
4.30	5.0

2. Transfer slides into plastic staining trough.

3. Rinse slides in distilled water 3 times.

4. Incubate slides in ATP incubation solution for 45 minutes at 37°C, in a temperature-controlled shaker.

5. Rinse slides in distilled water 2 times.

6. Incubate slides in 1% $CaCl_2.2H_20$ (Calcium Chloride) for 3 minutes at room temperature.

7. Rinse slides in distilled water 5 times.

8. Incubate slides in 2% $CoCl_2 \cdot 6H_20$ (Cobalt Chloride) for 3 minutes at room temperature.

9. Rinse slides with distilled water 5 times.

10. Incubate slides in 1% ammonium sulphide for 1 minute at room temperature.

11. Rinse slides in distilled water 5 times.

12. Dehydrate tissue for 2 minutes in each alcohol concentrations (70, 80, 90, 95 and 100% ethanol).

13. Clear sections with xylene. Do this twice in clean xylene @ 2 minutes.

14. Blot off excess xylene using Kimwipes. Mount the coverslips on slides using Permount (Fisher SP15-100). Allow Permount to dry (~1h). Store slides in the dark when not in use.

PART D: CAPTURING IMAGES / IMAGE ANALYSIS

1. Turn on camera and microscope and allow to warm up for 5min.

2. Focus image at 4x magnification.

3. Refocus image at 20x magnification to calculate fibre area (μm^2).

4. Open SPOT Advanced software.

5. Click "Get Image" icon to capture image.

6. Click "Focus" icon to refocus image.

7. Save image as .jpg file.

8. Capture 3-4 images per sample.

9. When finished with microscope and camera:
   a. Remove slide, lower platform, turn off camera, then microscope.
   b. Replace lens and dust covers.

10. Analyze Images using ImagePro Plus to determine fibre area (μm^2).

APPENDIX 11

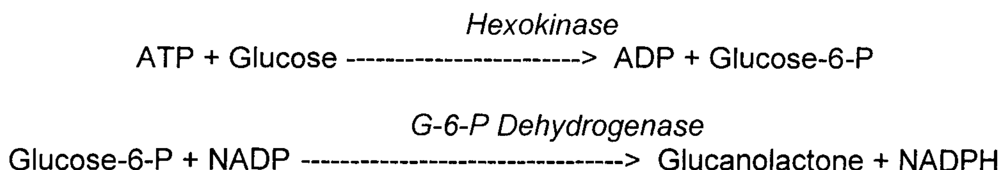
PLASMA GLUCOSE AND INSULIN ASSAYS

MUSCLE AND BLOOD GLUCOSE AND GLYCOGEN ASSAY

Reference:

Passoneau, J.A., and O.H. Lowry. Enzymatic Analysis: A Practical Guide, Totawa, NJ: Humana Press, 1993, pp. 157-158

Principle:



Reagents:

1. Tris pH 8.1 (1M stock solution); fridge
2. Magnesium Chloride (1M stock solution); freezer
3. DTT (0.5 M stock solution); freezer
4. ATP, disodium salt, Sigma A-2383 (MW = 551.1)
5. NADP, Roche 128 058 (MW = 787.4)
6. D-(+)- Glucose; dextrose: corn sugar, Sigma G-8270 (MW = 180.2)
7. Glucose-6-Phosphate Dehydrogenase; from yeast, grade 1
   Roche 127 035 (1 mg in 1 ml; ~350 U/mg)
8. Hexokinase (from yeast), Roche 1 426 362 (1 ml; ~1500 U/ml)

Stock Solutions:

1. ATP, 300 mM: dissolve 33.0 mg in 200 µl dH_2O.
2. NADP, 50 mM: dissolve 39.4 mg in 1 ml dH_2O.

Buffer:	Conc.	50 ml	100 ml	150 ml
Tris, pH 8.1	1 M	2.5 ml	5.0 ml	7.5 ml
$MgCl_2$	1 M	50 µl	100 µl	150 µl
DTT	0.5 M	50 µl	100 µl	150 µl
ATP	300 mM	50 µl	100 µl	150 µl
NADP	50 mM	50 µl	100 µl	150 µl

-- pH to 8.1 with NaOH, bring to volume then add G-6-P-DH --

G-6-P-DH	350 U/ml	5 µl	10 µl	15 µl

Enzyme:

Dilute 10 µl of Hexokinase in 1000 µl of assay buffer. Mix by inversion.

Concentrations in Assay Mixture:

Tris: 50 mM
$MgCl_2$: 1 mM
DTT: 0.5 mM
ATP: 300 µM
NADP: 50 µM
G-6-P-DH: 0.02 U/ml
Hexokinase: 0.3 U/ml
Glucose: ~0.15 – 18.0 µM

Standards:

Glucose (1.0 mM): Dissolve 36.04 mg Glucose in 200 ml dH_20 (may be stored frozen @ -80°C in 1 ml aliquots)

Std. #	Conc. (µM)	Stock B (µl)	dH_2O
1.	5	10	1990
2.	10	20	1980
3.	20	40	1960
4.	40	80	1920
5.	60	120	1880
6.	80	160	1840
7.	100	200	1800
8.	200	400	1600
9.	400	800	1200
10.	600	1200	800

Procedure:

Settings for Hitachi F-2500: Ex = 5, Em = 5, Voltage = 700, delay = 5 sec, int = 5 sec

1. Prepare buffer and pipette 1 ml into each cuvette.

2. For muscle and blood glucose assay, add 30 µl dH_2O (blank), standard or muscle extract to cuvette (samples run in duplicate)

   **NB: For glycogen assay, use 10 µl extract + 20 µl dH_20 and run samples in duplicate.

3. Mix, wait 5 min for equilibration, then take first reading @ 30 sec intervals.

4. Add 20 µl of Hexokinase to each tube.

5. Mix, wait 15 min and take second reading.

NOTE: The dilution factor for blood glucose will be 6.

INSULIN

Coat-A-Count TKIN1: Inter Medico

A. SPECIMEN COLLECTION
200 µL of either serum or heparinized plasma. Store - 20°C for up to 3 months.

B. REAGENTS and SUPPLIES

1. Insulin Ab-Coated Tubes
Store refrigerated for up to 1 year

2. [I^{125}] Insulin
To each vial of a concentrate add 100 ml of distilled H_2O. Store refrigerated: sable at 2-8°C for 30 days

3. Insulin Calibrators
One set of seven vials labeled A through G of lyophilized human serum. Reconstitute at least 30 minutes prior to use with 6 ml of distilled water for Calibrator A and with 3 ml of distilled water for all Calibrators B through G. Store frozen for up to 30 days. Avoid freezing and thawing.

Calibrator A: 0 µIU/mL Insulin
Calibrator B: 5 µIU/mL Insulin
Calibrator C: 15 µIU/mL Insulin
Calibrator D: 50 µIU/mL Insulin
Calibrator E: 100 µIU/mL Insulin
Calibrator F: 200 µIU/mL Insulin
Calibrator G: 300 µIU/mL Insulin

Note: these calibrators are only approximate concentrations. Check the vials for exact concentrations.

C. PROCEDURE

1. Label 4 plain uncoated tubes T (Total counts) and NSB (non-specific binding) in duplicate
2. Label 14 Insulin Ab-Coated Tubes A (maximum binding) and B through G in duplicate. Pipet 200 µL of the zero calibrator A into the NSB and A tubes and 200 µL of each of the remaining calibrators into the tubes labeled B through G. Pipet directly to the bottom of the tube.
3. Label addition Ab-coated tubes, also in duplicate for sample and control. Pipet 200 µL of the sample into the labeled tubes
4. Set the T tubes aside for counting at step 9 they require no further processing.

They can be capped and left.

5. Add 1 mL of $[I^{125}]$ insulin to every tube. Vortex
6. Incubate for 18-24 hrs at room temperature. (Alternatively, incubate for 3 hrs at 15-28°C, omitting the 5μIU/mL calibrator (B).
7. Decant thoroughly or aspirate the tubes. Remove all visible moisture. Remember not to decant the T tubes.
8. Count for 1 minute in a gamma counter.
9. Calculate insulin concentration from logit-log representation of the calibration curve.

Net Counts = Average CPM – Average NSB CPM

Then determine the binding of each pair of tubes as a percent of maximum binding (MB), with the NSB-corrected counts of the A tubes taken as 100%

$$\text{Percent Bound} = \frac{\text{Net counts}}{\text{Net MB counts}} * 100$$

APPENDIX 12

PREPARATION OF BLOOD/PLASMA FOR HPLC ANALYSIS

PLASMA AMINO ACID ANALYSIS BY HPLC

PREPARATION OF BLOOD/PLASMA FOR ANALYSIS BY HLPC:

NB: KEEP ALL SAMPLES AND REAGENTS ON ICE.

1. Allow appropriately labeled microcentrifuge (eppendorf) tubes to chill on ice (0-4°C).

2. Add 500μL of **ICE COLD** 0.6M Perchloric Acid (PCA) to microcentrifuge tube.

3. Add 200μL of plasma (100μL of whole blood) to PCA. Mix using vortex and cool on ice.

4. Centrifuge at 15,000rpm for 2 minutes.

5. Add 250 μL of **ICE COLD** 1.25M $KHCO_3$ (prepared FRESH daily) and let sit for 10 min. on ice **with lids open**.

6. Centrifuge at 15,000rcf (15,000g) for 2 min. at 0-4°C.

7. Remove supernatant and transfer into an appropriately labeled 13x100mm glass tube.

8. Dry samples in rotary evaporator (SpeedVac).

REAGENTS

1.25M $KHCO_3$

1.25g $KHCO_3$ in a 16x100mm disposable glass tube, add 10mL H_2O. Prepare **FRESH** daily

0.6M Perchloric Acid (PCA)

51.5mL 70% Perchloric Acid, bring up to 1000mL. Store 0-4°C for 2 months.

ANALYSIS OF AMINO ACIDS BY HPLC USING THE AccQ-Tag METHOD

Waters contact info if there are problems:
Machine is making all sorts of funny noises no matter what I do:
John Woods (800)252-4752 x 6731

To talk to people over the phone (800)252-4752
They will ask for some of these numbers:
Total Assurance Plan number: MLOSO1626
Serial number for 2690: B00SM4 848M
Serial Number for 474 detector: B00474699M

Micheal Shilling is good if you need someone to go over how to use the Millenium software: x 6727
Linda Schofield x 6385 knows the AccQ-Tag method

Eluent Preparation

REAGENTS

Reagent	Vendor	Order number
Sodium Acetate Trihydrate	Any HPLC grade	S-9513
Acetonitrile	Any HPLC grade	
Methanol	Any HPLC grade	
Phosphoric Acid	Any HPLC grade	
Sodium Azide	Sigma	S-8032
Trietylamine	Sigma	T-0886
Filters	Waters Corp.	P/N WAT200537

METHOD

Cleaning the Glassware

TWO HANDS ON GLASSWARE AT ALL TIMES

A. Cleaning the Eluent Bottles

1. Rinse each bottle four times with Milli-Q water
2. Fill bottle with Milli-Q, and pour Milli-Q through filter
3. Check filter for discoloration

4. If filter is clean after filtering then it is clean, if the filter shows discoloration wash bottle with Milli-Q two more times

Preparation of ELUENT A and B concentrate:

1. Eluent A and B should be prepared in a fumehood
2. Weigh 296 g of sodium acetate trihydrate into a 4 L beaker in the fume hood
3. Add 2 L of water and dissolve acetate
4. Add 10 g of sodium azide
5. Add 19.34 ml (14.12 g) of triethylamine
6. Add 5 ml of concentrated phosphoric acid to A and 1 ml of concentrated phosphoric acid to B to the bottle (this will reduce odor of TEA)
7. Dilute 300m ml of concentrate with 3L Milli-Q water to yield the working eluent.
8. Eluent A should have a pH of 4.97, and B should be adjusted with the phosphoric acid to a pH of 7.40 (adjust pH with a 85% HPLC grade phosphoric acid solution, being careful noto lower the pH too far, as back titration will increase the ionic strength.)
9. Vacuum Filter the eluents through a 0.45nm filter (after filtration check the filter for discolouration) → when filtering stop filtration before it reaches the neck of the bottle and funnel eluent in the bottle into a cleaned eluent bottle MAKE SURE THAT YOU HAVE A FIRM HOLD ON BOTH PARTS OF THE FILTRATION APPARATUS AT ALL TIMES

See section: Setting Up and Using the Filtration Apparatus for instructions on filtration.

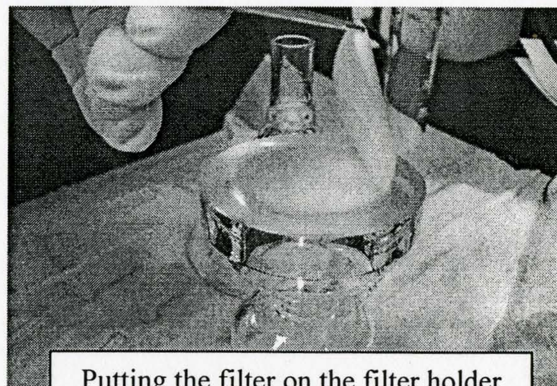
10. Properly clean all glassware before using (see section on cleaning glassware) and change filter when changing from B to A, the filter does not have to be changed when switching from A to B.

Extra Notes On Using/Making up the Eluent Dilute

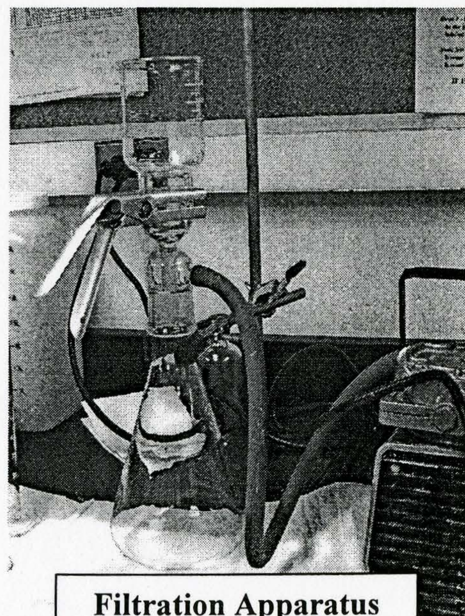
1. Make sure that when the eluent bottles are attached to the HPLC machine the sinker is not touching the bottom of the bottle → the sinker should be floating just above the bottom of the bottle
2. DO NOT top up the old bottles of eluent that are attached to the HPLC machine with new eluent; replace the old eluent with fresh eluent that is in a new bottle. The remaining eluent should be re-filtered into a new clean bottle.
3. DO NOT make up too much eluent at any one time. It is recommended that you make up two bottles of Eluent A at one time and one bottle of B.
4. Make up the A before you make up the B. This is because the glassware doesn't need to be cleaned and the filter doesn't need to be changed when going from making Eluent A to making Eluent B.

Setting Up and Using the Filtration Apparatus

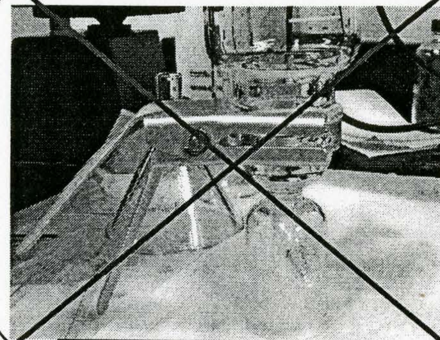
1. Place a "diaper" on the desk to absorb any spills
2. Rinse all glassware with Mili-Q water before using it
3. Set up the retort stand with the clamp
4. Clamp the large flask to the retort stand so that the apparatus will be stabilized
5. Attach the filter holder to the top of the flask (while the flask is clamped to the rod)
6. Take a filter from the box (using tweezers, and ensuring to minimize contact with the filter and only touching the edge of the filter when necessary), place the filter on the filter holder with the side that was up in the box also facing up on the filter holder. Ensure that the filter is centered on the filter holder.


Putting the filter on the filter holder

7. Once the filter is on take the top piece of the apparatus in one hand and the clamp in the other. Place the top on the filter holder, making sure that the filter is still properly centered. With the other hand (while still holding on to the top part) clamp the top to the filter holder.
8. Attach one end of the vacuum hose to the motor and the other to the tip on the top piece.
9. The filtration apparatus is now set up and ready to use.
10. To filter, pour the solution into the top and turn on the motor. Continue to refill the top with the solution as it is filtered, until the solution in the flask reaches the neck.
11. Once the solution has reached the neck of the flask turn of the motor and remove the hose from the spout of the top.
12. With one hand grasp and hold onto the flask and the filter holder, and with the other hand unclamp the apparatus from the retort stand.
13. Hold onto the flask with one hand and with the other hand gently twist and pull up on the filter holder. This will remove the top and the filter holder from the flask so that you can pour the filtered solution.


Filtration Apparatus

14. Hold the filter holder and the top with one hand and pour the filtered solution into a clean bottle. NEVER put the filter holder and the top down, or stand it up on the desk!!!



Never Put On Desk!!

15. With the funnel in the bottle that the eluent is going in, and holding the top part of the filtration apparatus pour the filtered eluent into the clean eluent bottle.

16. Reassemble the filtration apparatus and continue to filter the rest of the freshly made eluent.

17. Once all of the eluent has been filtered and poured into the proper bottles properly clean all the glassware, as per the glassware cleaning section.

Eluent C

100 % Acetonitrile

Eluent D

Milli-Q Water

Seal and Needle wash solutions

~10% Acetonitrile, 5% Methanol in Milli-Q water

Setting up the Detector (Fluorescence)

Excitation Wavelength	250 nm
Emission Wavelength	395 nm
Filter	0.5
Gain	100

Chromatographic Method

Set column temperature to 34°C
Set sample temperature to 15°C
Make sure the degasser is on continuous
Wet Prime all the lines when the machine has been off

- Always equilibrate and run a condition column

- Be sure that the degasser is set to continuous

Gradient Table

Note: if the pH of eluent A is off by as little as 0.05 pH units, the chromatogram will be off

Note: This gradient table has been modified from the original one given to us by Waters entitled 'Analysis of amino acids in cell culture media and supernatants using a modification of the AccQ-Tag method'.

Run time = 66 minutes

Pressure Limits:

High Limit (psi): 4000 Low Limit (psi): 0.0

Programmed Flow

Pump Mode: gradient Accelerate to 10mL/min: 2.00min (mL/min/min)

	Time	Flow	%A	%B	%C	%D	Curve
1	0.01	1.00	100.0	0.0	0.0	0.0	6
2	0.50	1.00	99.0	1.0	0.0	0.0	11
3	28.00	1.00	98.0	1.0	0.0	1.0	6
4	29.00	1.00	95.0	5.0	0.0	0.0	6
5	37.00	1.00	0.0	11.0	59.0	30.0	6
6	38.50	1.30	0.0	13.5	84.5	2.0	11
7	44.80	1.30	0.0	12.5	87.5	0.0	6
8	49.00	1.30	0.0	13.0	87.0	0.0	6
9	56.00	1.30	0.0	15.0	85.0	0.0	6
10	56.40	1.30	0.0	60.0	0.0	40.0	6
11	62.00	1.00	100.0	0.0	0.0	0.0	11

Preparing Standards

REAGENTS

Reagent	Vendor	Order number
L-Asparagine Monohydrate	**Sigma**	**A-8381**
L-Cysteine	**Sigma**	**C-7755**
L-Glutamine	**Sigma**	**G-3126**
L-Taurine	**Sigma**	**T-0625**
Amino Acid Standard Solution	**Sigma**	**A-9656**

METHOD

1. Prepare Stock Solutions

All of the following are made up in 0.1 N HCl

L-Asparagine Monohydrate (Sigma A-8381)
Stored on shelf in wet lab
Molecular weight: 150.1 g/mol (L-Asparagine = 132.1)
Solution required: 2.5 mmol/L
Weigh 37.53mg/100mL (i.e. 18.76 mg/50mL)

L-Cysteine (Sigma C-7755)
Stored on shelf in wet lab
Molecular weight: 121.2 g/mol
Solution required: 2.5 mmol/L
Weigh 30.3 mg/100mL (i.e. 15.15 mg/50mL)

L-Glutamine (Sigma G-3126)

Stored on shelf in wet lab
Molecular weight: 146.15 g/mol
Solution required: 2.5 mmol/L
Weigh: 36.52 mg/100 mL (i.e. 18.26 mg/ 50 mL; **9.13 mg/ 25 mL**)
Save about 10 mL and transfer to eppendorf tubes. Store at -20°C (~3 months)

L-Taurine (T-0625)

Stored on shelf in wet lab
Molecular weight: 125.1 g/mol
Solution required: 2.5 mmol/L
Weigh 31.28 mg/100mL (i.e. 15.64 mg/50mL)

2. Sigma standard has been discontinued;
- Sigma – A 9856 – Amino Acid Standard Solution – for food Hydrolysates
    - Solution of 0.1N HCl:10 μg per ml except L-cystine at 20 μg per ml
    - Stored at 2 to 8 degrees Celsius

To prepare *standard for discontinued Sigma – A 9856*:

200 μL of Amino Acid Standard Solution (Sigma A-9656)
15 μL of 2.5 M asparagine
5 μL of 2.5 M cysteine
30 μL of 2.5 M glutamine

5 μL of 2.5 M taurine
245 μL ddH2O

3. New standard:
   - Pickering Laboratories – 012506H (5ml) – Amino Acid Protein Hydrolysate Standard
     - *Usage:* Intended to be used to establish relative retention times and concentrations of amino acids. The calibration standard is a mixture of acidic, neutral and basic Amino Acids and related compounds. Each ml of solution contains 0.25μmol (equivalent to 0.25 mmol/μl) of each component in 0.2 N Sodium Citrate Buffer pH 2.20 with 0.008*N* Phenol as preservative.
     - *Storage:* Upon receipt, it is best to divide the standard into aliquots and store in the freezer (at least - 4 degrees Celsius), until ready for use. When the standard is needed thaw one aliquot at a time, and keep the thawed aliquot in a refrigerator (ca. 4 degrees Celsius). Before opening the vile, allow standard to reach room temperature to avoid condensation and possible contamination.

To prepare standard for Pickering Laboratories – 012506H:

128 μL of the standard
30 μL Asn
10 μL Cys
60 μL Gln
10 μL Tau
762 μL milli-Q water

= 1.0 ml

Amino Acid Amounts in Standard

	Component	Value	Value	Value	Value	Value	Units (Vial)
1	ASP	11.429000	19.048000	26.667000	38.095000	76.190000	pmol
2	SER	11.429000	19.048000	26.667000	38.095000	76.190000	pmol
3	ASN	26.786000	44.643000	62.500000	89.286000	178.571000	pmol
4	GLU	11.429000	19.048000	26.667000	38.095000	76.190000	pmol
5	GLY	11.429000	19.048000	26.667000	38.095000	76.190000	pmol
6	GLN	53.571000	89.286000	125.000000	178.571000	357.143000	pmol
7	HIS	11.429000	19.048000	26.667000	38.095000	76.190000	pmol
8	TAU	8.929000	14.881000	20.833000	29.762000	59.524000	pmol
9	THR	11.429000	19.048000	26.667000	38.095000	76.190000	pmol
10	ARG	11.429000	19.048000	26.667000	38.095000	76.190000	pmol
11	ALA	11.429000	19.048000	26.667000	38.095000	76.190000	pmol
12	PRO	11.429000	19.048000	26.667000	38.095000	76.190000	pmol
13	CYS	8.929000	14.881000	20.833000	29.762000	59.524000	pmol
14	TYR	11.429000	19.048000	26.667000	38.095000	76.190000	pmol
15	VAL	11.429000	19.048000	26.667000	38.095000	76.190000	pmol
16	MET	11.429000	19.048000	26.667000	38.095000	76.190000	pmol
17	ILE	11.429000	19.048000	26.667000	38.095000	76.190000	pmol
18	LEU	11.429000	19.048000	26.667000	38.095000	76.190000	pmol
19	LYS	11.429000	19.048000	26.667000	38.095000	76.190000	pmol
20	PHE	11.429000	19.048000	26.667000	38.095000	76.190000	pmol

Preparing Samples

Blood Samples:

1. add 0.5 ml (500 µl) of cold 0.6 M PCA to a microfuge tube, keep on ice
2. add 0.1 ml (100 µl) of whole blood to this. Mix and cool on ice
3. Centrigure at 15,000 rpm for 2 min in a refrigerated microguge (0 – 4 °C)
4. Add 0.25 ml (250 µl) of 1.25 $KHCO_3$ (made fresh daily) and let sit for 10 min.
5. Centrifuge at 15 000 rpm for 2 min in a refrigerated microfuge (0-4 °C).
6. Remove supernatant and store frozen in appropriately labeled microfuge tubes.

Muscle Samples: **1.** Use PCA extract protocol

Derivatization of Standards and Samples

Reconstituting AccQ-Fluor Reagent

Reagents:

(10mM AccQ-Fluor in acetonitrile)

Vial 2A
Vial 2B

Method:

1. Preheat a heating block to 55°C
2. Tap Vial 2A lightly before opening to ensure all reagent powder is at the bottom of the vial.
3. Rinse a clean micropipettor by drawing and discarding 1 mL of reagent diluent from vial 2 B (acetonitrile is flamable and toxic)
4. Transfer 1 mL of reagent diluent from 2B to the reagent powder in vial 2A. Cap the vial tightly.
5. Vortex for 10 seconds
6. Heat Vial 2A on top of heating block until the reagent powder is dissolved. Do not heat the reagent for longer than 10 minutes.

Storage: store reconstituted reagent in dessicator at room temperature for up to one week.

Derivatizing the Calibration Standard or sample

This procedure converts the amino acids to highly stable derivatives

1. Preheat a heating block to 55°C
2. Add 55 μL of AccQ-Fluor Borate Buffer (Reagent 1) to the sample tube. Vortex briefly.
3. Deliver 25 μL of calibration standard or sample to a glass insert placed in the glass tube.
4. Add 25 μL of reconstituted AccQ-Fluor Reagent to the sample tube. Vortex immediately for several seconds
5. Let stand for one minute at room temperature
6. Heat the vial in a heating block or oven for 10 minutes at 55°C

- *Let sit for at least 10h before running in the machine.*
- *Make two standards for each run*
- *Make sure to ONLY use standards that are made upon the same day as the sample was made*

Derivatizing a blank

1. Place 85 μL of AccQ-Fluor Borate Buffer in a sample tube
2. Add 15 μL of Acc-Q Fluor Reagent and vortex
3. Wait one minute for excess reagent to hydroglyze to AMQ

Note: heating is not necessary for the derivatization blank

Preparing a run table and gathering data

Loading a run table:

1. Enter Millenium
2. In Run Samples right click in 'run samples' window and highlight 2690_satin.
3. Under the 'file' menu choose 'load samples'. A box will appear choose 'Load using a previously created sample set method'. Click enter. Choose your sample set method (For blood use 'Amino Acid Template' for muscle use 'Amino Acid Template Muscle'). ALTERNATIVELY, you can make up your own sample set. (Remember always equilibrate for at least 10 minutes, purge injector, clear calibration and condition the column one or more times by choosing the function of 'condition column' (make sure the run time for condition column is 66 minutes and not the default of 10 minutes).
    - For blood samples you will need to inject 15 ul

- For muscle samples you will need to make two injections (1 and 15 ul) because of the really high concentrated amino acids (Gln, Tau, Arg) and lower concentrated amino acids.

4. Modify sample set with your actual sample names and correct vial number
5. Under instrument method click 'Amino Acid Method' and press the setup button if the HPLC is not already running.
6. When samples are loaded in appropriate vials click the green circle to run samples

Daily HPLC Checklist – Machine Running

Put in for each item refer to which section of problem solving area to refer to

- Check to ensure that the machine is running
- Empty waste
- Check levels of Eluent A and Eluent B, and replace with new eluent if necessary (refer to section ______ for info (do not top up, when change use new bottle and filter remaining before adding to the bottle,
- Make sure sinker is suspended above the bottom of the bottles
- Check water level, refill if necessary
- Check acetonitrile level, refill if necessary
- Check pressure (pressure should not exceed 2000psi), and make sure that the delta pressure is not above 20psi (put in note about how to check delta) → notes about why delta might change (new flow rate, change in components from all salt to actectonitrile or the other way around) → if changing refer to problem solving section for why and how to fix
- Check column temperature (34°C is normal) and the sample temperature (15°C is normal)

Starting the HPLC

- Turn on the HPLCs with the switch on the side.
- Turn on the lamps: for the 474 this is flicking the switch on the front of the detector, and for the 2475 on detector press "Shift" then "Lamp" then "Shift" then "Lamp"
- Allow time for the detector lamps to warm up before running any samples.
- While the lamps are warming run 60% Acetonitrile and 40% Water through the machines for 20 to 30 minutes at a flow rate of 1 mL/min.

- Using the screen and the button pad on the HPLC, set the column temperature to 34°C and the sample temperature to 15°C, also turn on the degasser.
- After the HPLC has been run at 60% Acetonitrile and 40% Water for the 20 to 30 minutes start running the HPLC at 100% A for at least 10 minutes before a run is started. This can be done either manually, or by setting the first line of the sample set to be run as "Equilibrate" and by setting the time to 10 minutes.

Daily HPLC Checklist – Checking Chromatograms

- **JUST BECAUSE MACHINE IS RUNNING DOESN'T MEAN THAT YOU ARE GETTING GOOD DATA, THIS SECTION IS VERY IMPORTANT EVERYDAY THAT THE MACHINE IS RUNNING. THIS SECTION MUST BE FOLLOWED EXACTLY**
- Section on how to work software → refer to this section in checklist
- Bring up in review window all channels that need to be checked
- Uncheck all checks so that you can overlay
- Check for peak separation and shift in retention times
- Overlay standard 2s and make sure that retention times and peak heights and areas are similar → if not see section ....
- Overlay each sample with one std 2 and check for separation, shift in retention times
- BIG PROBLEM SOLVING SECTION

Software Use

Logging on to Empower software and Opening a Project

- Double click the Empower Icon on the desktop
- Click on "Log in" on the window that opens and type in you User Name and Password and press Ok.
- Click on "Run Samples"
- Select the project that you want to open and select the HPLC that the project has been run on (either the 2475 or the 474)
- Once this has been done click "Use Quickstart" and the project will open

To view Chromatograms

- Click on browse project, and select the sample set that you wish to view. Right click on the sample set; go to V*iew As* and select C*hannels* (see picture below)

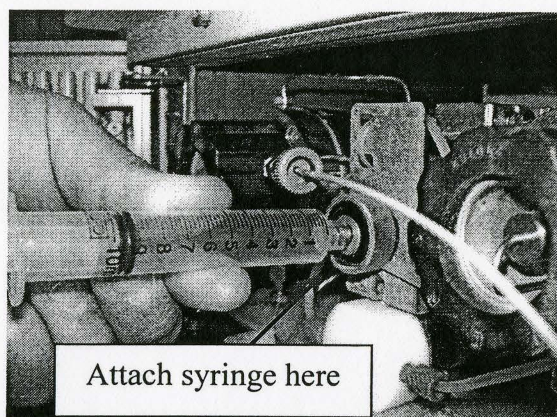
- Select the channels that you want to view (hold down Ctrl to select multiple channels); right click on one of the channels and select *Review – Replace Data*

(will just show the newly selected channels) or select *Review – Append Data* (will add newly selected channels to the data that is already being viewed)

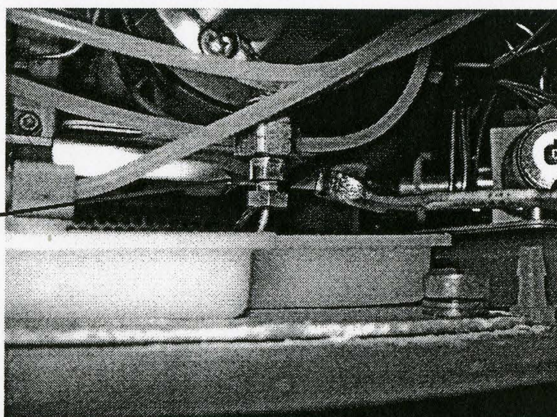
- Chromatograms are viewed under the *View Data* tab on the middle left hand part of the screen

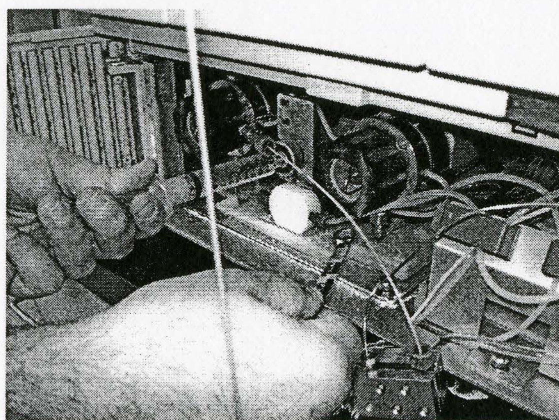
How to Get Back Prime

1. Attach the syringe to the valve
2. On the HPLC select "Direct Function", then select wet prime. Set the wet prime for 2 minutes.
3. Open the valve that the syringe is attached to by turning it to the left.
4. Pull the syringe out slowly, make sure that you **NEVER** allow the air or the liquid in the syringe to go back into the HPLC.
5. While holding the syringe out tap on the valve on the bottom of the holding tanks on both sides of the HPLC with the end of a wrench.



Tap wrench here and at the same spot on the other side





6. Continue tapping on the valves until liquid starts to flow into the syringe at a steady rate. Once this happens continue to hold the syringe out and close the valve that the syringe was attached to.
7. If liquid doesn't start to flow, continue to tap on the valves a little harder then before. If this still doesn't work, change the flow concentrations to 25% of A,B,C, and D, and repeat the priming process from the beginning. **If this still doesn't work Call John at Waters.**

APPENDIX 13

PREPARATION OF BLOOD/PLASMA FOR ANALYSIS BY GCMS

PREPARATION OF MUSCLE FOR ANALYSIS BY GCMS

AMINO ACID ISOLATION/PURIFICATION

DERIVATIZATION OF PROTEIN HYDROLYSATE AND AMINO ACIDS

PREPARATION OF BLOOD/PLASMA FOR ANALYSIS BY GCMS:

NB: KEEP ALL SAMPLES AND REAGENTS ON ICE.

9. Allow appropriately labeled microcentrifuge (eppendorf) tubes to chill on ice (0-4°C).

10. Add 500µL of **ICE COLD** 0.6M Perchloric Acid (PCA) to microcentrifuge tube.

11. Add 200µL of plasma (100µL of whole blood) to PCA. Mix using vortex and cool on ice.

12. Centrifuge at 15,000rpm for 2 minutes.

13. Add 250 µL of **ICE COLD** 1.25M $KHCO_3$ (prepared FRESH daily) and let sit for 10 min. on ice **with lids open**.

14. Centrifuge at 15,000rcf (15,000g) for 2 min. at 0-4°C.

15. Remove supernatant and transfer into an appropriately labeled 13x100mm glass tube.

16. Dry samples in rotary evaporator (SpeedVac).

REAGENTS

1.25M $KHCO_3$

1.25g $KHCO_3$ in a 16x100mm disposable glass tube, add 10mL H_2O. Prepare **FRESH** daily

0.6M Perchloric Acid (PCA)

51.5mL 70% Perchloric Acid, bring up to 1000mL. Store 0-4°C for 2 months.

PREPARATION OF MUSCLE FOR ANALYSIS BY GCMS:

1. Cut a portion of frozen muscle sample in -20°C cryostat and transfer cut piece in appropriate labeled eppendorf tube with a punctured lid.
2. Freeze-dry samples overnight.
3. Record dry weight.
4. Manually powder dried muscle and remove any visible connective tissue.
5. Transfer powdered muscle into an appropriately labeled 13x100mm glass disposable tube. May store at -80°C until further analysis.
6. To extract intracellular amino acids:
   KEEP ALL SAMPLES ON ICE.

   a. Add 500µL of ICE COLD 0.6M PCA.

   b. Gently shake or vortex to ensure powdered muscle has come into contact with PCA. **NOTE: Agitate carefully to minimize muscle adherence to the tube wall.**

   c. Let samples sit for 10min.

   d. Centrifuge at 4500rpm for 2 minutes.

   e. Using a P200 pipette, extract supernatant into an appropriately labeled eppendorf tube. Cap glass tube and save pellet for further analysis

   f. Neutralize supernatant as blood/plasma samples:

      i. Add 250 µL of ICE COLD 1.25M $KHCO_3$ (prepared FRESH daily) and let sit for 10min on ice **with lids open.**

      ii. Let samples sit for 10min.

      iii. Centrifuge at 15000g for 2min.

      iv. Carefully decant supernatant into 13x100mm tube

      v. Rotary evaporate samples for derivatization.

PREPARATION OF MUSCLE FOR ANALYSIS BY GCMS: AMINO ACID ISOLATION/PURIFICATION

Note: The muscle for this procedure can be either wet (must be maintained frozen) or dry.

REAGENTS

1. Dowex AG 100-200 mesh H^+ form cation exchange resin
2. 4N NH_4OH
3. 1N HCl
4. 1N NaOH
5. pH paper (pH range 4-10)
6. Fisher Screening Column 11-387-50

COLUMN PREPARATION

1. Add ~2cm of resin (rinsed with DDI water) to commercial column – make sure the column has a **fritted filter** in the end prior to introducing resin.

2. Rinse the resin with **1** head volume of **DDI water**.

3. Rinse the resin with **1** head volume of **2N NaOH**.

4. Rinse the column with at least **3** head volumes of **DDI water**, or until **pH is neutral**. **Check for neutrality with pH paper.**

5. Rinse column with **2** head volumes of **2N HCl**.

6. **Neutralize** the column with at least **4-5 volumes of DDI water** - CHECK for neutrality with pH paper.

ABSORPTION/DESORPTION

1. Add sample (either 10% TCA intracellular extract, SSA precipitated blood, or **6N HCl muscle pellet hydrolysate**) to column.
   Note: Add 0.5mL of hydrolysate, if dry weight of muscle is 1-2mg and 1mL of hydrolysate if <1.0mg.

2. Rinse the column with 7-8 head volumes of DDI water. **Wash vigorously to agitate beads and check for neutrality.**

3. Allow all fluid to escape for resin.

4. Desorb (release) the amino acids from the resin using 4N NH_4OH (~5mL).

5. Begin collecting the eluate from the columns immediately (i.e. have the collection tubes set up underneath the columns).

6. Evaporate sample using a rotary evaporator (approx. 12hrs). Be sure to use a BORIC ACID trap!

7. Store dry pellet – usually black/yellow in colour – at room temperature until ready for derivatization.

DERIVATIZATION OF PROTEIN HYDROLYSATE AND AMINO ACIDS

1. Add a mixture of 50μL MTBSTFA + 1% TBDMCS (Pierce P48925 or REGIS RT270142) + 50μL acetonitrile (HPLC grade) to dried samples.
   Use Hamilton microliter glass syringe to transfer derivatizing agents.

2. Heat samples at 90°C for 1 hour.

3. Remove samples from heating block and allow cooling to room temperature.

4. Transfer derivative into appropriately labeled glass vials with inserts and cap. Use methanol to remove pen markings.

5. Place vials onto auto-sampler tray and run protocol.

6. Using Selected Ion Monitoring (SIM) mode, extract desired chromatographs.

7. Manually integrate peaks.

8. Record m/z ratios of selected ions using area under peak.

Notes on the operation of GC/MS

1. Rubber septa located under autoinjector may need to be replaced if target pulsed pressure value is not reached at the start of sample run.
2. Autotune before a series of samples or if GC/MS has been in use.
3. Clean syringe with methanol.
4. Change selected ions (m/z) in MS SIM/Scan Parameters.