NITROGEN BALANCE AND LEUCINE KINETIC APPROACHES

TO THE EXAMINATION OF

PROTEIN REQUIREMENTS AND PROTEIN METABOLISM

IN RESISTANCE ATHLETES

By

MARK A. TARNOPOLSKY, BPE., MD.

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AUTHOR: Mark A. Tarnopolsky, BPE. (McMaster University)
MD. (McMaster University)

SUPERVISOR: Dr. S.A. Atkinson

CO-SUPERVISOR: Dr. J.D. MacDougall

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ABSTRACT

Previous work has demonstrated that resistance exercise may increase dietary protein (PRO) requirements to a varying degree. Much of these discrepancies probably arose from differences in training intensity, classification of exercise, habituation to a training stimulus, and methodological considerations such as: 1. inadequate dietary adaptation periods; 2. failure to measure routes of nitrogen (N) loss (i.e., sweat); 3. failure to measure the dietary PRO (nitrogen (N)) content; 4. collection periods too short to measure an impact of exercise (i.e., delay in N excretion due to dehydration); and 5. the use of measurements insensitive to short-term alterations in PRO metabolism (i.e., lean body mass estimate from skinfolds to assess changes in muscle mass over a week of treatment). In addition, there is a paucity of information quantitating the influence of acute and chronic resistance exercise on PRO turnover. The overall purpose of this thesis was to examine N metabolism and the PRO requirements of resistance athletes, under well controlled conditions, with three discrete, but related studies. The first investigation was a randomized, cross-over study designed to determine the dietary PRO requirements of young men performing intensive body building resistance exercise during the early stages of training (novice) and to determine whether the consumption of excessive amounts of PRO had an

ergogenic (work enhancing) effect upon muscle mass/strength gains. Twelve inexperienced, young, male volunteers received either a PRO supplement (total PRO_{IN}=2.62 $g \cdot kg^{-1} \cdot d^{-1}$) or a carbohydrate (CHO) supplement (total PRO_{IN}=1.35 $g \cdot kg^{-1} \cdot d^{-1}$) each for a period of 4 weeks during intensive (1.5 $h \cdot d^{-1}$, 6 $d \cdot wk^{-1}$) circuit weight training. Nitrogen balance (NBAL) measurements taken over the last 3 days on each diet were used to determine that the PRO intake (PRO_{IN}) for zero NBAL was 1.43 $g \cdot kg^{-1} \cdot d^{-1}$ (+1SD=1.62 $g \cdot kg^{-1} \cdot d^{-1}$ (recommended intake)). Pre- and post-training measurements of strength and estimates of muscle mass (density, creatinine excretion, and biceps muscle N content) were not different between diet treatments in spite of a significant training effect (increased strength and lean body mass) due to the exercise programme. It was recommended that young males performing body building-type resistance exercise require a dietary PRO_{IN} of 1.62 $g \cdot kg^{-1} \cdot d^{-1}$ during the first 2 months of training and that there are no greater increases in strength or muscle mass by consuming PRO in excess of this amount. The acute effects of resistance exercise upon leucine oxidation and whole body PRO synthesis (WBPS) were studied using stable isotope methodology in the second study. L-[1-^{13}C]leucine was used as a tracer to calculate these variables in 6 healthy, fed, male athletes in response to a 1 h bout of circuit-set resistance exercise. The measurements were

performed prior to, during and for 2 h after exercise and corrections were made for the background $^{13}CO_2/^{12}CO_2$ breath enrichment and bicarbonate retention factor (*c*). Results demonstrated significant increases in the background $^{13}CO_2/^{12}CO_2$ breath enrichment at 1 and 2 h after exercise and in *c* during exercise. At 15 min after exercise *c* was significantly lower than at rest. There were no effects of exercise on leucine oxidation, WBPS, nor the rate of appearance of endogenous leucine or total leucine flux. We concluded that circuit-set resistance exercise did not affect the measured variables of leucine metabolism. In addition, large errors in calculating leucine oxidation and WBPS during resistance exercise can occur if background $^{13}CO_2/^{12}CO_2$ breath enrichment and *c* are not accounted for. In the final study, leucine kinetics and NBAL were used to determine the dietary PRO requirements of sedentary (S) and resistance trained (BB) subjects. Each subject was randomly assigned to each of 3 dietary PRO_{IN} (LP=0.86; MP=1.40; HP=2.40 g PRO·kg^{-1}·d^{-1}) for a total period of 13 days. Over the last 3 d, NBAL measurements were completed and on the last day WBPS and leucine oxidation were determined from L-[1-^{13}C]leucine turnover. Regression analysis of the NBAL data was used to determine the PRO_{IN} for zero NBAL for S=0.69 g·kg^{-1}·d^{-1} and BB=1.41 g·kg^{-1}·d^{-1} and a recommended intake (ZERO intake +1 SD) for S=0.89 g·kg^{-1}·d^{-1} and BB=1.76 g·kg^{-1}·d^{-1}.

For BB the LP diet did not provide adequate PRO and resulted in an *accommodated* state (↓ WBPS vs MP and HP), the MP diet resulted in a state of *adaptation* as evident by the increase in WBPS (vs LP) and no increase in leucine oxidation (vs LP), while the HP diet did not result in increased WBPS compared to the MP diet but leucine oxidation did increase significantly indicating a *nutrient overload*. For S the LP diet provided adequate PRO and increasing PRO_{IN} did not increase WBPS. Leucine oxidation increased for S on HP diet. Taken together, these results indicated that the MP and HP diets were *nutrient overloads* for S. There were no effects of dietary treatment on indices of lean body mass (creatinine excretion, body density) for either group. Overall, this research provided evidence that the PRO requirements of resistance athletes are greater than for sedentary individuals and are above current Canadian and U.S. recommended daily PRO_{IN} for young, healthy males. The second study demonstrated that acute resistance exercise did not affect whole body leucine turnover and, in contrast to endurance exercise, there was no significant increase in leucine oxidation during exercise. In addition, this study demonstrated that changes occur in *c* and background breath enrichment during and after resistance exercise that can have an impact on leucine oxidation measurements. The final study also demonstrated that the positive NBAL that

occurs at high PRO_{IN} does not appear to represent a physiological entity (i.e., no increase in WBPS or lean mass) but is probably an error in the NBAL method.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF FIGURES (cont.)

LIST OF TABLES

LIST OF ABBREVIATIONS

a	arterial
ADP	adenosine diphosphate
amu	atomic mass unit
ANOVA	analysis of variance
APE	atom percent excess
ATP	adenosine triphosphate
B	protein breakdown
BB	body builder
BCAA	branched chain amino acid
BCKAD	branched chain keto acid dehydrogenase
BSTFA	N,O-bis(trimethylsilyl) trifluoroacetamide
c	bicarbonate retention factor
^{13}C	stable isotope of carbon (m.w. = 13)
°C	degree Celsius
CHO	carbohydrate
CV	coefficient of variation
d	day
df	degrees of freedom
δ ‰	delta per mil
EI	electron impact ionization
E_{IN}	energy intake
f	fraction of exogenous amino acid removed
F	F distribution statistic
FAT	dietary fats
GC	gas chromatography
GC/MS	gas chromatography/mass spectrometer
HCl	hydrochloric acid
I	dietary intake
IE	isotopic enrichment
IRMS	isotope ratio mass spectrometer
α-KIC	α-keto isocaproic acid
KMV	keto methylvaleric acid
LEU	leucine
min	minute
mol	gram molecular weight
mL	millilitre
m/z	mass/charge
M	mol/Litre
3-MH	3-methylhistidine
MPS	(mixed) muscle protein synthetic rate
MS	mean square
%MUA	percent motor unit activation

MVC	maximal voluntary contraction strength
n	number of subjects
NBAL	nitrogen balance
N m	newton meter
NS	not statistically significant
O	amino acid oxidation
o-TMS	*o*-trimethylsilyl-quinoxalinol derivative
P	theoretical probability of event *x*
PDB	Pee Dee *Belemnitella americana*
PRO	protein
PTT	peak twitch (evoked) torque
Q	amino acid flux
r	Pearson correlation coefficient
r^2	coefficient of determination
RDA	recommended daily allowance (U.S.)
RER	respiratory exchange ratio (VCO_2/VO_2)
RNI	recommended nutrient intake (Canada)
s	second
S	protein synthesis
S	sedentary subject
SD	standard deviation
SS	sum of squares
t	Student's t distribution statistic
tRNA	transfer ribonucleic acid
TFA-iB	trifluoroacetyl-isobutyl ester derivative
TMCS	trimethylchlorosilane
v	venous
$\dot{V}CO_2$	volume of carbon dioxide consumed
$\dot{V}O_2$	volume of oxygen consumed
$\dot{V}O_{2max}$	maximal oxygen consumption
WBPS	whole body protein synthesis

Chapter 1
GENERAL INTRODUCTION

1.1 Introduction

1.1.1 Historical perspective and rationale for study.

The impact of habitual physical activity on dietary protein (PRO) requirements remains a controversial subject (Brooks et al, 1987; Butterfield, 1987; Lemon, 1987; Wolfe, 1987; Paul, 1989). The ancient Greeks considered meat to be the sole source of energy for muscular contraction, thus, trainers promoted the consumption of large amounts of meat by their athletes during training and prior to competitions (Harris, 1966). The concept that PRO was the predominant fuel for muscular contraction continued until the 1800's (Liebig, 1842; Reviewed in Cathcart, 1925). One of the historical studies that contributed to the displacement of PRO as the preferred fuel for muscle was the account of a climb of the Faulhorn mountain (1956 m) in 1865 by Fick and Wislecenus (Fick and Wislicenus, 1866; Reviewed in Cathcart, 1925). These authors measured the urinary nitrogen (N) output during and after their climb and calculated that the oxidation of PRO could account for only a small portion of the total energy expenditure and concluded that, "the substances by the burning of which force is generated in the muscles are not the

albuminous [protein] constituents of those tissues but non-nitrogenous substances either fats of carbohydrates." (Fick and Wislicenus, 1866; Reviewed in Cathcart, 1925). By the 1900's it became generally accepted that fats and carbohydrates supplied most of the energy for the contraction of skeletal muscle and that PRO contributed minimally but measurably (Pettenkofer and Voit, 1866; Krogh and Lindhard, 1920; Cathcart, 1925) and this is still the currently accepted view (Felig and Wahren, 1975; Gollnick et al, 1985). In his review of the studies of PRO metabolism during exercise to date (1925), Cathcart concluded that, "...there is abundant evidence in support of the view that muscle work leads to a definite though perhaps limited increase in the output of nitrogen, i.e., to increased catabolism of protein and at the same time there is good evidence that the same muscle activity leads to the laying down of protein ... the existent evidence points to a definitely enhanced metabolism of protein as the result of muscle activity." (Cathcart, 1925). It was apparent from this review (Cathcart, 1925) that there was good evidence that PRO metabolism was altered due to physical activity and this may have an impact upon dietary PRO requirements.

More recently, textbooks (Åstrand and Rodahl, 1977), and expert consensus panels (FAO/WHO/UNU, 1985; U.S. Food and Nutrition Board, 1989; Health and Welfare, Canada, 1990) have

taken an even more conservative view and have concluded that exercise has an insignificant effect on PRO metabolism and subsequently on PRO requirements. Åstrand has stated that, "It is only when an individual is undernourished or fasting that proteins may be utilized as a source of metabolic energy." (Åstrand, 1977) and the U.S. Recommended Daily Allowance (RDA) for PRO makes no allowance for an effect of exercise on dietary PRO requirements and states that, "In view of the margin of safety in the RDA, no increment is added for work or training." (U.S. Food and Nutrition Board, 1989).

Many of the early studies concluding that PRO did not contribute to the energy cost of exercise were based solely upon urinary N excretion measurements (Cathcart, 1925). Urinary N (primarily urea N (UN)) measurements underestimate the oxidation of amino acids during exercise due to a reduction in splanchnic blood flow (Rowell, 1974) and in the slow rate of turnover of the urea pool (Golden and Waterlow, 1977). In longer term studies (including post-exercise recovery measurements), performed under more strict environmental and dietary controls, an increase in urinary UN consequent to endurance (Rennie et al, 1981; Tarnopolsky et al, 1988; Brouns et al, 1989a; Tarnopolsky et al, 1990) and weightlifting exercise (Dohm et al, 1982) has been demonstrated. By using amino acid tracers, a more direct

determination of PRO turnover including amino acid oxidation, PRO breakdown and whole body PRO synthesis (WBPS) is possible. Using these methods it has been demonstrated that endurance exercise increased amino acid oxidation and PRO breakdown and decreased WBPS (Rennie et al, 1981; Wolfe et al, 1982; Evans et al, 1983; Wolfe et al, 1984b; Knapik et al, 1991). These changes are probably the major determinant in the measured increase in the PRO requirements of endurance athletes (Gontzea et al, 1974; Tarnopolsky et al, 1988; Brouns et al, 1989a; Friedman and Lemon, 1989; Meredith et al, 1989). There is some evidence that resistance exercise also increases dietary PRO requirements (Consolazio et al, 1976; Torun et al, 1977; Tarnopolsky et al, 1988). This increase is probably due to an increase in the amino acid requirements to support muscle mass accretion (MacDougall et al, 1979; MacDougall, 1986)(discussed below).

Considering these more recent findings, several investigators have attempted to quantitate the PRO requirements of athletes (Gontzea et al, 1974; Gontzea et al, 1975; Consolazio et al, 1976; Torun et al, 1977; Evans et al, 1983; Tarnopolsky et al, 1988; Brouns et al, 1989a; Friedman and Lemon, 1989; Meredith et al, 1989) and several reviews have attempted to outline dietary PRO intake guidelines for athletes (Brotherhood, 1984; Lemon et al, 1984; Lemon, 1987;

Paul, 1989). In spite of the accumulating evidence in the last decade that certain types of exercise have an impact on dietary PRO requirements, it is indeed surprising that national bodies have largely ignored such evidence (U.S. Food and Nutrition Board, 1989; Health and Welfare, Canada, 1990). The U.S. National Research Council (U.S. Food and Nutrition Board, 1989) have rationalized their position on the effect of exercise on dietary PRO requirements in the statement, "Vigorous activity that leads to profuse sweating, such as in heavy work and sports, and exposure to heat increases nitrogen loss from the skin, but with acclimatization to a warm environment, the excessive skin loss is reduced and may be partially compensated by decreased renal excretion (WHO, 1985)." The statement on PRO in the Canadian Nutrition Recommendations (RNI)(Health and Welfare, Canada, 1990) makes no reference to a possible effect of exercise on PRO requirements. It is disturbing that dietary recommendations for sedentary individuals are based upon careful examination of the published literature (Pellett, 1990b); however, statements about exercising individuals appear to have been based upon a paucity of information (WHO/FAO/UNU, 1985; U.S. Food and Nutrition Board, 1989) or ignored altogether (Health and Welfare, Canada, 1990). Many recent, controlled studies (Tarnopolsky et al, 1988; Brouns et al, 1989; Friedman and

Lemon, 1989; Meredith et al, 1989) appear to have been ignored in the (U.S. Food and Nutrition Board, 1989; Health and Welfare, Canada, 1990) analysis of the literature on the subject of PRO requirements for athletes.

Young and Torun (1981) have stated in an extensive review of the effects of physical activity on PRO requirements that, "...exercise in adult subjects results in an increase in the minimal physiologic requirement for specific indispensable [essential] amino acids and/or protein.". Several review articles have suggested an increased PRO requirement for athletes (Brctherhood, 1984; Lemon, 1987; Paul, 1989) and the most recent edition of the authorative textbook of Astrand and Rodahl (1987) contains the statement, "Athletes may perhaps have a somewhat greater protein requirement during periods of intense training, especially that involving muscle strength.". Astrand and Rodahl (1987) go on to state that, "...the exact protein requirements of different categories of athletes in training have, so far, not been established by scientifically controlled metabolic balance studies.".

In spite of statements discounting an effect of exercise on PRO requirements (Astrand and Rodahl, 1977; FAO/WHO/UNU, 1985; Food and Nutrition Board, U.S., 1989), most athletes consume PRO in excess of established requirements (Laritcheva et al, 1978; Short and Short, 1983; Ellsworth et al, 1985;

Faber et al, 1986; van Erp-Baart et al, 1989; Bazzare et al, 1990). In particular, athletes involved in resistance exercise, habitually consume large amounts of PRO (Laritcheva et al, 1978; Faber et al, 1986; Tarnopolsky et al, 1988; Bazzarre et al, 1990). The mean PRO_{IN} for resistance athletes from the data of six studies was 2.34 $\pm$ 0.51 $g \cdot kg^{-1} \cdot d^{-1}$ (Celejowa et al, 1970; Laritcheva et al, 1978; Faber et al, 1986; Tarnopolsky et al, 1988; van Erp-aart et al, 1989; Bazzarre et al, 1990), which is 272 % greater than the 1990 Canadian RNI for PRO for males and females over 19 years of age (Health and Welfare, Canada, 1990). A 1979 survey of football coaches and trainers found that 51 % felt that a high PRO_{IN} was the most important factor for increasing muscle mass (Bentivegna et al, 1979). The results of a 1981 survey of American varsity level athletes found that 98 % of respondents felt that a high PRO_{IN} improved performance and 80 % felt that it was necessary for gaining muscle mass (Grandjean et al, 1981). A survey of American football players found that 18% consumed PRO supplements in addition to a habitual PRO_{IN} already greater than 2.0 $g \cdot kg^{-1} \cdot d^{-1}$ (Short and Short, 1983). Many athletes involved in resistance exercise consume expensive commercial PRO supplements or amino acid preparations (Short and Short, 1983; Faber et al, 1986; Slavin, 1988) and select foods high in PRO (i.e., egg whites,

milk, meats)(Short and Short, 1983; Faber et al, 1986; Tarnopolsky et al, 1988). Thus, there appears to be a disparity between "expert" scientific opinion and the practice of many athletes with respect to PRO supplementation in excess of national (Food and Nutrition Board, U.S., 1989; Health and Welfare, Canada, 1990) and international (FAO/WHO/UNU, 1985) recommendations.

It is important to continue a scientific investigation of the PRO requirements of athletes to provide a background upon which recommendations can be based. Some individuals feel that since many athletes consume PRO in excess of current recommendations (Butterfield, 1987) it is merely an academic exercise to examine the impact of exercise on PRO requirements. However, I feel that it is important to be able, with strong scientific evidence, justify to athletes whether or not their activities have an impact on their dietary PRO requirements for three reasons: 1. Protein is a very expensive (in economic terms) food group to produce and subsequently to purchase (Slavin, 1988). In the U.S. amino acid supplements of arginine, ornithine, and lysine (advertised to increase growth hormone release) cost about \$10.50 d^{-1} (Slavin, 1988) and in Canada casein or soy PRO hydrolysates cost between \$10.00 and \$25.00 per can which would last about one week according to the manufacturers

instructions; 2. The long term consequences of a high PRO_{IN} in otherwise healthy individuals has not been conclusively shown to result in negative health consequences, but, there is evidence that a high PRO_{IN} can have a negative impact on kidney function (Brenner et al, 1982), result in hypercalciuria (Hegsted et al, 1981), and in rats alterations of unknown significance (megamitochondria) have been observed in hepatocytes (Zaragoza et al, 1987); and 3. In contrast to demonstrating that excessive PRO_{IN} are not ergogenic, it is very important to determine whether exercise elevates PRO requirements above those for sedentary individuals for a chronic PRO deficiency may adversely affect immune function (Eskola et al, 1978; Chandra, 1981; Roberts et al, 1986; Keast et al, 1988), result in "runner's" anemia (Yoshimura et al, 1980), or in sub-optimal adaptations to those usually induced by the stress of exercise (increased lean body mass (resistance exercise), increased oxidative enzymes/mitochondria (endurance exercise)).

Although many athletes consume excessive amounts of PRO (Laritcheva et al, 1978; Short and Short, 1983; Faber et al, 1986; Tarnopolsky et al, 1988; van Erp-Baart et al, 1989; Bazzarre et al, 1990), these latter concerns are valid for a significant number of athletes may habitually consume PRO at or below current national recommendations for sedentary humans

(Nutrition Canada Survey, Health and Welfare, Canada, 1983; Deuster et al, 1986; Grandjean, 1989). For days or weeks of pre-competition preparations both body builders ("getting cut")(Walberg et al, 1988; Bazzarre et al, 1990) and wrestlers (attempting to make a weight class)(Grandjean, 1989; Bazzarre et al, 1990; Steen and Brownell, 1990) consume low intakes of PRO and energy. In addition to the possibility of an inadequate absolute PRO_{IN} a hypocaloric diet has a negative effect on PRO turnover (Waterlow, 1986; Hoffer and Forse, 1990; Knapik et al, 1991) and under these conditions dietary PRO requirements in athletes may be further increased (Butterfield, 1987; Walberg et al, 1988).

It is no longer appropriate to discount the importance of PRO requirements for exercising humans for the participation in regular physical exercise has become very popular (Participaction, 1982; Canadian Fitness Survey, Health and Welfare, Canada, 1983). In the Canadian Fitness Survey (1983) it was found that greater than 50% of the population over the age of 20 exercised three or more hours per week for greater than nine months.

The type of exercise needs to be clearly defined in an investigation of the PRO requirements of athletes. Exercise may be considered a continuum from resistance exercise, where 1 (or several) repetition(s) of the maximal (or a high % of

it) weight(s) lifted (1 RM or 7-10 repetitions at 75 % 1RM, respectively) would be at one end, and endurance exercise, where an athlete may perform 20,000 low intensity contractions of the hamstrings during a marathon, at the other. This thesis shall focus on resistance exercise and the athletes shall be referred to as resistance athletes or body builders (BB). The major type of exercise will be circuit weight training on a multi-station apparatus for this is the most popular form of resistance exercise among the population. Most of the athletes studied in this study also engaged in other activities such as rowing, rugby, football and jogging. Subjects were only included if weight training comprised greater than 60 % of their weekly training schedule. Again, this type of athlete was studied for the results would be more applicable to the general population who generally participate in multiple activities (Canadian Fitness Survey, 1983). Consideration will be given to data on endurance exercise as it contributes to the knowledge of PRO metabolism and dietary PRO requirements where knowledge of resistance exercise is lacking.

1.1.2 Factors involved in determining the protein requirements of athletes.

It is very difficult to give a single requirement for "athletes" in general due to the many factors which may

influence the magnitude of an increased PRO requirement under a given physiological state. The major factors to consider include: 1. the type of exercise (endurance and resistance) and the mechanism of impact on PRO turnover; 2. the duration and intensity of exercise; 3. the state of training of an individual; and 4. the macronutrient and energy content of the diet. The PRO requirement of athletes will have to be addressed through a number of studies of different types of exercise and under different physiological states.

a) *Potential mechanisms of increased protein requirements for endurance athletes.*

There is good evidence that endurance exercise increases the oxidation of amino acids based on data from both UN excretion (Lemon and Mullin, 1980; Dohm et al, 1982; Calles-Escandon et al, 1984; Tarnopolsky et al, 1990) and amino acid tracer (Rennie, 1981 et al; White et al, 1981; Wolfe et al, 1982; Evans et al, 1983; Wolfe et al, 1984b; Knapik et al, 1991). Although the relative contribution of amino acids to total energy expenditure is relatively low at between 2-15 % (Dohm et al, 1977; Lemon and Mullin, 1980; Rennie et al, 1981; Evans et al, 1983), the increased amino acid oxidation (Rennie et al, 1981; Wolfe et al, 1982; Evans et al, 1983; Wolfe et al, 1984b; Knapik et al, 1991) may have a negative impact on PRO requirements and in the capacity for PRO accretion

particularly if essential amino acids are limiting. It has been known for some time that skeletal muscle has the metabolic capacity to oxidize the amino acids leucine, isoleucine, valine, aspartate, asparagine, and glutamate (Goldberg and Chang, 1978). Since the branched chain amino acids (leucine, isoleucine and valine) are also essential amino acids, their oxidation would likely have the greatest impact on dietary PRO requirements. The increase in leucine oxidation at only 35 % $\dot{V}O_{2max}$ (Wolfe et al, 1982) and 45 % $\dot{V}O_{2max}$ (Knapik et al, 1991) is in the range of 200-300 % and whole body lysine oxidation is also increased slightly at 76 % (Wolfe et al, 1984b). Evans et al. (1983) have demonstrated that 90 % of the current dietary leucine requirement (FAO/WHO/UNU, 1985) is oxidized during 2 hours of cycling at only 60 % $\dot{V}O_{2max}$. These data support the thesis that dietary PRO requirements would be increased in humans who perform endurance exercise as has been described (Tarnopolsky et al, 1988; Brouns et al, 1989a; Friedman and Lemon, 1989; Meredith et al, 1989).

The observed increases in amino acid oxidation are not surprising since exercise increases the activity of branched chain α-keto acid dehydrogenase (BCKAD), in both liver (Shimomura et al, 1990) and skeletal muscle (Kasperek, 1989; Wagenmakers et al, 1989; Shimomura et al, 1990). This enzyme

is rate limiting in the oxidation of the essential branched chain amino acids (leucine, isoleucine and valine), and an increased activity would increase the dietary requirement for these particular amino acids. Wagenmakers and colleagues (1989) have demonstrated that BCKAD activity is increased in humans to a greater extent when muscle glycogen levels are low. The increased amino acid oxidation observed when carbohydrate intake is limiting (Lemon and Mullin, 1980; Brouns et al, 1989b) is probably linked to the need for amino acids (from protein breakdown) to fulfil an anapleurotic function in maintaining Kreb's cycle intermediates (Brooks, 1987).

In addition to increased amino acid oxidation, it is well known that endurance exercise results in an increase in mitochondrial volume density (with a resultant increase in total oxidative enzymes), capillarization, and myoglobin (Pattengale and Holloszy, 1967; Saltin et al, 1976; Henriksson and Reitman, 1977; Blomstrand et al, 1986; Holloszy and Coyle, 1984; Gollnick and Hodgson, 1986). Together, these adaptations would indicate an increase in the body PRO stores although the impact of these changes on PRO requirements would likely be much smaller than the increase in amino acid oxidation. An increase in muscle protein synthesis (MPS) has been documented following endurance exercise in humans

(Carraro et al, 1990b). The increase in MPS probably restores PRO lost during exercise through the processes of rhabdomyolysis (O'Reilly, 1987; Viru, 1987; Milne, 1988) and net whole body PRO degradation (Lemon and Mullin, 1980; Rennie et al, 1980; Millward et al, 1982; Wolfe et al, 1982) for endurance exercise does not usually result in muscular hypertrophy as does resistance exercise (Alway et al, 1988). In addition, WBPS is increased both acutely (Devlin et al, 1990) and chronically (Lamont et al, 1990) following endurance exercise.

b) Potential mechanisms of increased protein requirements for resistance athletes.

To date there have not been any studies of amino acid turnover during resistance exercise, nor have there been any studies of the activation of BCKAD during resistance exercise. Until such studies are done one can only speculate whether this occurs from indirect evidence. In contrast to the many studies of endurance exercise mentioned above that demonstrated increases in either urine UN or sweat UN excretion, there has only been one study that has demonstrated increased urinary UN excretion in humans performing "powerlifting" (a type of resistance exercise)(Dohm et al, 1982). Two well controlled studies of resistance exercise (circuit and free weight training) have not found an increase

in urinary UN excretion (Hickson et al, 1986; Tarnopolsky et al, 1988). The one study that measured sweat UN losses during resistance exercise did find significant increases compared to an age matched control group of sedentary subjects (Tarnopolsky et al, 1988); however, the impact of this on total N excretion (and NBAL) was small. This suggests that resistance exercise would probably increase amino acid oxidation to some degree but the absolute increase would be less than for endurance exercise. This is a question that needs to be addressed using stable isotope methodology to directly measure amino acid oxidation.

In contrast to endurance exercise (Alway et al, 1988), chronic resistance exercise results in hypertrophy of skeletal muscle (Costill et al, 1979; Goldberg et al, 1979; MacDougall et al, 1979; Laurent and Millward, 1980; Gollnick et al, 1981; Young and Torun, 1981; MacDougall et al, 1982; McDonagh and Davies et al, 1984; Tesch et al, 1984; MacDougall, 1986; Alway et al, 1988). Exercise induced hypertrophy has also been documented in human females (Bell and Jacobs, 1990; Staron et al, 1991), elderly individuals (Frontera et al, 1988), and in hypophysectomized, diabetic and starved rats (Goldberg et al, 1975).

The mechanism behind the hypertrophic process consequent to resistance exercise has traditionally been studied either

by weighting the wing of fowl (Laurent et al, 1978a, 1978b; Laurent and Millward, 1980) or by synergist ablation to induce compensatory hypertrophy of the remaining muscle (Jablecki et al, 1977; Goldspink et al, 1982; Morgan and Loughna, 1989; DeVol et al, 1990). A novel *in vivo* method of electrically induced weightlifting in rat plantar and dorsi flexors has recently been described which more closely mimics the intensity and repetitions that a human resistance athlete would perform (Wong and Booth, 1988; Wong and Booth, 1990a, Wong and Booth, 1990b). Using this model, it was found that MPS was elevated in the tibialis anterior 17 and 41 h after eccentric exercise (Wong and Booth, 1990a) and in the gastrocnemius 17 and 41 h after concentric exercise (Wong and Booth, 1990b).

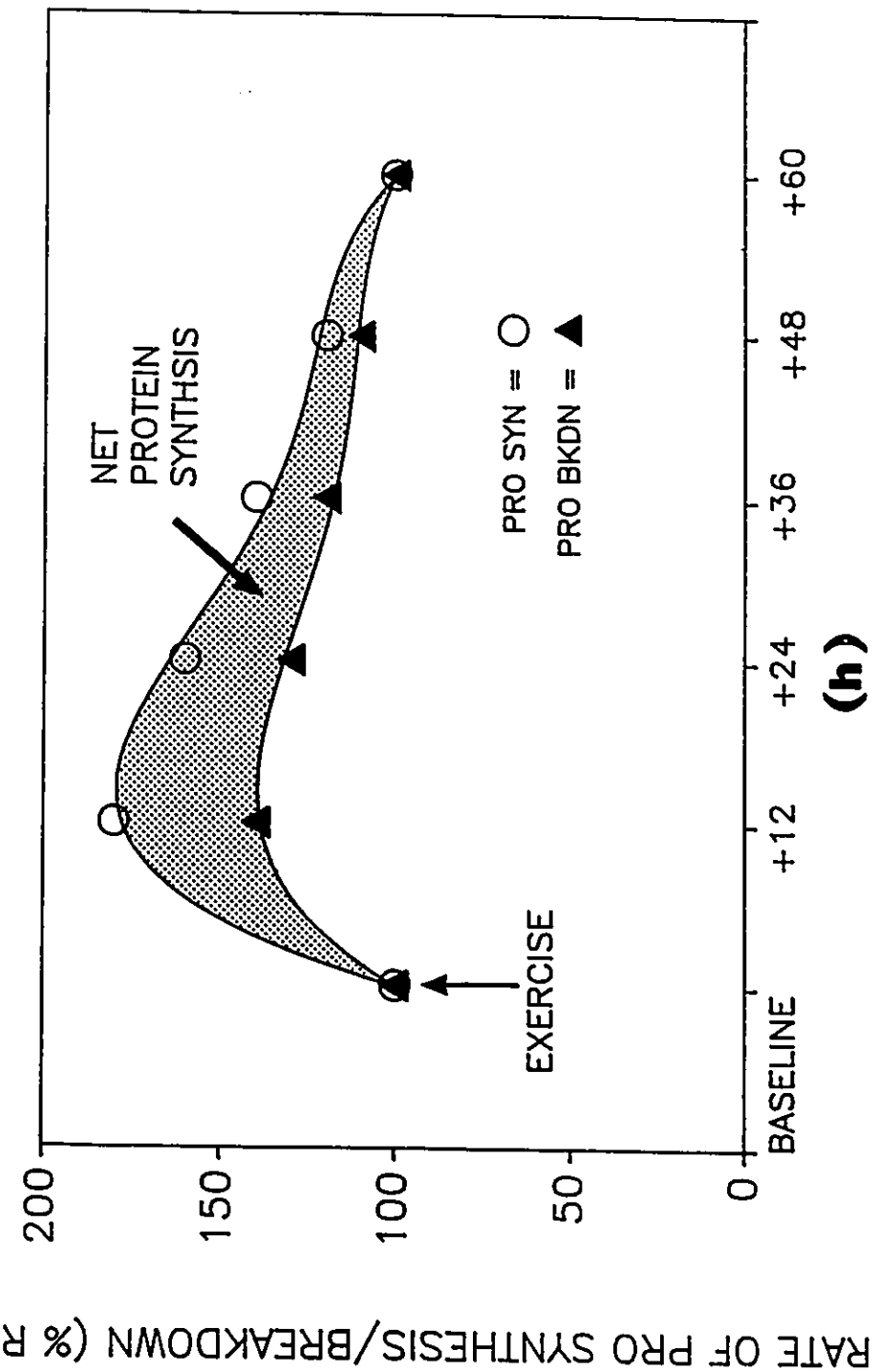
Using these animal models it has been demonstrated that hypertrophy due to resistance exercise is due primarily to an increase in myofibrillar MPS rates (Laurent et al, 1978b; Laurent and Millward, 1980; Booth and Watson, 1985; Wong and Booth, 1990a, 1990b) and partially (<4 %) to increased collagen synthesis (Laurent et al, 1978a; Laurent and Millward, 1980). Somewhat paradoxically, there appears to be a simultaneous increase in muscle PRO breakdown during hypertrophy, but net PRO synthesis is increased (Laurent et al, 1978a; Laurent and Millward, 1980; Goldspink et al, 1982).

The linking of synthesis and degradation appears to be a recurring motif in the modelling of skeletal muscle (Laurent and Millward, 1980; Goldspink et al, 1982; Millward, 1984; Frontera et al, 1988). Laurent and Millward (1980) have reviewed many of the animal model studies of hypertrophy and have summarized the effects of resistance exercise on muscle PRO turnover and how this results in net muscle hypertrophy (Figure 1).

In the human there have been very few reports of MPS changes consequent to resistance exercise (Yarasheski et al, 1990; Chesley et al, 1991). Yarasheski et al. (1990) found an increased quadriceps MPS in young men lifting weights for 12 weeks and others have stated that resistance exercise increased MPS in young men after 3 weeks but the actual results were not given in the paper (Rennie et al, 1980). Chesley et al. (1991) found large increases in MPS in the biceps of an exercised compared to rested arm in young men lifting weights at both 4 (increased 43 %) and 24 h (increased 80 %) after exercise. Increased MPS in humans has been demonstrated using electrical stimulation under conditions of paraplegia (Pacy et al, 1988) and immobilization (Gibson et al, 1988).

In humans performing resistance exercise there have not as yet been any tracer studies of muscle PRO breakdown.

Figure 1. Protein turnover and muscle hypertrophy. Theoretical prediction from the data (Laurent et al, 1978b; Wong and Booth, 1990a,b).


RATE OF PRO SYNTHESIS/BREAKDOWN (% REST)
200
150
100
50
0
BASELINE
+12
+24
+36
+48
+60
(h)
EXERCISE
NET
PROTEIN
SYNTHSIS
PRO SYN = ○
PRO BKDN = ▲

Several studies have however, used 3-methylhistidine (3-MH) as an indicator of myofibrillar PRO breakdown (Hickson et al, 1985; Hickson et al, 1986; Frontera et al, 1988; Horswill et al, 1988; Pivarnik et al, 1989). 3-MH is produced in skeletal muscle actin and fast-myosin proteins as a posttranslational modification of histidine residues (Young and Munro, 1978). Since it is not re-utilized for PRO synthesis nor oxidized after PRO breakdown, its excretion in urine has been demonstrated to be an indicator of myofibrillar PRO breakdown (Young and Munro, 1978; Lukaski et al, 1981). In spite of controversies over the validity of 3-MH in quantitatively measuring myofibrillar PRO breakdown (Rennie and Millward, 1983; Thomas and Ballard, 1983), a recent study has demonstrated that most of the urinary 3-MH does come from skeletal muscle and can be used as an valid indicator of myofibrillar PRO breakdown (Sjolin et al, 1989). The acute effect of resistance exercise on 3-MH excretion is controversial with some studies finding no exercise effect (Hickson et al, 1986; Horswill et al, 1988) and another finding a positive effect (Dohm et al, 1982). On the other hand, it appears that chronic resistance exercise does increase PRO breakdown as determined by 3-MH measurements (Hickson and Hinkelmann, 1985; Frontera et al, 1988; Pivarnik et al, 1989). Thus, in humans, it appears that the muscular

hypertrophy due to chronic resistance exercise (MacDougall et al, 1979; MacDougall, 1986; Alway et al, 1988) is due to an increased MPS (Yarasheski et al, 1990; Chesley et al, 1991) with a lesser increase in myofibrillar PRO breakdown (Hickson and Hinkelmann, 1985; Frontera et al, 1988; Pivarnik et al, 1989). This supports the concept that skeletal muscle hypertrophy is a coupled process of both increased synthesis and degradation (increased net synthesis).

The molecular mechanisms which modulate hypertrophy are complex and a detailed examination is beyond the scope of this thesis and the reader is referred to several reviews for a full discussion (Booth and Watson, 1985; Booth, 1989).

There is evidence that endocrine factors may influence the adaption of skeletal muscle to resistance exercise (Hakkinen et al, 1988; Boone et al, 1990; Kraemer et al, 1990). Testosterone administration to sedentary individuals resulted in an increase in MPS and in lean body mass (Griggs et al, 1989). Both total (Hakkinen et al, 1988; Kraemer et al, 1990) and free (Hakkinen et al, 1988) testosterone elevations have been found in humans following acute resistance exercise. Another endocrine change induced by resistance exercise that would have a positive effect on muscle accretion would be increased growth hormone and insulin-like growth factor (IGF-1) levels (Kraemer et al,

1990). These cannot be the sole mechanisms involved in muscular hypertrophy for females (low testosterone) experience hypertrophy when performing resistance exercise (Bell and Jacobs, 1990; Staron et al, 1991) and rats can demonstrate compensatory hypertrophy after hypophysectomy (↓ sex hormones, ↓ thyroid hormones, ↓ growth hormone (and IGF-1)), diabetes (↓ insulin) and starvation (Goldberg et al, 1975).

The primary stimulus for hypertrophy remains elusive but stretch, damage/repair, or cellular energy charge may be involved (Palmer et al, 1983; Booth and Watson, 1985; Booth, 1988; Yamada et al, 1989; Chesley et al, 1991). Evidence is accumulating to suggest that paracrine and autocrine factors are more important regulators of hypertrophy than are endocrine factors (Palmer et al, 1983; Jennische and Hansson, 1987; Booth, 1988; Booth, 1989; Yamada et al, 1989; DeVol et al, 1990; Kraemer et al, 1990). Fibroblast growth factor (Yamada et al, 1989), IGF-1 (Jennische and Hansson, 1987; DeVol et al, 1990), and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Palmer et al, 1983; Smith and Rennie, 1991) are among those involved in increasing MPS and hypertrophy of skeletal muscle.

Irrespective of the signals involved in hypertrophy, there is increased expression of the genes encoding for specific myofibrillar proteins (myosin and actin), which results in muscle hypertrophy (Pette and Vrbova, 1985; Booth,

1989; Matthew and Loughna, 1989; Wong and Booth, 1990a,b). The available evidence has suggested that acute resistance exercise increases MPS through translational and post-translational mechanisms, while chronic exercise results in increased gene transcription (Pette et al, 1984; Booth, 1988; Morgan and Loughna, 1989; Wong and Booth, 1990a,b). The effect of nutrition (Rucker and Tinker, 1986) and exercise (Pette et al, 1984; Booth, 1988; Booth, 1989; Morgan and Loughna, 1989; Wong and Booth, 1990a,b) on the expression of specific genes (including protooncogenes (Booth, 1988; Booth, 1989; Schonthal, 1990)) is an exciting area for future investigation, but it is important first to examine the effects of diet and exercise on the overall process of PRO synthesis prior to examining these more specific control loci.

In summary, endurance exercise could likely exert its major impact on PRO requirements by increasing the oxidation of certain essential amino acids, while resistance exercise would exert its effect through a need for amino acids to support muscular hypertrophy.

c) Duration and intensity of exercise.

It is well known that endurance exercise results in intra-muscular glycogen depletion (Hultman, 1989). The effects of this depletion on the activity of BCKAD (Wagenmakers et al, 1989) and urea excretion (Lemon and

Mullin, 1980; Brouns et al, 1990b) have been discussed above. Since significant glycogen depletion at an exercise intensity of 70-85 % $\dot{V}O_{2max}$ would be expected to occur after about 70-90 min (Hultman, 1989), amino acid oxidation should increase in proportion to the decrease in muscle glycogen (Wagenmakers et al, 1989). Plasma UN appeared to increase significantly at about 70 min during prolonged exercise (Haralambie and Berg, 1976) and pre-exercise muscular glycogen depletion resulted in large increases in sweat UN excretion (Lemon and Mullin, 1980). Since repeated daily endurance exercise can result in a progressive depletion of muscle glycogen (Costill et al, 1971), this may further increase dietary PRO requirements for an athlete who trains on a daily basis.

There have not been any studies examining the duration of resistance exercise on glycogen depletion or amino acid oxidation. Resistance exercise can deplete muscle glycogen (Tesch and Kaiser, 1984; Costill, et al, 1991), and if the subject does not consume CHO, continued exercise would increase BCKAD activity and amino acid oxidation (Wagenmakers et al, 1989). In the fed state, glycogen depletion over a bout of resistance exercise would likely be minimal for resynthesis could occur during the rest period (Reed, et al, 1989). A study of the effect of resistance exercise on PRO requirements needs to state the duration of the exercise bout,

exercise duration and the nutritional status of the subjects.

BCKAD activity increases in proportion to exercise intensity and inversely with cellular energy charge (Kasperek et al, 1985; Kasperek and Snider, 1987; Kasperek, 1989). In addition, the rate of glycogen breakdown increases with the intensity of exercise (Hultman, 1989). These observations may suggest that the amino acid oxidation should increase with the intensity of exercise. It has been shown in rats that amino acid oxidation increases with the intensity of endurance exercise (White et al, 1981; Lemon et al, 1982; Henderson et al, 1985), however, there have been no studies with resistance exercise. It would seem logical that resistance exercise would show a similar positive effect for a contraction at a high percentage of the 1 RM would stress cellular energy stores to a greater extent than a less forceful one and activate BCKAD to a greater degree (Kasperek, 1989). In addition, resistance exercise performed at a higher percentage of 1 RM (70%) resulted in greater glycogen depletion than did contractions at a lower percentage of 1 RM (30%)(Costill, et al, 1991). Further evidence to support an effect of intensity on amino acid comes from the observation that urea excretion was significantly increased in "powerlifters" (Dohm et al, 1982) and not in body builders (Tarnopolsky, 1988). It is assumed that the "powerlifters" performed exercise at a higher

intensity (% 1RM) than the body builders but this could not be ascertained from the data given for the "powerlifters" (Dohm et al, 1982). Again, this demonstrates the paucity of information about resistance exercise and the need for future studies to define the intensity of exercises performed.

d) The state of training of an individual.

One of the earliest studies to suggest that training influenced PRO turnover was one in which NBAL measurements were made over a 20 d period in men performing endurance exercise (Gontzea et al, 1975). The observed large, initial negative NBAL was progressively less negative as training continued (Gontzea et al, 1975). Several other studies have been conducted at low exercise intensities of endurance exercise and similar results have been obtained (Butterfield and Calloway, 1983; Todd et al, 1984). The interpretation of such studies has been that training resulted in adaptive changes which limited the negative effect of exercise on PRO requirements (Todd et al, 1984; Butterfield, 1987). However, in the studies mentioned above (Gontzea et al, 1975; Butterfield and Calloway, 1983; Todd et al, 1984), the intensity of the exercise was not increased to match the training induced increases in $\dot{V}O_{2max}$, and as such, the exercise load would have resulted in a progressively lower relative exercise intensity.

Since endurance exercise results in an increased mitochondrial volume density (Holloszy and Coyle, 1984), it would follow that the capacity for leucine oxidation via BCKAD (a mitochondrial enzyme) would be increased. In rats, the oxidation of leucine is increased in trained compared to control rats (Dohm et al, 1977; Henderson et al, 1985). In humans who have been training for many years the PRO requirements remain significantly elevated above those for sedentary individuals (Tarnopolsky et al, 1988; Friedman and Lemon, 1989; Meredith et al, 1989) which suggests that little adaptive decrease in amino acid oxidation occurs when subjects maintain exercise at the same relative intensity as training adaptations occur.

The pattern of adaptation in PRO requirements for resistance exercise may go in an opposite direction to those described for endurance athletes and result in greater PRO needs in the early stages of training (novice) as compared to an elite athlete who has trained for several years. It is known that greater myofibrillar hypertrophy occurs over the first few months of resistance exercise (MacDougall et al, 1979; McDonagh and Davies, 1984) than is possible in subjects who have trained for many years (Hakkinen et al, 1988). In an animal model of wing weighting the greatest muscle PRO accretion occurred in the early stages of the weight bearing

(Laurent et al, 1978). PRO requirements may be greatest for those who are in the earlier (months) rather than the later (years) stages of training due to the greater ability to accrete muscle mass. The resistance athlete who has trained for many years may be at a "plateau" with a limited ability to further increase muscle mass. Support for this comes from the fact that the calculated PRO requirements for elite (trained for > 3 y) body builders was only 16 % greater than for sedentary controls (Tarnopolsky et al, 1988). There have not been any prospective trials that have investigated how PRO requirements change during the adaptation to resistance exercise. It is likely that the novice body builder would have greater PRO requirements than the elite/habitual body builder. It is important to state the training history and the current state of training of the subjects in any study determining the PRO requirements of resistance athletes for these reasons.

e) The energy and macronutrient content of the diet.

It has been known for quite some time that energy intake has a positive influence on NBAL (Calloway and Spector, 1959). At low energy intakes there is a larger positive effect on NBAL with additional energy as compared to higher energy intakes (Chiang and Huang, 1988; Welle et al, 1989; Elwyn, 1990). At inadequate energy and PRO_{IN}, NBAL is more positive

when additional energy is provided as CHO compared to fat but both are as effective at or above adequate intakes of energy and PRO (Elwyn, 1990). One study examined the effect of a moderate PRO (0.80 $g \cdot kg^{-1} \cdot d^{-1}$)/high CHO (70 % of energy intake)/low energy (18 $kcal \cdot kg^{-1} \cdot d^{-1}$)(MP/HC) diet compared to a high PRO (1.6 $g \cdot kg^{-1} \cdot d^{-1}$)/ moderate CHO (50 % of energy intake)/low energy (18 $kcal \cdot kg^{-1} \cdot d^{-1}$) (HP/MC) diet on NBAL in young male athletes performing circuit weight lifting exercises (Walberg et al, 1988). As expected the HP/MC group had a more positive NBAL (+ 4.13 $g \cdot d^{-1}$) than the MP/HC group (- 3.19 $g \cdot d^{-1}$)(i.e., PRO was more effective than CHO in maintaining NBAL when energy intake was low)(Walberg et al, 1988). It would have been a more informative study had PRO_{IN} remained constant and the % energy intake from CHO been manipulated, but further evidence was provided that a PRO_{IN} close to Canadian RNI (Health and Welfare, Canada, 1990) and the US RDA (U.S. Food and Nutrition Board, 1989) was inadequate for young men performing body building exercises even when consuming a high CHO intake.

In a study to determine the PRO requirements for resistance athletes or to examine aspects of metabolism, it is important to first determine the habitual energy and % CHO intake of the target study population. If the habitual energy intake of the subject group is under-estimated, the PRO

requirements derived from NBAL results would be falsely elevated. Conversely, if the energy intakes are over-estimated, the PRO requirements would be falsely under-estimated. Changes in the percent of energy derived from CHO would be a less important factor at adequate energy intakes.

One method to estimate the energy requirements for resistance athletes is to add the measured energy cost of a usual exercise bout to an estimate for an active, but not athletic, individual. The U.S. RDA recommends that men aged 18-49 y consume 39 $kcal \cdot kg^{-1} \cdot d^{-1}$ (US Food and Nutrition Board, 1989; Pellet, 1990a). A recent study of 11 men performing body building exercises (3-4 sets of 10 repetitions of each exercise at 70 % of the 1 RM for 30-35 min) found that the total energy cost was 2.4 $kcal \cdot kg^{-1} \cdot d^{-1}$(Pivarnik et al, 1989). This would indicate that the energy intake for body builders would be about 41 $kcal \cdot kg^{-1} \cdot d^{-1}$. The data from several dietary analyses of body builders has demonstrated that the habitual energy intakes ranged from 38 to 60 $kcal \cdot kg^{-1} \cdot d^{-1}$ with a mean intake of about 44 $kcal \cdot kg^{-1} \cdot d^{-1}$ (Faber et al, 1986; Tarnopolsky et al, 1988; Grandjean, 1989; van Erp-Baart et al, 1989). Prior to a competition, energy intakes of 25 $kcal \cdot kg^{-1} \cdot d^{-1}$ have been observed in elite athletes to "get cut" (Bazzarre et al, 1990).

It is important that the energy intake of the phase of training be determined and reported with the calculated PRO requirements in a given study. Protein requirements determined while subjects are consuming energy above or below habitual intake levels will not be accurate.

1.2 Methods of determining protein requirements.

The factorial method to determine PRO requirements involves placing humans on a PRO free diet and measuring the obligatory nitrogen (N) losses in urine, feces, and skin. To these losses are added adjustments for the inefficiency in PRO utilization, the quality of the PRO consumed, and for growth requirements (Pellett, 1990b). It is now apparent that the factorial approach under-estimates the safe dietary PRO_{IN} (Garza et al, 1977; FAO/WHO/UNU, 1985; U.S. Food and Nutrition Board, 1989; Pellett, 1990b) and the NBAL method is considered to be the current gold standard (FAO/WHO/UNU, 1985; U.S. Food and Nutrition Board, 1989; Pellett, 1990b).

The NBAL method involves the measurement of the difference between total dietary N intake and losses via urine, feces, sweat and other miscellaneous routes of excretion. A response curve is determined from subjects fed two or more different PRO_{IN} and regression analysis is used to determine the zero intercept by extrapolation or ideally, interpolation. To meet the PRO needs for the majority (>97.5

%) of the population, two standard deviations (SD) are added to the zero intercept to determine a safe intake value (Pellett, 1990b). Given ideal technical conditions, it is obvious that the accuracy of the calculation will be a function of the number of subjects and number of PRO_{IN} examined as well as how close the NBAL PRO_{IN} are to the estimated zero NBAL level (the latter is usually known only after a study is completed but a reasonable estimate can be obtained from previous studies). The NBAL derived recommended safe PRO_{IN} for young males over 19 y of age is 0.86 $g \cdot kg^{-1} \cdot d^{-1}$ in Canada (Health and Welfare, Canada, 1990) and 0.85 $g \cdot kg^{-1} \cdot d^{-1}$ in the U.S. (US Food and Nutrition Board, 1989).

From NBAL data it is known that the body adapts to a new PRO_{IN} over a period of several days (Rand et al, 1976; Oddoye and Margen, 1979). This adjustment of N output to a new PRO_{IN} occurs within eight days and is about the same for both a decreasing intake or an increasing intake (Rand et al, 1976; Oddoye and Margen, 1979). NBAL studies should therefore have an adaptation period of at least this duration or be performed at a pre-determined habitual PRO_{IN}.

It is known that the slope of the NBAL response is not linear across a broad range of PRO_{IN} and decreases at intakes close to the zero intercept (Pellett, 1990b). The implications of this observation are that the accuracy of the

prediction of PRO requirements based on NBAL data will be greater if several PRO_{IN} close to the zero NBAL level are studied. The observed slope makes physiological sense at low PRO_{IN} where the efficiency of PRO utilization is increased (Waterlow et al, 1978; Waterlow, 1986). At PRO_{IN} in excess of physiological requirements, PRO utilization decreases and the slope of the response curve does not approach zero as expected (Hegsted, 1975; Oddoye and Margen, 1979). This positive NBAL at high PRO_{IN} may indicate a slight increase in splanchnic protein accretion (Lin and Huang, 1982); however, the calculated net accretion of PRO expected at high PRO_{IN} far exceeds the amount required for gut hypertrophy and probably indicates a methodological error at high PRO_{IN} (Hegsted, 1975). This is a particularly disturbing problem for those using this method for, as yet, there is no good explanation for the observation. Hegsted (1975) has suggested that this observation could be a combination of several factors including: 1. a bias in favour of over-estimating intake and under-estimating losses (magnifying effect at higher N intakes); 2. unmeasured losses (ie. N_2 gas, NH_3 gas, sweat N losses, and misc. losses); and 3. insufficient adaptation periods. In addition to these concerns about the NBAL method, it has recently been suggested that other limitations to the NBAL method include: 1. N equilibrium can be obtained at

potentially sub-optimal PRO turnover dynamics (ie. decreased WBPS); 2. excess energy intakes improve NBAL and underestimate requirements at maintenance energy intakes; and 3. no satisfactory validation is available for NBAL studies. (Young et al, 1989).

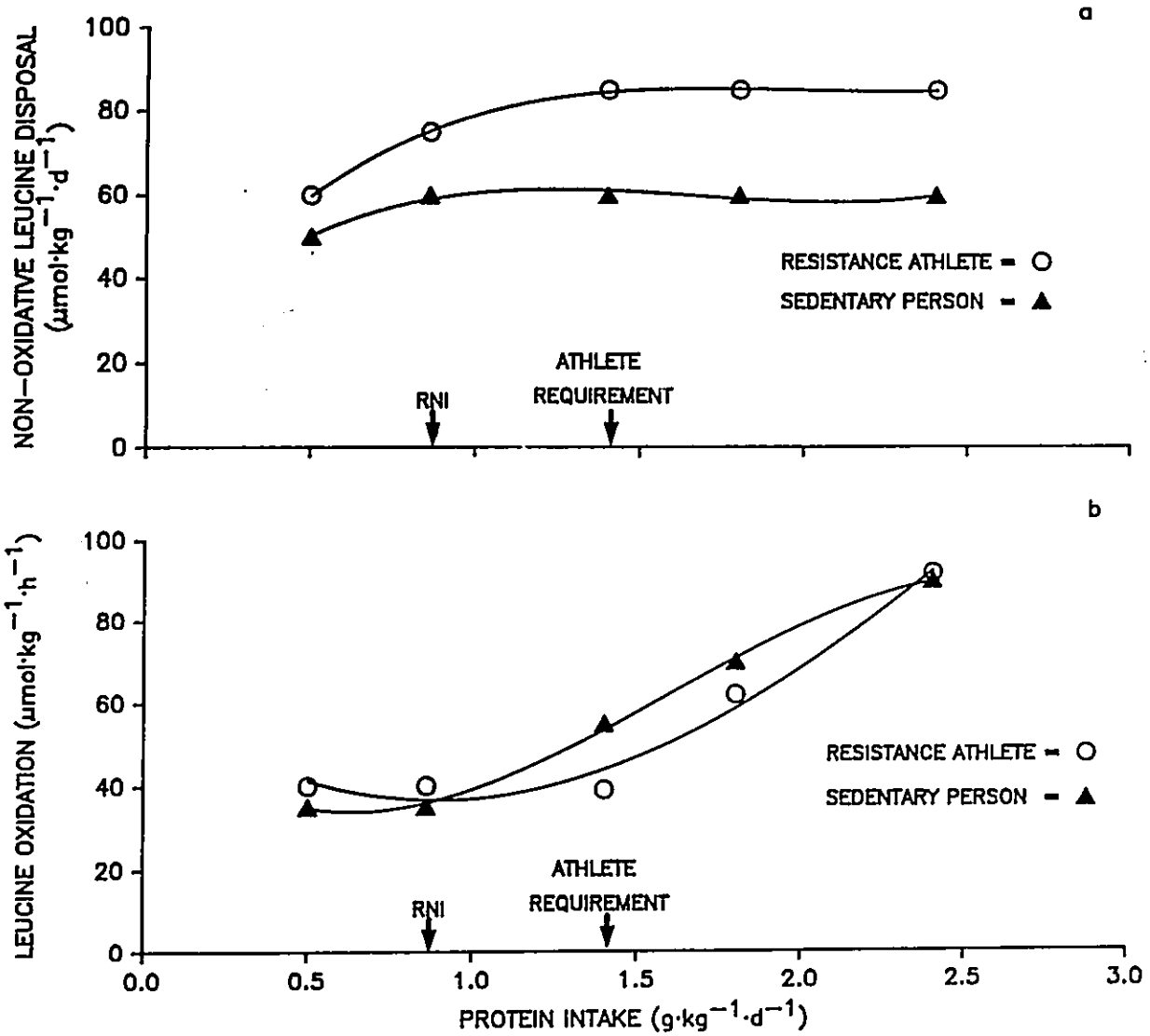
In an attempt to provide physiological evidence in support of NBAL studies some investigators have measured certain plasma proteins (i.e. albumin, pre-albumin, retinol binding protein, Waterlow and Stephen, 1969; Waterlow et al, 1978) or liver enzymes (i.e. aspartate aminotransferase and alanine aminotransferase, Garza et al, 1977) at different PRO_{IN} to determine the adequacy of a given PRO_{IN}.

It had been observed for some time that PRO synthesis decreased at low PRO_{IN} and that amino acid oxidation increased at excessive PRO_{IN} (reviewed in Waterlow and Stephen, 1969; Waterlow et al, 1978; Waterlow, 1986). From these observations, and given the advances in stable isotope methodology to measure PRO turnover in the late 1970's (Matthews et al, 1980; Halliday and Read, 1981), it was proposed that PRO turnover studies should be combined with NBAL studies to provide further insight into the processes involved in adapting to a given PRO_{IN} and to determine the adequacy of a given intake (Young et al, 1981a, 1981b). It was hypothesized that WBPS (and MPS) would be decreased at

sub-optimal PRO_{IN}, would plateau at intakes in excess of requirements and that amino acid oxidation would increase exponentially at excessive PRO_{IN} (Young et al, 1981a, 1981b) (Figure 2). Young and colleagues provided initial support for these concepts in sedentary subjects studied in the fed state (Motil et al, 1981).

It may be useful to conceptualize the PRO status of the human within a framework of: 1. *nutrient deficiency* - where there are obvious signs of PRO loss (ie. kwashiorkor); 2. *accommodation* - where there is a compromise of some physiological function in an attempt to maintain PRO homeostasis (ie. decreased MPS in an human on a diet low in PRO); 3. *adaptation* - where the body has ample reserve to deal with day to day stresses on PRO metabolism and still maintain optimal amino acid flux through biosynthetic pathways (ie. upper respiratory tract infection (increased glutamine flux in lymphocytes) and resistance athlete is able to maintain MPS); and 4. *nutrient overload* - amino acids are provided in excess of need and are oxidized for energy or converted to FAT or CHO and stored (ie. resistance athlete who consumes 100 g PRO powder in addition to a diet providing 2.0 g $PRO \cdot kg^{-1} \cdot d^{-1}$) (Beaton, 1986; Young and Bier, 1987). Stable isotope tracer studies of PRO turnover combined with NBAL methods may help to identify these physiological states in an athlete and

Figure 2. a) Theoretical plateau in NOLD (an estimate of WBPS) for a resistance athlete (BB) and a sedentary person (S). Plateau occurs around 0.86 $g \cdot kg^{-1} \cdot d^{-1}$ for S (Pellett, 1990b) and 1.4 $g \cdot kg^{-1} \cdot d^{-1}$ (Tarnopolsky et al, 1988). b) Theoretical increase in leucine oxidation occuring at about the same dietary PRO_{IN} as for NOLD.


a
NON–OXIDATIVE LEUCINE DISPOSAL (μmol·kg⁻¹·d⁻¹)
100
80
60
40
20
0
RESISTANCE ATHLETE
SEDENTARY PERSON
ATHLETE REQUIREMENT
RNI
b
LEUCINE OXIDATION (μmol·kg⁻¹·h⁻¹)
100
80
60
40
20
0
RESISTANCE ATHLETE
SEDENTARY PERSON
ATHLETE REQUIREMENT
RNI
0.0
0.5
1.0
1.5
2.0
2.5
3.0
PROTEIN INTAKE (g·kg⁻¹·d⁻¹)

provide objective evidence for an optimal PRO_{IN}.

To date there have not been any studies of PRO turnover in human resistance athletes using stable isotope tracers, nor have there been any studies combining PRO turnover studies with NBAL measurements in this group. Since the major influence of resistance exercise is on the process of PRO synthesis and this process seems to be the predominantly regulated process in PRO turnover (Smith and Rennie, 1990), I feel that this process would be the most valid to measure in determining the PRO requirements for resistance athletes. Using L-[1-^{13}C]leucine as a tracer to measure whole body PRO turnover in resistance athletes it would be expected that PRO_{IN} in excess of requirements would result in a plateauing of WBPS and at deficient intakes WBPS would be reduced.

1.3 Objectives and Hypotheses

The overall objective of this thesis was to provide information on PRO turnover in the human performing resistance exercise to further understand the physiological adaptations involved and to provide a determination of dietary PRO requirements. In order that the results of the studies presented in this thesis provided valid information from which objective conclusions could be drawn, care was taken to consider a number of important factors not always controlled for in past studies examining the PRO requirements of

athletes, such as: 1. training type, intensity, volume, and frequency; 2. habituation to a training stimulus; 3. precise measurements of protein metabolism (amino acid turnover) and lean body mass (ie. densitometry); and 4. adherence to established NBAL techniques (adaptation periods adequate for PRO_{IN}, attempt to account for all routes of loss (ie. sweat), biochemical determination of diet N and energy content, dietary and collection compliance verified, and collection periods made during habitual activities for three days).

It was hypothesized, *a priori*, that humans performing resistance exercise would have higher PRO requirements than sedentary humans and these differences would be reflected in differences in whole body PRO turnover as determined by NBAL and stable isotope methods. The second chapter in this thesis will discuss the theories and development of stable isotope methodology. Following this, 3 studies are presented individually in 3 chapters and each study has specific objectives and hypotheses.

1.3.1 Objectives and hypotheses of study 1 (Chapter 3).

The objective of this study was to determine the PRO requirements of athletes performing intensive resistance exercise in the early stages of training (novice) and to determine whether a very high PRO_{IN} (PRO supplement = 2.6 $g \cdot kg^{-1} \cdot d^{-1}$) would result in greater strength and/or lean body mass

accretion compared to a lower PRO_{IN} (1.35 $g \cdot kg^{-1} \cdot d^{-1}$).

The hypotheses were: 1. During the early phases of an advanced body building training programme, a dietary PRO_{IN} of 2.6 $g \cdot kg^{-1} \cdot d^{-1}$ would not result in increased strength or lean body mass when compared to a PRO_{IN} of 1.35 $g \cdot kg^{-1} \cdot d^{-1}$; 2. The nitrogen balance (NBAL) derived PRO requirements for these subjects would be greater than current Canadian and U.S. recommendations, but would be lower than the PRO_{IN} habitually consumed by elite resistance athletes; and 3. The NBAL derived PRO requirements for the novice body builder subjects would be greater than requirements previously determined for elite resistance athletes (Tarnopolsky, et al, 1988).

1.3.2 Objectives and hypotheses of study 2 (Chapter 4).

The objective of this study was to determine the impact of circuit weight (resistance) exercise on amino acid turnover to gain insight into the mechanism behind the elevated PRO requirement for athletes performing this type of exercise.

The hypotheses were: 1. Leucine oxidation during circuit weight exercise would be increased but the magnitude of the increase would be less than that observed for endurance athletes; 2. WBPS would be decreased during the exercise bout and increased after exercise; and 3. The bicarbonate retention factor (*c*) would be increased during exercise and background breath $^{13}CO_2/^{12}CO_2$ enrichment would decrease slightly

during exercise.

1.3.3 Objectives and hypotheses of study 3 (Chapter 5).

The objective of this study was to determine the PRO requirements for both sedentary (S) and resistance-trained (body builders (BB)) men by examining NBAL and leucine turnover in response to three levels of dietary PRO_{IN} (low PRO (LP) = 0.86 $g \cdot kg^{-1} \cdot d^{-1}$; moderate PRO (MP) = 1.40 $g \cdot kg^{-1} \cdot d^{-1}$; and high PRO (HP) = 2.4 $g \cdot kg^{-1} \cdot d^{-1}$).

The hypotheses were: 1. WBPS would be greater for BB than for S; 2. WBPS would be decreased for BB on a dietary PRO_{IN} at the Canadian R.N.I. (LP)(*accomodation*), increased on a PRO_{IN} previously recommended for BB (MP)(Tarnopolsky, 1988), and would not increase any further on an high PRO_{IN} (HP) (*nutrient overload*), while for S WBPS would not be influenced by dietary PRO_{IN} for the LP diet would already be above their physiological requirement (*adaptation*); 3. Leucine oxidation would increase for BB from the MP to the HP diet while it would increase for S from the LP to MP and HP diets; and 4. The NBAL determined PRO requirements for BB would be greater than the Canadian R.N.I. and greater than for S and these requirements would be close to those predicted using the leucine kinetic approach.

Chapter 2

MODELS AND METHOD DEVELOPMENT

2.1 Stable isotope model.

2.1.1 General isotope terminology.

A tracer is an element or molecule that can be introduced into a physiological system to study the kinetics of a certain biochemical pathway or physiological process. Tracers are distinguished from the natural element or molecule (tracee) in the system based upon some distinctive physical property. Radio-active tracers have been used to study the kinetics of several biochemical pathways and processes for many years. However, there are concerns about the potential long-term health consequences of radio-active tracers in human metabolism research (Wolfe, 1984c). With the recent developments in mass spectrometry technology (Halliday and Read, 1981), an interest in the use of stable isotopes has emerged. Isotopes of a given element have the same atomic number (number of protons) but differ in atomic mass (due to differences in the number of neutrons). A stable isotope is one that does not undergo spontaneous decay, hence it does not emit potentially hazardous rays (ie. gamma rays) or particles (ie. alpha or beta particles) as radio-active tracers do.

There are a number of naturally occurring isotopes for most of the elements. Carbon for example, has seven isotopes with the isotopic abundance of the two most common isotopes being: ^{12}C (98.9 %); and ^{13}C (1.1 %)(Schoeller et al, 1980). The relatively high natural "background" abundance of certain isotopes provides one of the challenges to their use for the measuring instrument must resolve differences above the natural abundance or background level of a particular isotope (an important point discussed below).

In the following discussion, stable isotope terminology will be used but in most cases the terminology is interchangeable with that of radio-tracers. For example, the presence of a radio-active tracer in a system is measured by a scintillation counter and is expressed as specific activity in units of counts or disintegrations per minute (c.p.m. or d.p.m.). A stable isotope tracer is measured by mass spectroscopy and is expressed as enrichment in units of atom percent excess (APE) or delta per mil ($\delta^{13}C(‰)$). With isotope ratio mass spectroscopy (IRMS, discussed below) the $^{13}CO_2/^{12}CO_2$ ratios are expressed as delta per mil (part per thousand, $\delta^{13}C(‰)$):

$$^{13}C = [(r_{sa} - r_{st})/r_{st}] \cdot 1000\ ‰$$

where r_{sa} is the sample isotope ratio, r_{st} is the isotope ratio of the standard, and 1000 ‰ increases the result for

convenient expression. The convention is to express the delta per mil values relative to the international standard, PDB (*Belemnitella americana*)(‰ $\delta_{PDB}{}^{13}C$) which is 1.1237 % ^{13}C (Wolfe, 1984c; Barstow et al, 1989). In metabolic studies the convention is to express enrichments as atom percent excess (APE), for the results can therefore be directly related to radio-isotope results (Wolfe, 1984c). One APE refers to an increase in the enrichment of a sample by 1 molecule in 100. To determine the APE a baseline value is required to which subsequent samples can be compared during the infusion. Conversion from $\delta^{13}C$ ‰ to APE can be made from the equation (Barstow et al, 1989):

$$1\ \delta^{13}C\ ‰ = 1.123 \cdot 10^{-3}\ APE$$

In this thesis the APE terminology will be used in all calculations and $\delta^{13}C$ ‰ will be used only to show the effect of diet on background breath ^{13}C enrichment.

2.1.2 Model of protein turnover.

In general the estimations of whole body protein turnover using isotope tracers have used one of two approaches. One method is the flooding-dose method where a large dose of tracer amino acid is infused into the system and the decay response kinetics are measured by sampling the amino acid in some compartment. This method has the theoretical advantage that the enrichment of the tracer amino acid in the sampling

compartment (ie. plasma) is in rapid equilibrium with the immediate intra-cellular precursor for both protein synthesis and amino acid oxidation (Garlick, 1989; Obled et al, 1989). However, the amount of amino acid delivered using this method is likely to perturb the kinetics of protein synthesis (Schwenk et al, 1987) and amino acid oxidation (Tessari et al, 1985) and give falsely elevated values.

The most common approach is the primed-continuous infusion of an amino acid (usually L-[1-^{13}C]leucine) to achieve an isotopic "steady state" (Matthews et al, 1980; Motil et al, 1981; Matthews et al, 1982; Wolfe et al, 1982; Wolfe et al, 1984b; Cortiella et al, 1988; Thompson et al, 1988; Bier, 1989). Steady state is defined as the time at which the rate of appearance (Ra) of unlabelled amino acid (tracee) into the sampling pool is equal to the rate of disappearance (Rd) of the tracee out of the pool (Wolfe, 1984c). This dilution of the tracer by tracee to measure the rate of appearance is referred to as total flux (Q). The steady state is really a "quasi-equilibrium" state for true equilibrium between all pools would require several months due to the very long turnover of structural proteins such as collagen in bone and tendons; however, for the purposes of amino acid turnover it is only necessary to achieve an isotopic plateau or quasi-equilibrium (Waterlow and Jackson, 1981; Wolfe, 1984c). It

is important to note that isotopic steady state is not synonymous with physiological steady state, and as such the tracers can be used to determine protein turnover under conditions of altered metabolic states provided a new isotopic steady state is achieved (Wolfe, 1984c). For example, measurements can be made during exercise (Wolfe et al, 1982), intra-venous amino acid infusions (Bennet et al, 1989), and during insulin infusions (Castellino et al, 1987).

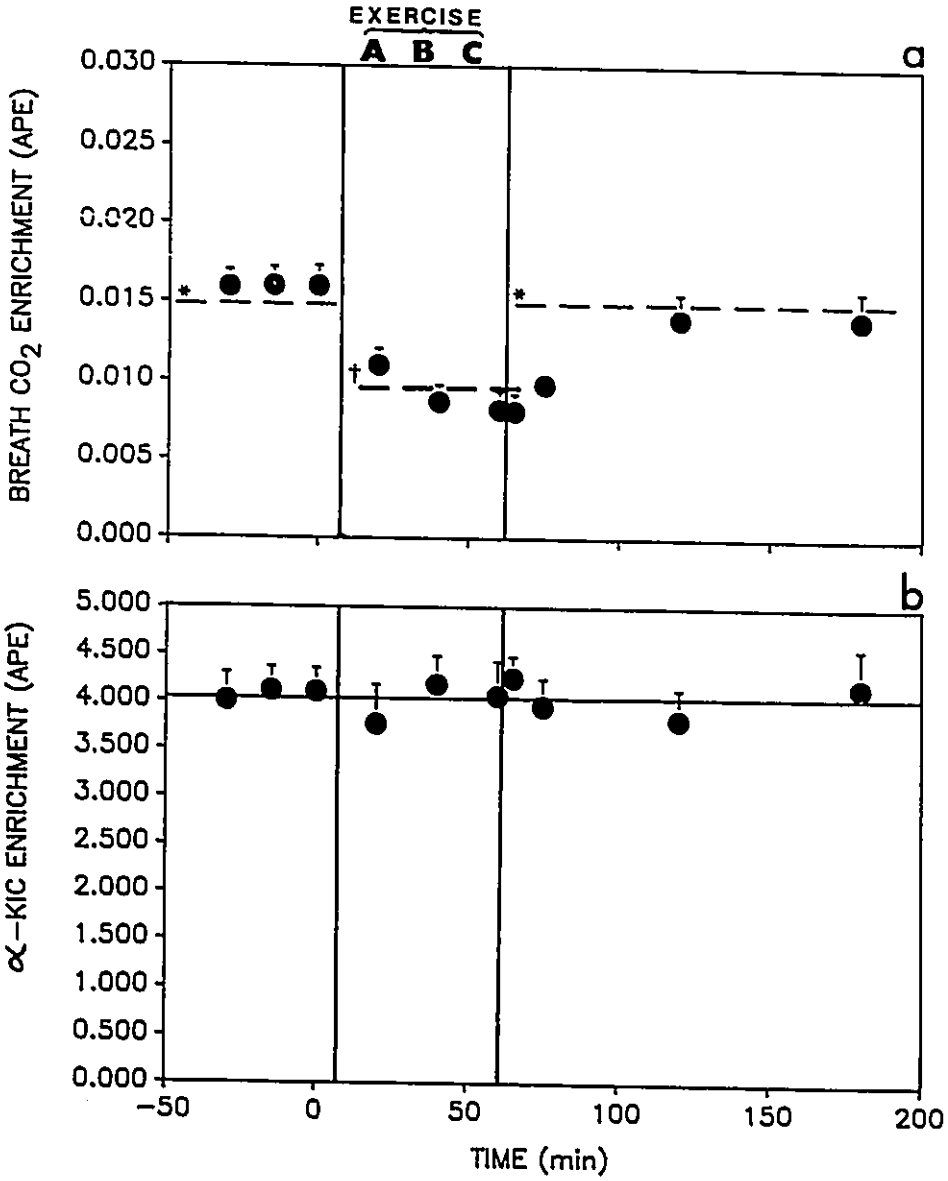
Even with a priming dose of amino acid at least 7-8 hours are required to achieve a plateau in breath $^{13}CO_2/^{12}CO_2$ enrichment due to the mixing of the $^{13}CO_2$ with the unlabelled CO_2 in the bicarbonate pool (Allsop et al, 1978; Wolfe, 1984c). The time to achieve plateau can be substantially reduced (< 2 hours) by simultaneous administration of a priming dose of [^{13}C]bicarbonate together with the tracer prime (Allsop et al, 1978; Wolfe, 1984c). Studies have found that the time to isotopic plateau for both breath $^{13}CO_2/^{12}CO_2$ and plasma ^{13}C-amino or α-keto acid enrichment using this method ranged from 45 to 120 minutes (Matthews et al, 1980; Wolfe et al, 1982; Wolfe et al, 1984b; Bennet et al, 1989; Devlin et al, 1990). An isotopic plateau has been quantitatively defined as the point at which the coefficient of variation (CV) in the values over time is less than 10 % (Thompson et al, 1988) and the regression equation joining the points has

a slope that is not significantly (P>0.05) different from zero (Hoerr et al, 1991) (Appendix I.1.C).

We have found that an isotopic plateau is clearly achieved in both the breath and plasma α-ketoisocaproic acid (α-KIC) enrichments within 90 minutes of the start of a primed-continuous infusion of L-[1-^{13}C]leucine (Figure 3)(Tarnopolsky et al, 1991). During resistance exercise α-KIC enrichment remained at the pre-exercise plateau level while the breath enrichment re-established a new, lower isotopic plateau level as previously described (Figure 3) (Wolfe et al, 1982). In addition, in the last study described in this thesis, an isotopic plateau was evident for the CV of breath $^{13}CO_2/^{12}CO_2$ enrichment at plateau was 3.8 % and for plasma α-KIC enrichment it was 6.4 % and for both of these the probability of the slope joining the points being greater than zero was less than 1.0 % ($P < 0.01$). Therefore, two of the pre-requisites to using the steady-state equations (described below) to measure leucine turnover have been demonstrated.

The model used to determine protein turnover has been described by Waterlow et. al. (1978) and represents a form of stochastic analysis. In this analysis the overall inflow-outflow processes into a single, common metabolic pool are measured as opposed to compartmental analysis where the immediate pools and their exchanges between individual tissues

Figure 3. Breath CO_2 enrichment (corrected for background and *c* changes)(a) and plasma α-KIC enrichment changes (b) during the L-[1-^{13}C]leucine infusion protocol. All points joined with the thin (*) line are not significantly different from each other but are significantly different ($P<0.01$) from those joined with the thicker line (†).


EXERCISE
A B C
a
b
BREATH CO_2 ENRICHMENT (APE)
α-KIC ENRICHMENT (APE)
0.030
0.025
0.020
0.015
0.010
0.005
0.000
5.000
4.500
4.000
3.500
3.000
2.500
2.000
1.500
1.000
0.500
0.000
−50
0
50
100
150
200
TIME (min)

are measured (Waterlow et al, 1978; Matthews et al, 1980; Wolfe, 1984c). In the whole body there exist two inter-related cycles of nitrogen (amino acid) metabolism. One is that between the body and environment and consists of input (diet, infusion (I)) and output (breath $^{13}CO_2$ (O), urinary ^{15}N (C)) and the other at the cellular level is between protein synthesis (*S*) and protein breakdown (B) (Waterlow et al, 1978; Matthews et al, 1980; Waterlow, 1984). With the single pool model, using L-[1-^{13}C]leucine as a tracer, it is assumed that all amino acids enter the pool either from the diet (I) or from protein breakdown (B) and exit from the pool as protein synthesis (*S*) or oxidative disposal (O). The processes involved in amino acid/protein turnover are complicated and it is well accepted that the models of protein turnover are quite simple. Although more complex models of amino acid kinetics have been described (Cobelli et al, 1987; Bier, 1989), their acceptance will be limited until it can be demonstrated that the theoretical pools do indeed represent physiological entities (Waterlow, 1984; Bier, 1989).

The single pool model of protein turnover is described by the equation: $Q=S+C=B+I$; where Q is the flux or rate of appearance as mentioned above; *S* is the rate of incorporation of label into protein (also referred to as non-oxidative leucine disposal (NOLD)); C is the rate of catabolism of amino

acid (in the case of L-[1-^{13}C]leucine it is the rate of leucine oxidation (O); B is the rate of protein breakdown (also referred to as the rate of appearance of endogenous leucine (Ra end leu)). In the post-absorptive state B=Q (flux) for the only source of an essential, non-labelled amino acid in this case would be from protein breakdown; and I is the rate of exogenous leucine intake (dietary or infusion (ie. total parenteral nutrition))(Figure 4a).

The flux (Q) or total rate of appearance of leucine is calculated from the standard equation (Waterlow et al, 1978; Matthews et al, 1980):

$$Q=i[E_i/E_p-1]$$

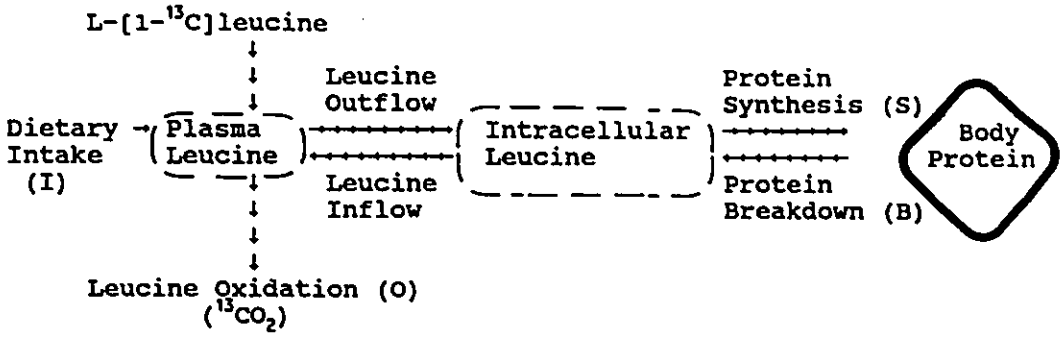
where, i=the infusion rate of L-[1-^{13}C]leucine ($\mu mol \cdot kg^{-1} \cdot h^{-1}$); E_i=the enrichment of the infused leucine (APE); E_p=the enrichment of plasma L-[1-^{13}C]leucine or [^{13}C]α-ketoisocaproic acid (α-KIC)(see below); and the term "-1" corrects for the contribution of the infused label to leucine turnover (Matthews et al, 1980).

2.1.3 Assumptions of the Model.

One of the pre-requisites to using the model equation is the need for isotopic plateau. Other assumptions inherent in the equation include:

Figure 4. a) Model of whole body leucine metabolism. b) Intracellular leucine metabolism to demonstrate reciprocal pool model. * - ^{13}C label (L-[1-^{13}C]leucine).

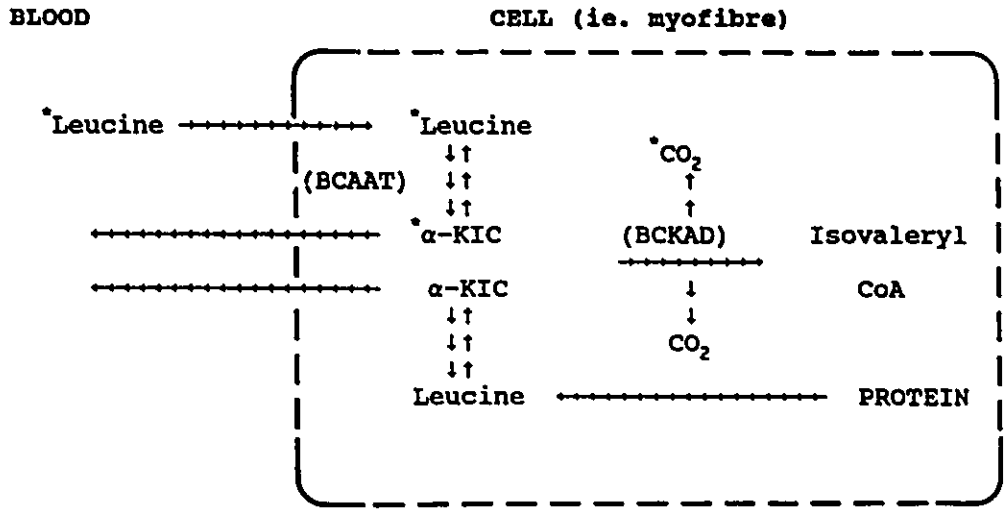
a) Model of whole body leucine metabolism.



Leucine Flux (Q)	=	Leucine Oxidation (O)	+	Protein Synthesis (S)	=	Leucine Intake (I)	+	Protein Breakdown (B)

Q = Rd(Rate of Disappearance) = Ra(Rate of Appearance)

b) Reciprocal pool model (cellular level).



1. The enrichment of the tracer in the plasma compartment represents that of the intra-cellular pool for both protein synthesis and oxidation; 2. There is rapid and complete mixing of the tracer within the body pool; 3. Over the time course of the experiment there is no isotope re-cycling (ie. L-[1-^{13}C]leucine taken up into protein via synthesis is not returned to the metabolic pool via protein breakdown); 4. The tracer (^{13}C) is not distinguished to any great extent from the tracee (^{12}C) in biochemical processes (ie. no carbon isotope effect occurs); 5. The amount of CO_2 that remains in the body (bicarbonate retention factor) during the course of the experiment remains constant; and 6. The natural background enrichment of $^{13}CO_2/^{12}CO_2$ remains constant during the course of the experiment. These first four assumptions appear to be met sufficiently to provide validity to the calculations and under certain conditions the latter two are also reasonable assumptions. However, quantitative values can be obtained for a given experimental protocol to correct for the latter two assumptions as described below.

There is evidence that the intra-cellular pools for protein synthesis and protein catabolism (oxidation for leucine) are not homogeneous (Schneible et al, 1981). Most of the leucine for protein synthesis appears to come from protein breakdown (Schneible et al, 1981) and is best

reflected by the enrichment of [^{13}C]leucyl-tRNA (Martin et al, 1981; Smith and Rennie, 1990). Unfortunately, the determination of [^{13}C]leucyl-tRNA requires several grams of tissue (Garlick, 1989; Watt et al, 1989) and it would be nearly impossible to obtain samples of this size from one tissue in human volunteers, much less from several tissues for an estimation of whole body turnover. Since the amino acid pool for oxidation appears to be derived primarily from the extra-cellular pool (Schneible et al, 1981) and the enrichment of plasma tracer reflects that of leucyl-tRNA (Everett et al, 1981), the enrichment of plasma leucine has been used to determine protein turnover (Golden and Waterlow, 1977; Matthews et al, 1980). However, it has been demonstrated that by using the plasma leucine enrichment with a primed-continuous infusion of L-[1-^{13}C]leucine, estimates of leucine turnover are underestimated (especially during exercise) for they do not accurately reflect intra-cellular tracer dilution (Wolfe et al, 1982).

One method to overcome this is to give a "flooding dose" of tracer (Garlick, 1989; Obled et al, 1989) which has the limitations mentioned previously. Another method is to use the enrichment of plasma α-KIC as a reflection of intra-cellular leucine enrichment (Matthews et al, 1982; Wolfe et al, 1982; Schwenk et al, 1985; Vazquez et al, 1986; Thompson

et al, 1988; Bennet et al, 1989; Horber et al, 1989)(Figure 4b). This "reciprocal pool" method makes intuitive sense for the only source of plasma α-KIC is via intra-cellular transamination of leucine. The tissue enzyme involved in this transamination (branched chain amino acid transaminase (BCAAT)) is not rate limiting in non-hepatic tissues, and there is a rapid release of α-KIC into plasma (Thompson et al, 1988). Following maximal exercise to exhaustion (~15 min) there is a delay in the diffusion of α-KIC into plasma (Fielding et al, 1986) but this is not likely to be limiting in circuit weight training due to its intermittent nature (20 s exercise: 2 min rest). In animal models (Vazquez et al, 1986; Layman and Wolfe, 1987; Horber et al, 1989) and in humans (Bennet et al, 1989), the correlation between α-KIC and intra-cellular leucine enrichment is very high. This relationship has been demonstrated not only for skeletal muscle but also for erythrocytes, heart, liver and jejunum (Horber et al, 1989). Until recently, it was theoretically argued that α-KIC enrichment best reflected the enrichment of leucyl-tRNA (Matthews et al, 1982; Nair et al, 1988; Bennet et al, 1989) but recent work has demonstrated that this relationship was very strong in skeletal muscle in pigs (Watt et al, 1989) and in humans during spinal surgery (Smith and Rennie, 1990). For the reasons mentioned above it was decided

a priori that the reciprocal pool model (α-KIC) would be the best for the determination of leucine turnover during (Wolfe et al, 1982) and after exercise (rest) in the studies described in this thesis. Therefore, in all calculations presented in the results of this thesis, the E_p values used to determine Q will be that of α-KIC.

The second assumption of rapid and complete mixing of tracer in the body pool has also been demonstrated to be a reasonable one. Thompson and colleagues (Thompson et al, 1988) infused a bolus dose of L-[1-^{13}C, ^{15}N]leucine into humans and found a rapid (<10 min) appearance of both [^{13}C]α-KIC and [^{13}C]leucine into plasma. These results demonstrated that the reversible transamination reaction in the body was rapid, as was the equilibration between the plasma and intra-cellular pool (Thompson et al, 1988). During exercise there is an augmentation of amino acid transport (via System L transporters (Christensen et al, 1990)) into muscle (Goldberg and Goodman, 1969) and the diffusion of α-KIC from muscle into plasma is fairly rapid even after continuous, intensive exercise (Fielding et al, 1986). Therefore, during exercise the mixing of the pools should be rapid, especially during weight lifting where a brief work task (~30s) is followed by a rest period (~2min).

There has been some debate about the optimal sampling sites to get a good representation of whole body kinetics (Katz et al, 1982; Matthews et al, 1982; Pell et al, 1983; Layman and Wolfe, 1987; Thompson et al, 1988). Ideally, one would like to infuse the tracer on the efferent side of all body tissues and sample the dilution of the tracer from a well mixed, afferent pool. This would require an infusion of tracer into the pulmonary artery (a) and sampling from the union of the superior and inferior vena cava (v)(right atrium). This is called the av method and would clearly not be practical for a study involving healthy humans (Layman and Wolfe, 1987). In human tracer studies the so called va method is used in which the tracer is infused into a deep forearm vein (v) and sampling occurs via an "arterialized" (a) hand vein (hand heated to ~65°C in a hot box to decrease the av transit time)(Layman and Wolfe, 1987). Although the av mode is optimal, in studies of leucine turnover using the reciprocal pool model, it has been shown that the enrichment of [^{13}C]α-KIC not only reflects tissue leucine enrichment, but it was also insensitive to sample site (Layman and Wolfe, 1987). Matthews and colleagues also found that the sample site did not affect the enrichment ratio of [^{13}C]α-KIC:L-[1-^{13}C]leucine under a range of dietary protein intakes (Matthews et al, 1982). In fact, a single deep venous catheter could

theoretically be used for tracer (L-[1-^{13}C]leucine) infusion and sampling ([^{13}C]α-KIC) given these results (obviously L-[1-^{13}C]leucine enrichments would be erroneous and variable under these conditions).

The third theoretical concern about tracer studies is that the recycling of the tracer label will lead to an unknown underestimation of flux (Q)(Wolfe et al, 1982). This underestimation is due to the labelled amino acid being incorporated into proteins and released back into the sampling pool over the course of the experiment. Since E_p is the denominator of the flux (Q) equation given above, a false increase in E_p will underestimate Q. The amount of recycling will be a function of the turnover rate of body proteins and their relative contribution to the whole body pool. The turnover of most muscle proteins is in the order of days (Waterlow et al, 1978; Clark and Zak, 1981) and of visceral proteins, hours (Waterlow et al, 1978; Carraro et al, 1990a). Since muscle protein turnover represents about 25-30% of whole body turnover (Lobley et al, 1980; Nair et al, 1988), it would be expected that recycling of label would become a problem with protocols longer than several hours in duration.

Schwenk and colleagues addressed this question using an ingenious approach (Schwenk et al, 1985). They infused two groups of human volunteers with a combination of isotopes

(group I= 4 h of [^{3}H]leucine + α-[^{14}C]-KIC; group II= 24 h of [$^{2}H_{3}$]leucine, with the last 4 h identical to group I) and calculated that the rate of appearance of [$^{2}H_{3}$]leucine back into plasma was 30 % greater for group II compared to group I. In addition, the decay kinetics of [$^{2}H_{3}$]-leucine and [$^{2}H_{3}$]-α-KIC were slower than for the kinetics of [^{3}H]-leucine and [^{3}H]-α-KIC for group II compared to group I (Schwenk et al, 1985). Together, these data indicated that in tracer studies of at least 4 hours in duration there is no isotope recycling but substantial (~30%) recycling occurs by 24 hours. In the present thesis the infusion protocols were of 5 and 3.5 hours in duration, so it is unlikely that the values were affected by recycling.

The fourth assumption mentioned above is that no carbon isotope effects occur. It is known that some carbon isotope effects do occur for the isotopic enrichment of ^{13}C in the end product of a biochemical pathway is lower than that for the reactants (probably due to a higher binding affinity of the "heavier" ^{13}C molecule)(Peronnet et al, 1990). However, for the purposes of protein turnover there does not appear to be any significant influence of the carbon isotope effect on flux values. Flux values determined using L-[1-^{13}C]leucine are identical to those using L-[$^{2}H_{3}$]leucine (Hoerr et al, 1991) as are those using L-[1-^{14}C]leucine and L-[^{3}H]leucine (Schwenk et

al, 1987). The isotope effects would be expected to be greater for ^{3}H and ^{2}H labelled compounds for they differ in weight from the natural element (H) by 200 and 100 % respectively whereas ^{14}C and ^{13}C labelled compounds differ from the natural element (^{12}C) by only 17 and 8 % respectively. In addition, the isotope effects, negligible though they are, would be less for ^{13}C compared to ^{14}C labelled compounds.

The fifth assumption mentioned above is that the bicarbonate retention factor (c) remains constant during the protocol. The bicarbonate retention factor corrects leucine oxidation measurements for the proportion of labelled $^{13}CO_2$ that is produced by oxidation of L-[1-^{13}C]leucine and is subsequently excreted in the breath during the time course of the experiment (Kien et al, 1989). This value is normally less than unity for a molecule of CO_2 is "lost" in slowly turning over metabolic pools such as bone and as metabolic intermediates (ie. oxaloacetate)(Irving et al, 1983). Leucine oxidation measurements require knowledge of c and are calculated from the equation:

$$\text{Leucine oxidation} = [(IE_{CO2} \cdot c^{-1}) \cdot IE_{\alpha\text{-KIC}}{}^{-1}] \cdot \dot{V}CO_2]$$

where IE_{CO2} = isotopic enrichment of breath CO_2 (APE); c = bicarbonate retention factor (described below); $IE_{\alpha\text{-KIC}}$ = isotopic enrichment of plasma α-KIC; and $\dot{V}CO_2$ = volume of carbon dioxide produced ($\mu mol \cdot min^{-1}$).

The bicarbonate retention factor (c) is determined in a separate experiment where $NaH^{13}CO_2$ is infused at a known rate and the proportion of $^{13}CO_2$ recovered is calculated according to the equation (Wolfe et al, 1984a; Kien et al, 1989):

$$c = \dot{V}CO_2 \cdot (IE^{13}CO_2 \cdot F^{-1})$$

where: $\dot{V}CO_2$ = volume of carbon dioxide produced ($\mu mol \cdot min^{-1}$); $IE^{13}CO_2$ = isotopic enrichment of expired CO_2 at plateau; and F = the rate of tracer ^{13}C infusion ($\mu mol \cdot min^{-1}$).

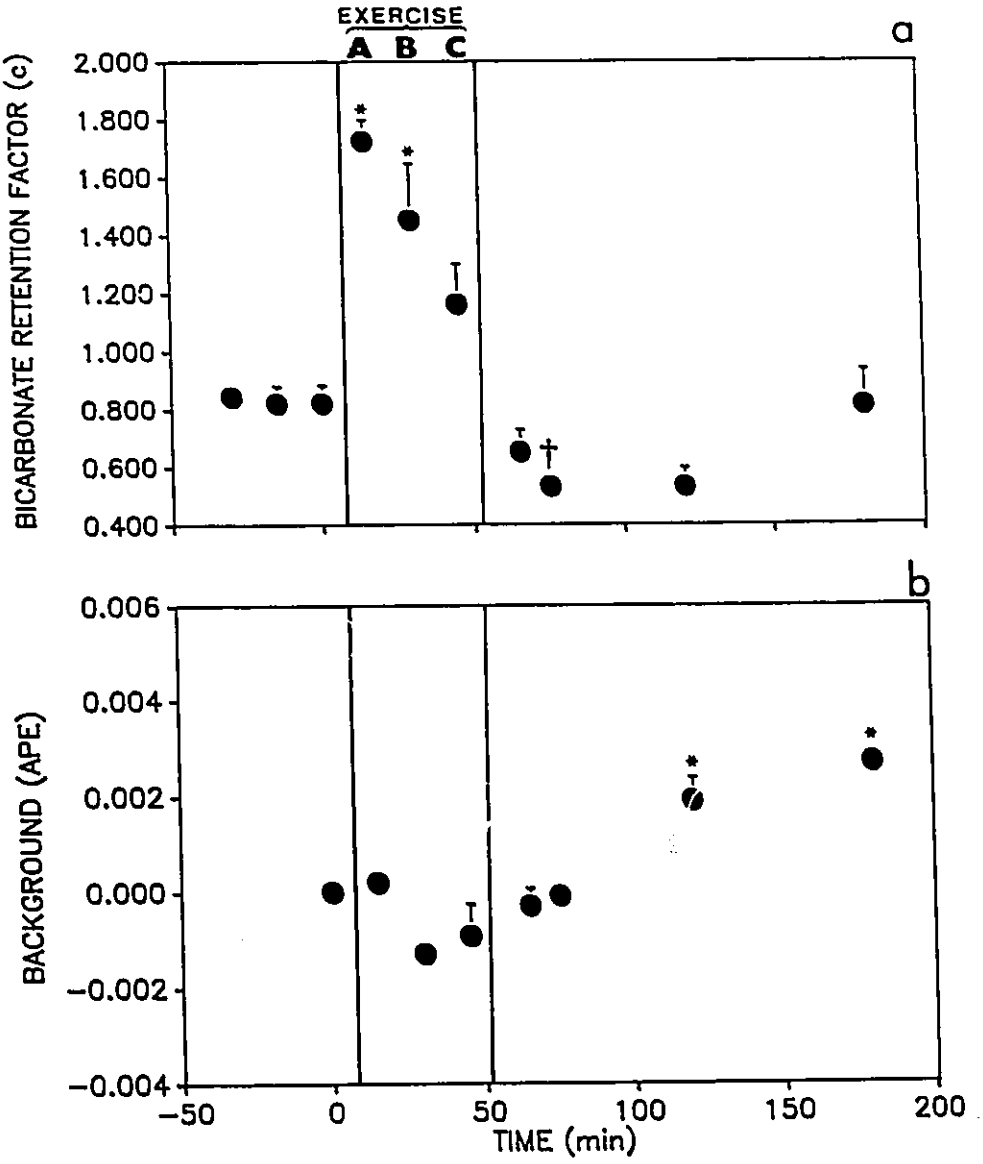
As for leucine kinetics, it is essential that an isotopic plateau be attained but there are a few methodological concerns in these experiments that require special mention. Firstly, it has been suggested that some loss of $^{13}CO_2$ during a $NaH^{13}CO_2$ infusion occurs in first pass loss in breath (Irving et al, 1983) but this has recently been shown to be negligible by measuring the difference between arterial and venous administration of tracer (Downey et al, 1986). Secondly, Wolfe (1984c) has suggested that some of the tracer label may be lost via isotopic exchange with air bubbles in a solution of $NaH^{13}CO_2$. To minimize this latter effect, we have mixed our bicarbonate solution immediately prior to infusion in all of the experiments described in this study and ensured that their were no visible air bubbles prior to infusion.

In the majority of studies the c value has been estimated at 0.81 and no attempt was made to determine it under the

conditions of the given experiment (Golden and Waterlow, 1977; Matthews et al, 1980; Motil et al, 1981; Matthews et al, 1982; Wolfe et al, 1982; Evans et al, 1983; Bennet et al, 1989; Knapik et al, 1991). This is probably a reasonable assumption under conditions in which the metabolic rate is not perturbed to any great extent (Armon et al, 1990), however, exercise in particular increases *c* (Wolfe et al, 1984b; A. Coggan, 1990 (personal communication)). It has been shown that during exercise at only about 35 % $\dot{V}O_{2max}$ the *c* values increased rapidly during the early stages of exercise to about 1.85 and fell exponentially to about 1.06 after 1 hour of bicarbonate infusion (Wolfe et al, 1984b). Given this information it was decided that a bicarbonate infusion study would be required to establish the retention factor during the fed state and in response to a weight-lifting protocol (Appendix I.2.B).

Bicarbonate infusion studies were performed in 4 subjects (study subjects, see experimental details below) using a primed-continuous infusion of $NaH^{13}CO_2$ (MSD Isotopes, Pointe Claire, PQ, 99% ^{13}C enrichment) with a prime:infusion ratio of 85:1 (Wolfe et al, 1984b, Wolfe, 1984c). It was found that the resting, fed state *c* value was 0.83 and the response to exercise is presented in Figure 5a. In a recent review of the literature it was found that the mean fed state *c* value from 6 studies was 0.84 $\pm$ 0.7 (Hoerr et al, 1989).

Figure 5. Bicarbonate retention factor (c) determined by primed-constant infusion of [^{13}C]sodium bicarbonate (n=4 subjects) (a). Background breath $^{13}CO_2/^{12}CO_2$ isotopic enrichment with no isotope infusion (n=4 subjects). Values are atom percent excess (APE) vs pre-exercise value (b). * $P<0.01$ compared to mean pre-exercise values. † $P<0.05$ compared to mean pre-exercise values.


EXERCISE
A B C
a
BICARBONATE RETENTION FACTOR (c)
2.000
1.800
1.600
1.400
1.200
1.000
0.800
0.600
0.400
b
BACKGROUND (APE)
0.006
0.004
0.002
0.000
−0.002
−0.004
−50
0
50
100
150
200
TIME (min)

The response to exercise found in this study was very similar to that observed by Wolfe et al. (1984b) and the reasons for the change from rest are discussed in the Discussion section of Study 2. In calculations of my results, the individual *c* values were used for the 4 subjects, and in all other cases the mean value (n=4) was used.

A failure to account for these changes results in an over-estimation of leucine oxidation during exercise and an under-estimation of WBPS (non-oxidative leucine flux). For this reason, the results of any tracer study during exercise that did not calculate *c* must be interpreted with this knowledge (Rennie et al, 1981; Millward et al, 1982; Wolfe et al, 1982; Evans et al, 1983; Knapik et al, 1991). In fact, the graph of breath $^{13}CO_2$ enrichment during exercise in the study by Rennie et al. (1981) was not corrected for *c* changes and the shape was very close to that obtained by Wolfe et al. (1984b) and to the graph of *c* presented in Figure 5a. This indicated that correcting for *c* would tend to decrease the oxidation values reported by Rennie et al. (1981).

The last assumption concerning the background $^{13}CO_2/^{12}CO_2$ breath enrichment changes during an experiment is only a concern for investigators using stable isotope tracers and not for those using radio-active tracers. This is because the body has a natural background abundance of ^{13}C of about

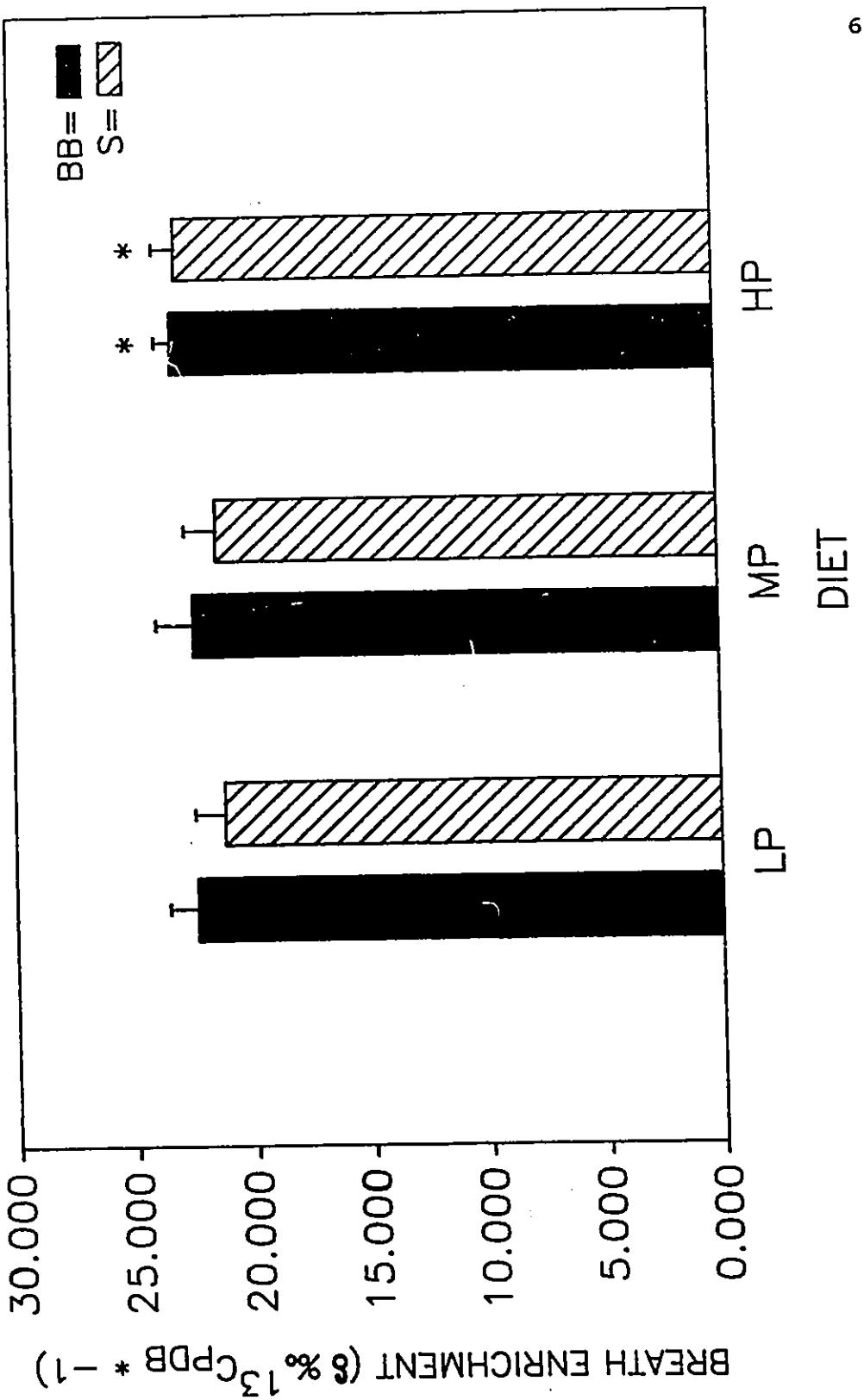
1.1 % and the enrichment in breath will change as a function of the metabolic fuels being used at a given time (Schoeller et al, 1980; Wolfe et al, 1984a; Perronet et al, 1990).

Metabolic fuels have different isotopic enrichments due to isotopic fractionation (ie. ^{13}C is handled differently by certain biochemical reactions differently than ^{12}C). For this reason carbohydrates, fats, and proteins have different abundances of ^{13}C. Carbohydrates usually have the highest enrichment (range = 1.0855 (honey) to 1.0995 $\%^{13}C$ (corn)), with fats the lowest (range = 1.0791 $\%^{13}C$ (soy oil) to 1.0950 (corn oil)) and proteins an intermediate value (range = 1.0843 (casein hydrolysate) to 1.0964 (ham)) (Schoeller et al, 1980). An interesting observation and example of fractionation is the difference in the ^{13}C enrichment between corn starch (high enrichment 1.0995 $\%^{13}C$), which is produced via a C_4 photosynthetic reaction compared to rice (low enrichment 1.0846 $\%^{13}C$) which is produced in a C_3 reaction (Schoeller et al, 1980). Due to carbon isotope fractionation, the enrichment of ^{13}C will be lower in products of metabolic pathways as compared to the reactants as it will in longer compared to shorter metabolic pathways (Peronnet et al, 1990).

The effect of an alteration in the isotopic enrichment of the diet due to different CHO, FAT and PRO intakes usually takes several days to become evident (Schoeller et al, 1980;

Barstow et al, 1989). Differences in dietary substrate intake have an influence on breath $^{13}CO_2$ enrichment for North Americans usually have higher ^{13}C breath enrichments compared to Europeans (due primarily to the higher amount of corn consumed by North Americans), and cows fed C_4 feed have higher milk ^{13}C enrichment compared to those fed C_3 feed (Schoeller et al, 1980). In the study presented in chapter 5 of this thesis the baseline breath enrichment of subjects while on a low protein (LP) and medium protein (MP) dietary treatment (both higher in corn starch (↑ $^{13}CO_2$)) for 13 d, had significantly greater ($P< 0.05$) breath enrichments than during the high protein (HP) dietary treatment (higher in whey and casein hydrolysates)(Figure 6). In humans infused with dextrose the enrichment of breath $^{13}CO_2$ increased as did the respiratory exchange ratio indicating a greater metabolic reliance on carbohydrate utilization (Wolfe et al, 1984a). During exercise, it has been found that breath $^{13}CO_2$ enrichment increases (Wolfe et al, 1984a), remains essentially the same (Massicotte et al, 1984; Schoeller et al, 1980), or decreases slightly (Barstow et al, 1989). These differences are probably due to differences in the exercise protocols and in subject dietary state so it is important to establish the change in background enrichment in any proposed study using an exercise protocol not previously described.

Figure 6. Effect of dietary intervention (LP, MP, HP; see text for definitions (Chapter 2 and 5)) on baseline breath isotopic enrichment for sedentary (S) and resistance athlete (BB) groups. * Significantly less enriched than for diets MP and LP ($P < 0.05$).


BB=
S=
*
*
HP
MP
LP
DIET
30.000
25.000
20.000
15.000
10.000
5.000
0.000
BREATH ENRICHMENT (δ‰ $^{13}C_{PDB}$ * −1)

In the second study described in this thesis (Chapter 4) the effect of exercise on breath background enrichment was studied in 4 subjects by not infusing any isotope during the weight-training protocol. The breath enrichment decreased during exercise (Figure 5b) and the oxidation and bicarbonate retention factor values were corrected for these changes.

In the study described in Chapter 5 of this thesis the effects of different dietary interventions upon breath background enrichment were studied in 2 subjects on both a high PRO diet (HP) and high CHO diet (LP) by consuming formula aliquots every 30 min (q30 min) for 3.5 h (the MP diet changes were taken as the mean of the changes for the HP and LP diets). The acute effect of q30 min formula aliquots was to significantly ($P< 0.01$) increase breath $^{13}CO_2$ enrichment (LP = ↑ 0.0018 APE (1st 120 min), ↑ to 0.0027 APE (by end of 210 min); HP = ↑ 0.0017 APE (1st 120 min), ↑ to 0.0024 APE (by end of 210 min)) with no between trial effect. Thus, there does not appear to be a differential change in breath isotopic enrichment over the short term (i.e., 3.5 h) when foods of different isotopic enrichment are ingested. This probably indicates that the breath enrichment is determined more from the prevailing diet rather than the isotopic composistion of the immediate diet (Schoeller, et al, 1980). These corrections for dietary intake effects were used in the

calculations of this thesis (Appendix I.2.C).

2.1.4 Validity of the model.

The question of the validity of the tracer studies to measure whole body protein turnover also needs to be addressed in addition to the technical/theoretical discussion above.

With respect to measurements of whole body protein synthesis (WBPS), the model described above predicts that the non-oxidative leucine disposal (NOLD) should be an indicator of WBPS for, other than oxidative loss, this is the only known route of leucine disposal. In theory leucine could be lost in urine under conditions of proteinuria but almost no tracer is lost via this route in healthy subjects (Golden and Waterlow, 1977). To investigate the validity of NOLD as a measure of WBPS, Schwenk et al. (1987) measured leucine turnover in post-absorptive dogs before and after the administration of emetine (a translational PRO synthesis inhibitor) and found a 70 % decrease in NOLD after the drug was administered. They concluded that NOLD was not a quantitative measure of PRO synthesis (since it did not decrease by 100 % after emetine) but that it provided a rapid estimate of synthesis (Schwenk et al, 1987). It is likely that the strength of the association between NOLD and true WBPS is greater since in this study the dose of emetine was only slightly greater than that used in humans for the

treatment of amoebiasis (Schwenk et al, 1987). This dose would not likely result in a complete block in WBPS and their conclusions were based upon this assumption (Schwenk et al, 1987). In addition, more direct measurements of leucine incorporation into skeletal muscle (muscle protein synthesis (MPS)) are well correlated to WBPS in humans (Nair et al, 1988) and animals (Lobley et al, 1980), which provides further support to the validity of WBPS measurements.

In order to make the conversion from NOLD to WBPS it must be assumed that the essential amino acid in question is handled in the same manner as all essential amino acids and an average tissue concentration of the representative amino acid must be used. Based upon the content of leucine in mammalian tissue, an average tissue content of 590 μmol·g PRO^{-1} was used in the calculations in the present thesis (Block and Weiss, 1956; Golden and Waterlow, 1977). These values would change if the contribution of certain proteins to WBPS changed during the infusion, but most tissues contributing to WBPS vary only slightly in their leucine content so this would not have a large effect (Block and Weiss, 1956; Golden and Waterlow, 1977). The validity of converting NOLD to WBPS has been questioned by several investigators who have found different values of WBPS using different essential amino acid tracers (Wolfe et al, 1984b; Obled et al, 1989). This means

that the WBPS values presented in this paper should be considered semi-quantitative, although directional changes would not be influenced by the results of the studies mentioned above (Obled et al, 1989).

During the post-absorptive state the only source of essential amino acids comes from PRO breakdown and under these conditions flux (Q) = protein breakdown (B). The significant, positive correlation between tissue essential amino acid content and the flux of the same amino acids provides strong evidence that under fasted conditions, flux is a good measure of protein breakdown (Bier, 1989). During periods of feeding there is both an exogenous (diet intake (I)) as well as an endogenous (B) source of leucine (ie. Q = I + B) and most studies have merely subtracted the estimated leucine content of the diet from Q to determine B (Ra END LEU)(Motil et al, 1981). Unless the amino acids are infused directly into a vein (Bennet et al, 1989), recent evidence suggests that the rate of appearance of exogenous leucine is not 100 % due to some loss in absorption and in first pass metabolism by the liver (Cortiella et al, 1988; Hoerr et al, 1991). The approximate loss of amino acid given by mouth (assuming no significant malabsorption) has been calculated using a simultaneous intra-gastric infusion of [$^{2}H_{3}$]leucine and an intra-venous infusion of [^{13}C]leucine at about 80 % using the

primary pool model and 90 % using the reciprocal pool model (α-KIC) (Hoerr et al, 1991). These results were based upon the intestinal absorption of a free amino acid (Hoerr et al, 1991) and it is known that amino acid absorption from a protein is slightly less (Silk et al, 1973). Therefore, the estimations of the amount of leucine provided by the liquid diet (casein and whey proteins) in the present studies were corrected to 85 % of total PRO_{IN} (94 % absorbed (FAO/WHO/UNU, 1985); 90 % released into portal vein as α-KIC) to account for these recent observations (Hoerr et al, 1991). In addition, it was decided to provide the diet in 30 minute aliquots rather than the usual 60 minute aliquots (Motil et al, 1981) so the rate of appearance into the body was more uniform.

2.2 Stable isotope methodology.

It is beyond the scope of this thesis to discuss the details of instrumentation in depth but a brief mention will be made of the essential aspects of isotope ratio mass spectroscopy (IRMS) and gas chromatography-mass spectroscopy (GC/MS) instrumentation. Technical reviews on instrumentation have been published (Halliday and Read, 1981; Wolfe, 1984c). The major focus of this section will be on the development of sample preparation and on the validity/reliability of the results.

2.2.1 Isotope ratio mass spectroscopy (IRMS).

For the determination of the enrichment of $^{13}CO_2$ in breath samples an instrument must be able to reliably detect enrichment changes in the range of 0.005 to about 0.025 APE (Scrimgeour and Rennie, 1988). This degree of sensitivity is achieved with an isotope ratio mass spectrometer (IRMS) for this instrument directly compares the sample gas to a reference gas several times during a measurement. The original IRMS instruments required 10-50 mL of pure gas to make a measurement but more recent versions can make reliable measurements on micromolar amounts (< 100μL) of gas (Scrimgeour et al, 1988b).

The IRMS machine operates essentially by ionizing (via an electron beam) the sample and reference gases and accelerating the then positively charged ions through a curved magnetic field. The ions are deflected by the magnetic field as a function of the square root of their mass with the lighter ions being more deflected relative to the heavier species (Halliday and Read, 1981; Wolfe, 1984c). The ions are collected into Faraday detector cups which create an electrical current proportional to the ion impact. The signal is then amplified and recorded. For example, a molecule of $^{12}CO_2$ at m/z (mass/charge) = 44 and $^{13}CO_2$ at m/z = 45 would create an electrical signal directly proportional to the

isotope ratio (Wolfe, 1984c).

2.2.2 Breath analysis with IRMS.

As mentioned, a sample for IRMS must be a pure gas. For the case of CO_2 collected from breath this can be achieved either by trapping the CO_2 in a NaOH solution and liberating it with concentrated phosphoric acid or by cryogenically trapping the CO_2 (Wolfe, 1984c). We have chosen to cryogenically trap the CO_2 on a vacuum line, which involves freezing down the H_2O and CO_2 in liquid nitrogen in a "U"-tube, pumping away the O_2 and N_2, and re-trapping the CO_2 in removable cylindrical glass finger tube while the water is trapped in the original "U"-tube using a dry ice-isopropanol slush (Wolfe, 1984c). This method has been used in many studies (Cortiella et al, 1988; Nair et al, 1988; Welle et al, 1989; Bennet et al, 1990; Devlin et al, 1990; Lamont et al, 1990; Hoerr et al, 1991) and cryogenic extraction of CO_2 has been shown to be convenient and reproducible (Scrimgeour and Rennie, 1988a).

The breath samples were collected into 150 L meteorological balloons, transferred into 20 mL Vacutainers (Becton Dickinson, Rutherford, NJ), and cryogenic extraction occurred within 7 days. One modification that we have made is to trap the CO_2 (and H_2O) twice to minimize the possibility of H_2O contamination (Scrimgeour, 1988b). Preliminary work

from our lab and from the lab of Steve Welle in Rochester, NY (Personal Communication, 1988) found that there is no effect of storage for at least 10 d on $^{13}CO_2$ enrichment levels. Others have reported that there is very little contamination of Vacutainers if stored at room temperature for up to one month (Milne and McGaw, 1988; Scrimgeour and Rennie, 1988a; Smith et al, 1988).

After CO_2 extraction the isotope ratios in this study were determined using a VG Isogas SIRA 10 IRMS (Cheshire, England). All values were corrected using the Craig formula (Craig et al, 1953) for the contribution of oxygen isotopes to the ^{13}C measurement. Using this method the inter-assay coefficient of variation (CV) was 1.25 % for 18 sample pairs with a range of enrichments from 0.0022 to 0.0155 APE. The inter-test regression equation was: Y (sample 1)= -0.000031 + 0.985(X)(sample 2) with a coefficient of determination (R^2) of 98.5 %. The intra-assay CV for 38 sample pairs was 0.889 % with a range of enrichments from 0.0022 to 0.0259 APE. The intra-test regression equation was: Y = -0.000083 + 1.00 (X) with R^2 = 99.7 %. Due to the superior intra-test reliability, each individual's samples were run at the same time.

2.2.3 Micromolar $^{13}CO_2$ enrichment determination.

To further test the minimum sensitivity of the extraction process/IRMS analysis and to assess the feasibility of

performing $^{13}CO_2$ measurements on human muscle biopsy samples, I performed an experiment to measure the recovery and reproducibility of $^{13}CO_2$ from authentic leucine samples. The modified Van Slyke procedure (Van Slyke et al, 1941) was used to liberate the carboxyl carbon from leucine as CO_2 and involved: addition of leucine standards (or separated amino acids from protein sample) to a 10 mL test-tube → addition of 1 mL of pH 2.2 citrate buffer → refluxing solution @ 120°C X 30 min → rapid replacement of de-gassed rubber septa (Pre-boiled in NaOH (2N) X 30 min → rinse → boil in pH 3.0 HCl X 5 min) → cool in ice slush → add solid ninhydrin (~20 mg) and replace septa (on ice) → de-gas solution to ~50 mT (on ice) → liberate CO_2 by heating tube in a boiling water bath X 30 min → cool for 1 hour → trap CO_2 as per procedure mentioned above (also trap H_2O in original sample tube)(Read et al, 1984; Smith et al, 1988). With this method the mean APE for 4 leucine samples of theoretical yield 1.7 μmol (~16 X smaller than that from a 20 mL breath sample) was 0.0198 ± 0.0027 which gave a CV of 13.6%. In order to measure muscle protein synthesis (MPS) it is necessary to have a CV of < 3 % (Halliday et al, 1988; Nair et al, 1988). Therefore, this CV was clearly unacceptable. Dr. C. Scrimgeour at the University of Dundee suggested that our poor CV's may be a result of the manual CO_2 extraction technique for he had similar problems

and found acceptable CV's with an automated CO_2 extraction procedure (Personal Communication, 1990). Since we did not have the automatic breath carousel on our IRMS this was not an option open to us. It was suggested to me by Yadu Moharir (post-doctoral fellow in organic chemistry, Personal Communication, 1990) that some CO_2 contamination of the sample may come from the citrate buffer for it contains carbon atoms (since all of our tubes are silylated no contamination could have come from the glass).

To confirm this I measured the major beam (m/z = 44) signal strength (A) from 3 samples of citrate buffer, cryogenically extracted on the above mentioned apparatus, and compared this to that of 7 authentic leucine standards (the signal strength of m/z = 44 is proportional to sample size)(Smith et al, 1988). The leucine standards gave a mean signal of 3.54×10^{-9} A and the citrate standards gave a signal strength of 1.99×10^{-10} A. Therefore, 5.6 % of the sample signal came from buffer contamination. I discussed this with K. Yarasheski (Post-Doctoral fellow with D. Bier at Washington State University, Personal Communication, 1990) and he suggested that I try a phosphate buffer.

I repeated the above experiment with 1.7 μmol of theoretical CO_2 and used 1 mL of phosphate buffer (0.2N NaH_2PO_4, to pH 2.2 with phosphoric acid) instead of a citrate

buffer. The amount of contamination CO_2 in 3 samples of buffer alone was undetectable and the CV for $^{13}CO_2$ enrichments (APE) for 5 leucine samples (1 lost in transfer) was 1.5 % (mean = 0.03418, SD = 0.000521). Therefore, with the set up that we have it is possible to get ^{13}C enrichment values with as little as 1.7 μmol of CO_2 that are almost as reliable as those from large breath samples (~27 μmol CO_2/20 ml breath). Thus, we should be able to determine MPS from muscle biopsy samples of about 50 mg in weight (~7.7 μmol ideal (after separation only about 2.3 μmol is left))(Smith et al, 1988).

2.2.4 Gas chromatography/mass spectroscopy (GC/MS) and GC.

A GC/MS is really 2 machines linked in series with the GC being used to separate compounds and the mass spectrometer being used to measure the molecular mass of fragmented compounds (a very sensitive detector for each fragmented compound has a unique fragmentation pattern (signature)).

Prior to separation by a GC, the compound(s) of interest is(are) extracted from a biological medium (ie. blood, urine, tissue) and derivatized to form a volatile compound that can be injected onto the GC column (Thompson et al, 1989). Most biological samples (and all amino acids) must be made volatile by derivatization in order to be carried through the GC column in a gaseous state (Wolfe, 1984c).

In general, a GC separates compounds my moving the volatilized sample over a stationary phase with a carrier gas. The sample will move through the GC column as a function of the sample partition coefficient (K) which is inversely proportional to the vapour pressure of the sample. The partition coefficient is equal to the weight of solute per mL stationary phase/weight of solute per mL mobile (gaseous) phase. Thus, a sample with a low K value (high vapour pressure) will elute early for more will remain in the gaseous carrier state compared to the stationary phase (Halliday and Read, 1981: Wolfe, 1984c).

The original separation columns were glass and the sorbent or stationary phase was either packed into the column (SCOT; Surface Coated Open Tubular) or onto the inside (WCOT; Wall Coated Open Tubular). The problem with these in interfacing with a mass spectrometer is that the gas flow rates are very high and a separator is required to reduce the amount of gas entering the mass spectrometer. One advantage is that a large sample can be injected into these columns for preparative purposes (discussed below)(Halliday and Read, 1981). In the separation of organic acids (α-KIC) for protein turnover studies these columns were initially used (Schwarz et al, 1980; Wolfe et al, 1982). The capillary column is now used (Roccicciolí et al, 1981; Ford et al, 1985) for it has

superior separation capabilities and can be directly interfaced with a mass spectrometer (Halliday and Read, 1981).

Once the sample of interest is separated by the GC it is eluted into the ion source of the mass spectrometer where the sample is ionized (and fragmented) by either chemical or electron impact ionization (CI and EI, respectively). The ions are then repelled through an oscillating magnetic field (quadrupoles) which controls the movement of certain molecules dependant upon the specified mass filter (radio-frequency). Thus, only ions of specified mass will pass through the field and reach the detector. Ion beam detection is usually enhanced by an electron multiplier which increases the ionic signal strength by a factor of about 10^6 prior to final amplification prior to data storage (Wolfe, 1984c).

The data acquisition can either be in the form of a total ion chromatogram/monitoring (TIM) or a selected ion chromatogram/monitoring (SIM). Data acquisition by TIM means that the computer records all of the mass spectral data from the compound of interest over a very wide range of atomic mass units (amu)(ie. 0 to 500 amu) whereas, SIM acquisition focuses the record over a specified amu range. In the extreme definition SIM focuses the machine on a specific amu, however, if sample size is not limiting, narrow scan acquisition (ie. over 10 or 20 amu around the fragment of interest) may provide

more information and the reproducibility is well within acceptable limits (see below).

2.2.5 Plasma α-KIC derivatization and GC/MS acquisition parameters.

For keto acids one of the most common derivatives is the o-trimethylsilyl-quinoxalinol derivative (Rocchiccioli et al, 1981) for the orthophenylenediamine (OPDA) used to make the quinoxalinol stabilizes the derivative and thus limits the number of fragmentation species and increases their mass spectral intensity (Wolfe, 1984c). I have developed a simplified derivatization process to make the *o*-TMS-OPDA derivative of α-KIC based upon standard techniques (Rocchiccioli et al, 1981; Wolfe et al, 1982; Wolfe, 1984c; Shangraw et al, 1988). Plasma proteins are extracted from 250-500 μL of plasma with 5 mL absolute ethanol, centrifuged at 10,000 rpm for 10 min and the supernatant is decanted to a silylated screw-top tube and dried under N_2. The residue is re-suspended in 1 mL of H_2O to which 1 mL of 2% OPDA in 4N HCl is added and heated for 1 hour at 100°C to derivatize the ketone group. After cooling the derivative is extracted twice with 2.5 mL of methylene chloride and the lower layer transferred to a clean screw-top tube. Final derivatization was performed by addition of 50 μL of *N,o*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) + 1 %

trimethylchlorosilane (TMCS)(Pierce Chemicals, Rockford, IL) and heating at 100°C for 30 min.

The α-KIC enrichments in this thesis were determined using the same GC/MS instrument (Hewlett Packard Model 5890 GC; VG-Trio-2 quadrupole mass spectrometer (VG, Cheshire, England). About 0.3 μL of derivatized sample was injected in the splitless mode directly onto a 15 m fused silica capillary column (DB5 J.W. Scientific, Rancho Verda, CA) with helium as the carrier gas (32 $cm \cdot sec^{-1}$). The GC oven was programmed at an initial temperature of 120°C, ramped to 160°C at 8°C·min^{-1}, and then ramped to 300°C at a rate of 20°C ·min^{-1} and held for 3 min to drive off the later eluting compounds. Ionization was by electron impact (70 eV, trap current 170 μA, source current 1.6 mA, ion source temp. at 200°C). The enrichments of the 233.1/232.1 amu species (enriched/unenriched) were monitored by scanning the machine over a narrow scan range (228 to 238 amu) and the listing of the mass spectra acquired at the apex of the chromatographic peak was used to determine sample enrichment (the results were the same for chromatogram areas).

Authentic α-KIC, ketocaproic acid, and ketomethylvaleric acid (KMV) derivatives were prepared using the procedure mentioned above to determine the optimal GC oven temperature programme for separation of samples and to determine the

elution time and mass spectra of α-KIC (Figure 7a). Identification of the α-KIC peak was confirmed by peak enhancement methods and by comparing the electron impact ionization spectra to that published by others (Wolfe et al, 1982; Wolfe, 1984c). As expected the ion species cluster at 232.1 amu gave the greatest intensity (this was the reason for monitoring these cluster ions)(Figure 7b).

The theoretical isotope cluster abundance calculations based on the known isotopes contributing to the derivative ($C_{12}H_{16}N_2OSi$) predicted that the intensity of the 233.1 amu species relative to the normalized 232.1 species should be 19.63 (Figure 8a). It has been suggested that the cluster ions should be 232.2/233.2 (Wolfe et al, 1982; Wolfe, 1984b). However, the theoretical species should be 232.1/232.1 as calculated and slight differences probably relate to different machines and day to day "wobble" in assigning mass to the fragments (Figure 8b). From a total of 57 baseline blood samples that I have analyzed under the conditions described above the 232.1/233.1 intensity was 19.38 $\pm$ 0.38. Therefore, the accuracy of the method relative to theory was 98.7 %. The intra-test CV of the method was 0.81 % (n=9) and the inter-test CV of the method was 0.42 % (n=14 pairs) over a variety of enrichments (2 to 9 APE).

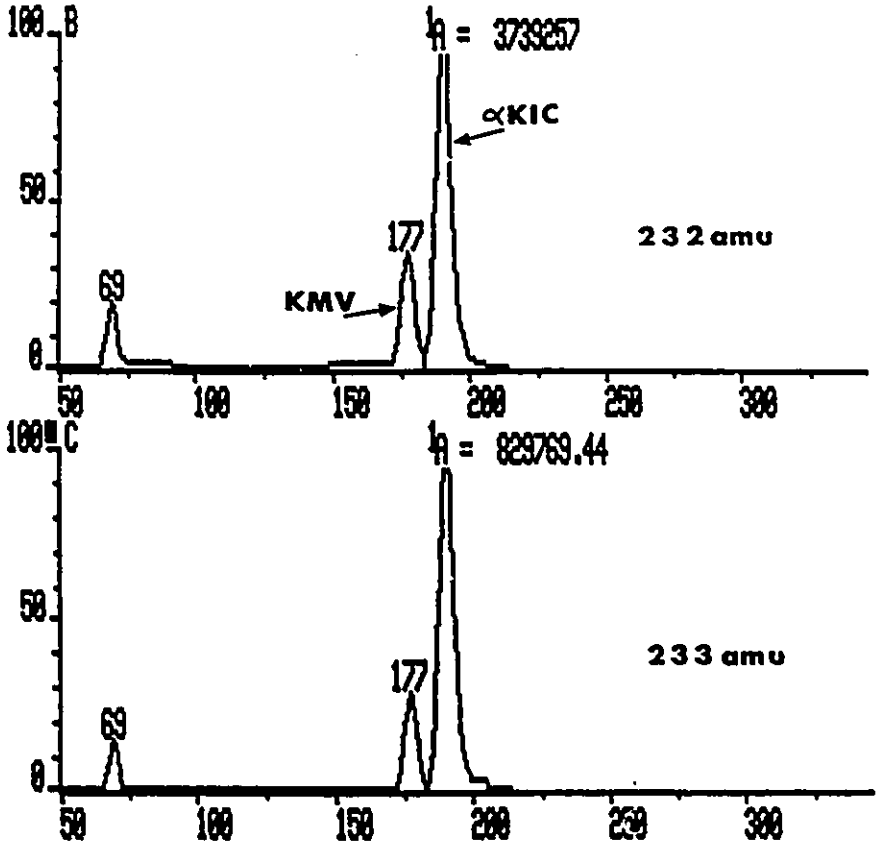
Figure 7. a) GC elution profile. KMV = ketomethylvaleric acid; α-KIC = α-ketoisocaproic acid; amu = atomic mass units. b) Mass spectrum of α-KIC derivative (narrow scan range, see text).

∝-KIC ENRICHMENT-

a. -CAPILLARY GC/MS

-QUINOXALINOL-TMS DERIVATIVE OF ∝-KIC

-ELECTRON IMPACT IONIZATION.



b. -MASS SPECTRA-

Figure 8. a) Theoretical α-KIC derivative isotope cluster abundance (19.63 %) and expected mass spectra of derivative.
b) Theoretical derivative mass calculation (amu).

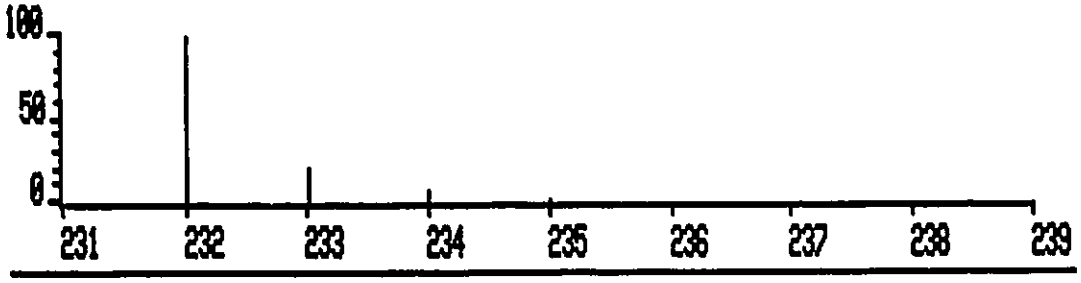
a.

Formula: (NOM,ACC,SPE) Mode: [NOM,ACC,SPE] NOM

Element	#Atoms	#Isotopes	Element	#Atoms	#Isotopes	Element	#Atoms	#Isotopes
C	12	2						
H	16	3						
N	2	2						
O	1	3						
SI	1	3						

Spectrum: (NOM) Nominal Mass = 232

Mass	%Int	Mass	%Int	Mass	%Int	Mass	%Int	Mass	%Int
232	100.00	235	0.62	238	0.00				
233	19.63	236	0.05						
234	5.29	237	0.00						



b.

Mass Calculation Mode

Formula	Mass
$C_{12}H_{16}N_2OSi$	232.1031916960
$^{13}CC_{11}H_{16}N_2OSi$	233.1065465350
$C_{12}H_{16}N_2O^{29}Si$	233.1027596960

2.2.6 GC separation of muscle amino acids.

In addition to the separation of α-KIC for the determination of whole body protein turnover I have also been developing the muscle amino acid isolation and amino acid GC separation/collection method described by other laboratories (Smith et al, 1988; Nair et al, 1988; D. Halliday (Personal communication, 1989)) for the determination of MPS in humans. Muscle samples (50-100 mg wet weight) are obtained by biopsy and lyophilized. The muscle is then ground to a powder in liquid nitrogen with a mortar and pestle and transferred to a test tube. The sample is washed twice with petroleum ether (5 mL) to extract fats and then twice with 3 mL of 0.2N perchloric acid to remove free amino acids. The protein is then hydrolysed to free amino acids with 3 mL of 6M HCl for 15-18 hours at 120°C. The sample is evaporated to about 200 μL with a stream of N_2 gas at about 130°C. The hydrolysate is re-constituted in 1 mL of 50% glacial acetic acid and applied to a 4 cm^2 cation exchange column (Dowex 50W-X8, H^+ form, 100-200 mesh resin (Sigma, St. Louis, MO)). The amino acids are eluted with 7.75 N NH_4OH (4 mL) + 1 mL H_2O after washing the column with 3 mL 1M HCl and 6 mL H_2O. The samples are dried with a stream of N_2 and the residue amino acids are esterified with 400 μL of 3N HCL-isobutanol at 110°C for 30 min (Kaiser

et al, 1974). The samples are blown down with a stream of N_2 and the final acylation is performed with 100 μL of trifluoroacetic anhydride (TFAA) and 250 μL of ethyl acetate at 120°C for 30 min. After drying under N_2, the TFA-iB amino acid derivatives are extracted into 1 mL of n-heptane which is concentrated (N_2 gas) to about 25 μL prior to GC injection. The leucine fraction is collected in a post-column splitter into a U-tube cooled in liquid nitrogen (Smith et al, 1988). The eluted compounds were detected with flame ionization.

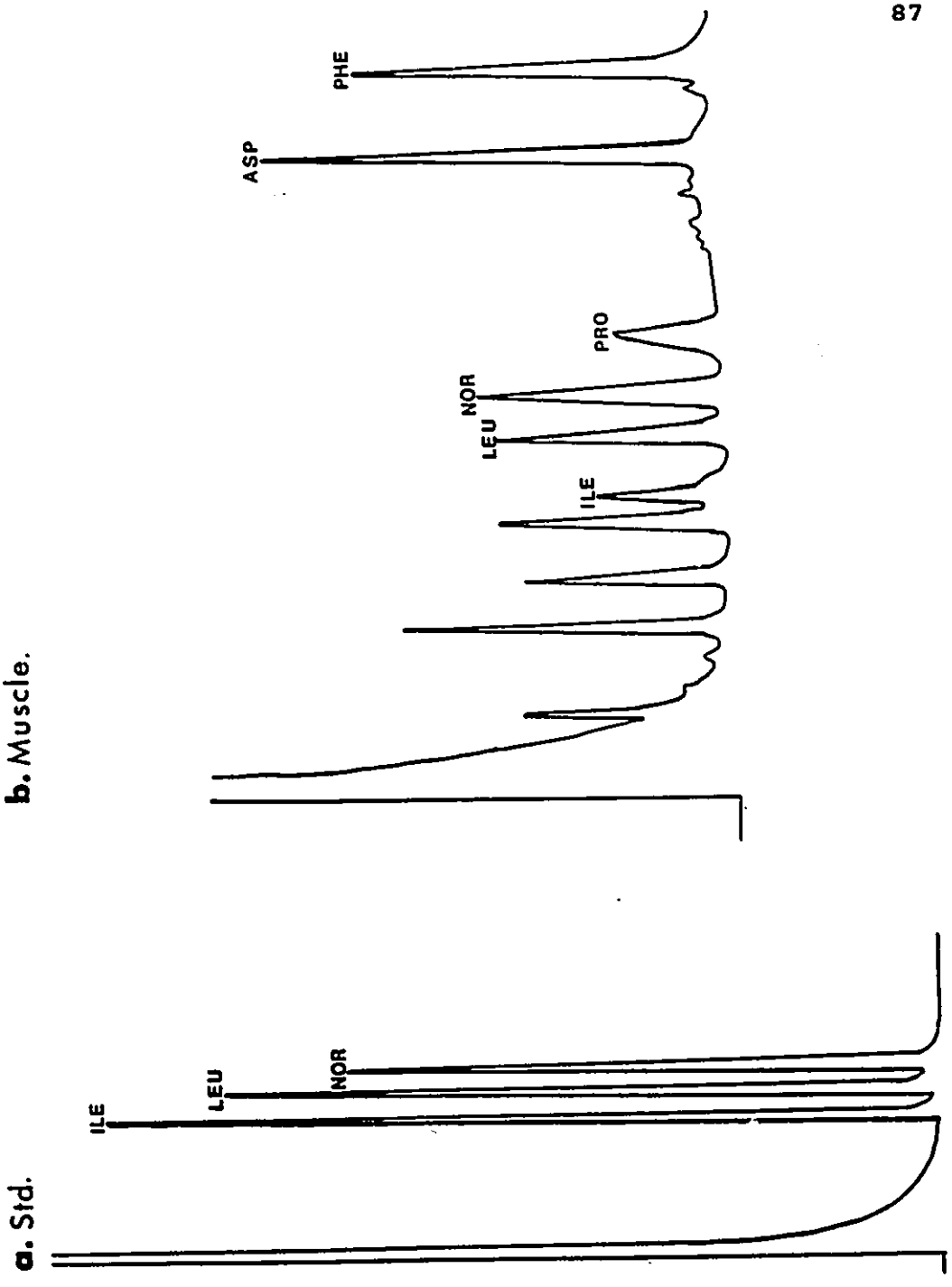
The GC used was a Hewlett Packard Model 5710A (Hewlett-Packard, Avondale, PA) fitted with a custom made glass column (6m, 4mm i.d.) and packed with Chromasorb W-AW (Mannville, Denver, CO) + 2 % ethylene glycol adipate (EGA)(Kaiser et al, 1974). The injector temperature was 250°C and the detector 200°C and the oven programme was kept at 170°C for 15 min and ramped to 220°C for 15 min to drive off the later eluting amino acids. Argon was the carrier gas at a rate of 30 ml$\cdot$ min^{-1} and all of the sample was injected. To collect the leucine fraction I built a stainless-steel, post-column, splitter interfaced with a glass collection tube and stop-cock from commonly available laboratory equipment. Initially, authentic leucine, isoleucine, and norleucine (internal standard) standards (Sigma, St. Louis, MO) were derivatized as described above to identify elution times (peak enancement

methods) and separation characteristics. The three amino acids were very well separated in spite of their identical atomic weights (Figure 9a). Muscle biopsy samples (pectoralis major) were obtained at autopsy (fresh samples from accident victim) and the amino acid isolation and derivatization procedures were performed on 50 mg wet weight samples. The chromatography profile of both leucine, isoleucine, and norleucine standards and a muscle hydrolysate are presented in Figure 9a,b. The isoleucine, leucine and norleucine peaks were identified by elution time and peak enhancement and tentative identification of a few other amino acids was made by comparison to the profiles reported by others (Smith et al, 1988).

The initial collection apparatus (ie. U-tube cooled in liquid nitrogen) did not work so it was decided to bubble the leucine fraction into 1 ml of methylene chloride. This latter modification resulted in quantitative recovery of the leucine fraction of 82 % (n=4) and 62 % (n=6) with a CV of 4.5 % which is somewhat better than the 50 % recovery described by Smith et al. (1988).

I plan to use these methods in the near future to measure MPS in humans (see Chapter 6, section 6.2).

Figure 9. a) GC elution profile of authentic amino acid standards (TFA-iB derivative). b) GC elution profile of human pectoralis muscle amino acids (TFA-iB derivative). ILE = isoleucine, LEU = leucine, NOR = norleucine (internal standard), PRO = proline, ASP = aspartic acid, PHE = phenylalanine.


a. Std.
ILE
LEU
NOR
b. Muscle.
ASP
PHE
PRO
NOR
LEU
ILE

Chapter 3

PROTEIN REQUIREMENTS AND LEAN BODY MASS DURING INTENSIVE RESISTANCE TRAINING IN NOVICE BODY BUILDERS

3.1 Introduction.

The question of the dietary PRO requirements for resistance athletes has been a subject of debate for many years (Fick and Wislicenus, 1866; Cathcart, 1925; Torun et al, 1977; Tarnopolsky et al, 1988). The Canadian Nutrient Intake (RNI)(Health and Welfare, Canada, 1990) and U.S. Recommended Daily Allowance (RDA)(U.S. Food and Nutrition Board, 1989) for PRO are 0.86 and 0.85 g $PRO \cdot kg^{-1} \cdot d^{-1}$ respectively for men >19 years of age, and make no allowance for the type and/or intensity of physical activity. However, evidence is growing to support the concept that both endurance- (Gontzea et al, 1974; Evans et al, 1983; Tarnopolsky et al, 1988; Merideth et al, 1989; Friedman and Lemon, 1989; Brouns et al, 1989a) and resistance-(Celojowa et al, 1970; Consolazio et al, 1976; Torun et al, 1977; Laritcheva et al, 1978; Marable et al, 1979; Dragan et al, 1985; Walberg et al, 1988; Tarnopolsky et al, 1988) type exercise increase PRO requirements.

The suggested safe PRO_{IN} for resistance athletes ranges widely; from levels just above (Consolazio et al, 1975; Torun et al, 1977; Tarnopolsky et al, 1988), to those 2-2.5 times (Laritcheva et al, 1978) and even 4 times (Celejowa et al, 1970) the Canadian R.N.I. and U.S. R.D.A. recommendations. This wide discrepancy may relate to differences in training intensity (Henderson et al, 1985; Lemon et al, 1982), energy (Walberg et al, 1988; Knapik et al, 1991)) and carbohydrate (Lemon and Mullin, 1980; Walberg et al, 1988; Brouns et al, 1989b) content of the diet, adaptation to a given training load (early training vs habitual)(Gontzea et al, 1975; Tarnopolsky et al, 1988; Pivarnik et al, 1989), and possibly to the confounding effect of anabolic steroids (Griggs et al, 1989; Boone et al, 1990).

Resistance/bodybuilder athletes habitually consume a calculated mean PRO_{IN} of 2.34 ± 0.51 $g \cdot kg^{-1} \cdot d^{-1}$ (Celejowa et al, 1970; Laritcheva et al, 1978; Faber et al, 1986; Tarnopolsky et al, 1988; van Erp-Baart et al, 1989; Bazzarre et al, 1990) in spite of the questionable effect of an excessive PRO_{IN} upon lean body/muscle mass accretion (Rasch and Pierson, 1962; Consolazio et al, 1975; Torun et al, 1977; Dragan et al, 1985; Tarnopolsky et al, 1988).

There is limited evidence suggesting that long-term, excessive PRO_{IN} may have potentially negative health consequences (Brenner, 1982; Zaragoza et al, 1987) but it has not been documented that the long-term consumption of PRO at greater than recommended levels in otherwise healthy resistance athletes would have negative health consequences. However, PRO is an expensive macronutrient (since costs of animal PRO are usually in excess of isoenergetic amounts of fat or carbohydrate food-stuffs) and a high PRO_{IN} may have a negative impact upon the percent energy intake ($\%E_{IN}$) from carbohydrates and increase the $\%E_{IN}$ from fats, both of which are associated with increased health risk (Health and Welfare, Canada, 1990). Thus, it is important to determine the PRO requirement for strength athletes to avoid a state of nutrient excess and conversely, to avoid a nutrient deficiency state with possible negative health consequences such as, suppressed immune function (Ardawi and Newsholme, 1983), sports anemia (Yoshimura et al, 1980)) and sub-optimal muscle growth.

The purpose of this study was to determine the PRO requirements for strength athletes performing intensive resistance exercise in the early stages of training and to determine whether a very high PRO_{IN} (PRO supplement) (2.6 $g \cdot kg^{-1} \cdot d^{-1}$) would result in greater strength and/or lean mass

accretion compared to a lower PRO_{IN} (CHO supplement) (1.4 $g \cdot kg^{-1} \cdot d^{-1}$).

The hypotheses of the present study were: 1. Protein supplementation to 2.6 $g \cdot kg^{-1} \cdot d^{-1}$ for one month during the early phases of an advanced body building training programme would not result in a functional increase in lean body/muscle mass accretion or strength when compared to one month at a PRO_{IN} of 1.35 $g \cdot kg^{-1} \cdot d^{-1}$ and thus would result in a nutrient excess. 2. Calculated PRO requirements from nitrogen balance data would be greater than the Canadian RNI and U.S. R.D.A. and less than PRO_{IN} habitually consumed by elite resistance athletes; 3. The calculated PRO requirements would be greater in the early stages of training (first 2 months) than requirements determined for experienced (habitual) resistance athletes in a previous study (Tarnopolsky, et al, 1988).

3.2 Methods.

3.2.1 Subjects.

Twelve, healthy, young males volunteered as subjects for the study after being informed of the risks and benefits of their participation and informed consent was obtained in accordance with the McMaster University Research Project Advisory Committee for Clinical Studies and the Kent State Ethics Committee. None of the subjects had participated in

a structured weight-lifting program for the 12 months prior to starting the study, yet all had some past experience with weight-lifting. None of the subjects reported use of anabolic steroids. The characteristics of the subjects are given in Table 1.

Table 1. *Subject characteristics*

n=12	
Age, yr	22.4 ± 2.4
Weight, kg	81.9 ± 11.3
Height, cm	180.8± 6.1
Body fat, %*	10.1 ± 6.1

Values are mean ± SD. * Determined from hydrostatic weighing and Brozek equation (Brozek et al, 1973).

3.2.2 Experimental protocol.

Each of the subjects completed two 1 mo experimental phases separated by a 7 d *ad libitum* diet washout period. For the entire 2 mo period each subject received a diet that was isoenergetic to their habitual energy intake as determined from 3 d computerized diet record assessment (Analyze, McMaster University, Hamilton, Ont) collected during the week prior to the first diet period. The treatments were randomized in a double-blind and counter-balanced design such that subjects received, in addition to their normal diet, an isoenergetic supplement (1.5 $g \cdot kg^{-1}$) of either carbohydrate (CHO treatment; maltodextrin in a

flavoured drink plus 30 gelatin capsules containing maltodextrin) or PRO (PRO treatment; calcium caseinate in a flavoured drink plus 30 gelatin capsules containing free amino acids).

During the final 3 d of each diet phase nitrogen balance (NBAL) was determined as described below. During the NBAL phase all subjects were given food in a pre-packaged form and were instructed to consume only the foods provided. Subjects recorded consumption of all foods and liquids and reported compliance was >97 %. Compliance was maximized by having the subjects check off, from a list, all foods immediately after consumption and by having them report to the testing centre at least twice over the NBAL period to deliver urine samples. To allow for dietary adaptation, the diet provided during the NBAL was based upon each individual's intake which was recorded for 3 d after 3.5 wk on each dietary treatment (Appendix IV.2, dietary adaptation was confirmed by lack of slope in day to day urinary UN excretion).

The subjects participated in an intensive (6 $d \cdot wk^{-1}$) body building (BB) type of weight-lifting programme that was supervised by professional bodybuilders. The training was a standard set, 3 d split-routine (day 1: chest/back; day 2: legs; day 3: shoulders/arms) repeated twice per week, with 1

d rest per week. The individual exercises on each day were: 1. chest/back - bench press, incline press, dumbbell flys, latissimus pulldown, seated row, dumbbell row, and situps; 2. legs - squats, knee ext., leg curl, calf raise, and situps/leg raise; 3. shoulder/arms - shoulder press, side laterals, upright row, triceps ext., triceps push-down, biceps curl, conc. curl, and situps. Each training day the athletes completed a warm-up set followed by 4 sets of 8-10 repetitions to failure at approximately 70-85% of the individual's one repetition maximum (1RM). Subjects trained with partners and were encouraged to increase weight as their strength progressed. Prior to the study the athletes received instruction in the proper technique of each lift and completed 1 wk of training with increasing resistance in an attempt to minimize soreness once the actual training began. In order to assess the effect of the supplement/training interaction on muscle directly, a single arm training model was used (each subject trained the elbow flexors of one arm while on CHO supplement and of the other while on PRO supplement).

Measurements of the effects of diet and/or training upon indices of muscular development were assessed before and after each 4 wk training period on each supplement and included: lean body mass (hydrostatic weighting); nitrogen

balance (NBAL); one repetition maximum (1RM) contraction strength for bench press and leg squat exercises; neuromuscular properties of the elbow flexors including peak twitch tension (PTT), post-tetanic PTT, maximal isometric contraction force (MVC), and percent motor unit activation (% MUA); and a muscle biopsy of the biceps brachii was obtained and analyzed for total nitrogen content. Measurements were also completed of the mid-arm and mid-thigh circumferences and computerized axial tomography (CAT) scans of the mid-arm and mid-thigh were also performed and reported (Lemon et al, 1990) but were not included in this thesis for I did not contribute substantially to these measurements. All of these measurements occurred before and after each of the two four week training blocks.

3.2.3 Lean body mass.

Lean body mass (LBM) was determined using the hydrostatic weighing technique and residual lung volume was determined using a helium dilution method (W.E. Collins, Braintree, MA) and percent body fat was estimated by the equation of Brozek (1967). Subjects were weighed nude using an electronic scale accurate to ± 10g (Mott Scales, Brantford, Ont.). Using this method the CV for determining lean body mass was 1.2 % for 31 subjects tested >10 days apart with 2 or 3 determinations for each subject.

3.2.4 One repetition maximum.

One repetition maximum strength was taken as the maximal weight (1 RM) lifted using a concentric contraction at a pre-determined starting joint angle of 90 degrees after a brief warm-up. The measurements were the bench press and squat manoeuvres. All measurements were performed using the same weight machine (Global Gym, Weston, Ont.).

3.2.5 Neuromuscular function.

This was determined for the elbow flexors (primarily biceps brachii) using a custom made dynamometer (see Blimkie, et al, 1990 and Tarnopolsky, et al, 1989 for details about the apparatus). The arm was firmly strapped to the recording device using Velcro straps at a fixed elbow joint angle of 110° and the shoulder at 90° of flexion. The apparatus and recording equipment have been previously described (Tarnopolsky et al, 1989). The initial arm to be trained was tested before and after the first four week training session and the contralateral arm was tested before and after the second training session. The measurements included PTT (motor point stimulation), post-tetanic PTT (PTT taken within 5 s of an MVC), MVC, and % MUA (the latter of the biceps brachii using the interpolated twitch technique (Belanger and McComas, 1981)).

3.2.6 Muscle biopsy.

A percutaneous needle biopsy sample (approx. 100mg) was obtained from the lower-lateral quadrant of the biceps brachii under local anaesthesia (Evans et al, 1982). A total of 4 biopsies were taken (2 from each muscle (1 before and 1 after each 4 wk period)). The sample was quenched in liquid nitrogen for subsequent analysis of total nitrogen (TN) content using an elemental analyzer (flash combustion-gas chromatography-thermal conductivity detection)(Perkin-Elmer, Eden Prairie, MN).

3.2.7 Nitrogen balance.

From the individual computerized analysis of 3 day food records collected one week prior to each of the NBAL periods, an individual diet was prepared for each subject to match their individual mean energy intake and to be representative of the percent energy derived from each of fat, carbohydrate, and PRO. Subjects continued to consume either their PRO or maltodextrin supplements and these were included in the determination of the energy intake of each subject. The subjects were issued their diet: 50% solid foods and 50% defined formula liquid supplement (Ensure, Ross Laboratories, Columbus, OH) in a pre-packaged form with each item weighed to $\pm$0.05g (OHAUS, E400D, Florham Park, NJ). The solid food component consisted of one of 5

distinct diets composed of varying amounts of the following foods: spaghetti, spaghetti sauce, whole wheat bread, jam, apples, apple juice, peanut butter, crackers, chocolate chip cookies, and butter. Twenty percent of each dietary component from each of the 5 diets was homogenized for 10 min, lyophilized, ground and analyzed for total nitrogen (TN) by the micro-Kjeldahl method and gross energy content by adiabatic bomb calorimetry. During each three day NBAL period urine was collected into 4L containers containing 5 mL glacial acetic acid and a 72 h fecal collection was made between carmine markers. Carmine markers (1 $g \cdot subject^{-1}$) were provided in gelatin capsules. Aliquots of urine were kept at -70°C until subsequent determination of TN, urea nitrogen (UN) and creatinine. Fecal samples were weighed, homogenized with an equal weight of deionized water, and an aliquot was lyophilized and analyzed for TN content. Sweat nitrogen loss estimates were made in 3 subjects on the CHO treatment and in 4 different subjects on the PRO treatment following a 2 h exercise period using the washdown method described by Lemon et al (1986). The resting sweat losses were estimated from the results of a recent study performed using similar protein intakes in BB athletes (Tarnopolsky et al, 1988). An aliquot of the sweat washdown was taken and kept at -70°C until analysis for UN content. The individual

sweat values were used for those tested and the mean values were used for the other subjects. Miscellaneous N losses (semen, tooth brush, toilet paper, plate losses, hair, N_2 gas) were estimated at 140 $mgN \cdot d^{-1}$ per subject in both groups (Calloway et al, 1972). Apparent NBAL was calculated as the difference between N_{IN} (diet) - N excretion (urine + feces + sweat + misc.) (Analytical precision for each component of NBAL is given in Appendix II.1.A).

3.2.8 Biochemical analyses.

Total N (TN) content of the diets, urines and feces were determined using the micro-Kjeldahl technique (Assoc. of Official Analytical Chemists, 1965). The intra-assay CV for diets, urines and feces was 4.4 %, 5.8 %, and 3.8 % while the inter-assay CV was 9.2 %, 1.1 %, and 5.0 % respectively (Appendix II.1.A). The mean ratio of the measured:calculated N was 0.91 ± 0.04 for the 5 standard solid food diets respectively (assuming mixed PRO are 16% N by weight)(Appendix II.1.B). The gross energy content of each diet was determined by adiabatic bomb calorimetry (Parr Instruments, Moline, IL). The intra-assay CV of the bomb calorimeter was 3.1 %. To convert from metabolizable energy (diet calculations) to gross energy the % metabolizable energy contribution of carbohydrate, fat and PRO were multiplied by 1.00, 1.03 and 1.43 (Merrill and Watt, 1973;

Buskirk and Mendez, 1980). The ratio of measured:calculated gross energy content of each of the 5 standard solid food diets was 0.94±0.05. All N and energy data given during the NBAL period in tables and figures are corrected for the measured values (Appendix II.1.A. + B.).

Urine UN was determined using the urease-phenol method (Kit #640, Sigma, St. Louis, MO). The intra and inter-assay CV's were 4.6 % and 9.0 % respectively. Creatinine was determined using a colorimetric picric acid method (Kit #555, Sigma, St, Louis, MO). The intra and inter-assay CV's for creatinine measurements were 3.0 % and 9.9 % respectively (Appendix III.2).

3.2.9 Statistical analyses.

The effect of diet (CHO vs PRO) on NBAL and creatinine excretion was determined using a paired t-test. Regression analysis of NBAL vs PRO_{IN} was performed to determine the PRO_{IN} for zero nitrogen balance. The effect of the training (pre-post variable) and diet treatment (CHO vs PRO variable) on various indices of muscle function (neuromuscular function, 1 RM strength tests) and anthropometry (body density, CAT scan data, limb circumferences) were determined using a repeated measures analysis of variance (ANOVA). A probability of $P \leq 0.05$ was taken to indicate significance. Values in text, tables, and figures are mean ± SD.

TABLE 2. *Diet summary*

PERIOD*	ENERGY INTAKE ($kJ \cdot kg^{-1} \cdot d^{-1}$)	(PRO)†	(CHO)	(FAT)	(ETOH)	PROTEIN INTAKE ($g \cdot kg^{-1} \cdot d^{-1}$)
Habitual	165 ± 11	14	52	32	2	1.44 ± 0.24
AD-PRO	172 ± 12	26	43	31	0	2.62 ± 0.33‡
AD-CHO	174 ± 12	13	55	30	2	1.35 ± 0.37
NBAL-PRO	165 ± 13	28	41	31	0	2.67 ± 0.34‡
NBAL-CHO	161 ± 39	10	60	30	0	0.99 ± 0.29§

Values are mean ± SD. * Periods: Habitual=habitual consumption in the week prior to starting study; AD-PRO and CHO=one month adaptation period on each of the respective diets; NBAL-PRO and CHO=3 d nitrogen balance period on each of the respective diets. † % contribution to total energy intake. ‡ Significantly ($P<0.001$) greater than other periods. § Significantly lower than AD-CHO or Habitual ($P<0.05$).

3.3 Results.

3.3.1 Dietary analyses.

The data for the dietary intakes during each phase are given in Table 2. Energy intakes were not significantly different between diet periods. The PRO_{IN} (and % contribution of PRO to total energy) were greater ($P \leq 0.001$) for the adaptation and NBAL PRO periods vs habitual intake, adaptation and NBAL CHO periods. PRO_{IN} was significantly lower during the NBAL CHO period than during the adaptation CHO period ($P \leq 0.05$) and the habitual PRO_{IN} ($P<0.01$). The habitual PRO_{IN} was not significantly different from that during the adaptation CHO period. The % energy contribution from carbohydrate during the NBAL and adaptation PRO periods were significantly ($P \leq 0.05$) lower than during the other periods. (Table 2).

3.3.2 Nitrogen balance.

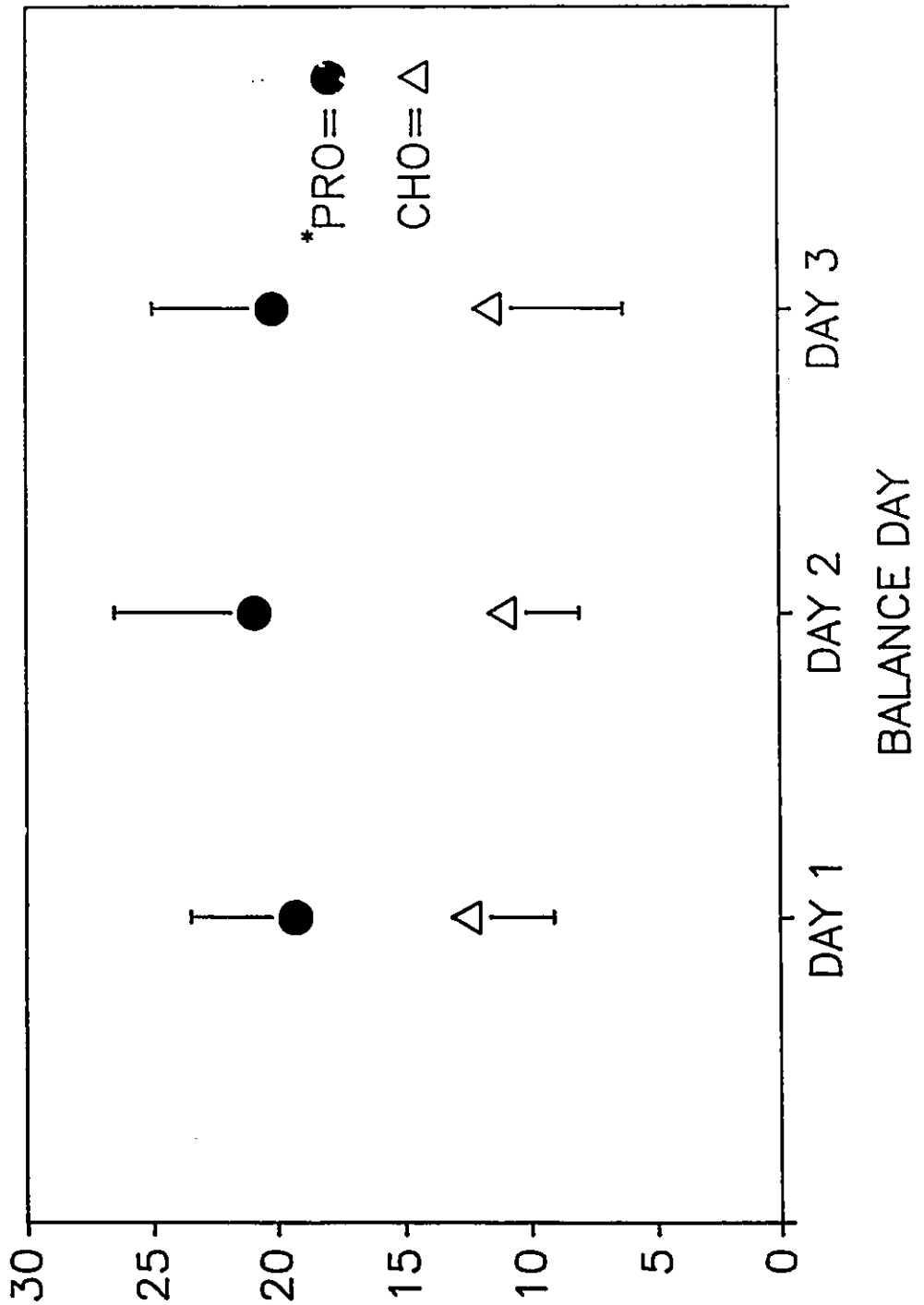
The nitrogen balance was significantly more positive for the PRO vs CHO periods (+8.9 vs -3.4 $g \cdot d^{-1}$, $P<0.001$) and all subjects were in negative NBAL while on the CHO treatment (Table 3). In addition, N intake, urinary N losses, sweat + misc. N losses, and total N losses were greater for the PRO vs CHO treatments ($P<0.001$)(Table 3). Urinary urea losses accounted for 92 % and 95 % of TN losses on the CHO and PRO treatments respectively. Adaptation to

TABLE 3. ***Nitrogen balance summary***

Treatment Group	N Intake ($g \cdot d^{-1}$)	N Excretion ($g \cdot d^{-1}$)				
		Urine	Feces	Sweat* + Misc†	Total	Balance‡
CHO	12.8±3.1	12.5±1.2	2.0±0.9	1.6±0.2	16.2±1.7	-3.4±1.9
PRO	34.8±4.6§	21.1±5.6§	2.2±1.0	2.5±0.3§	25.8±6.0§	+8.9±4.2§

Values are mean ± SD. * Sweat = measured exercise loss + rest est. (Tarnopolsky et al, 1988). † Estimate of miscellaneous losses (Calloway et al, 1971). ‡ Balance = N Intake - Total N Excretion. § Significantly greater for PRO vs CHO ($p<0.001$).

Figure 10. Effect of dietary treatment on daily urinary urea excretion. * Significantly greater than for CHO treatment, P<0.01. PRO = protein supplement; CHO = carbohydrate supplement.


URINARY UREA NITROGEN (g·d⁻¹)
30
25
20
15
10
5
0
DAY 1
DAY 2
DAY 3
BALANCE DAY
*PRO=●
CHO=△

the N intake during each of the NBAL periods is evident by the lack of day to day change in urinary UN excretion and the CV during the CHO and PRO treatments was 6.2 % and 4.1 % respectively (Figure 10).

3.3.3 Protein requirement.

The PRO_{IN} to achieve NBAL was interpolated from multiple regression analysis of N intake ($g \cdot kg^{-1} \cdot d^{-1}$) vs NBAL ($mg \cdot kg^{-1} \cdot d^{-1}$) (NBAL= -119 + 83.1($PRO_{IN}$); SD=9.58, R^2=0.774, $P \leq 0.001$)(Figure 11). The PRO requirement to achieve zero NBAL was 1.43 $g \cdot kg^{-1} \cdot d^{-1}$. With a margin of +1SD the requirement was computed as 1.62 g PRO$\cdot kg^{-1} \cdot d^{-1}$ and with a margin of +2SD the requirement was computed as 1.86 g PRO$\cdot kg^{-1} \cdot d^{-1}$ (Figure 11) (Appendix IV, A).

3.3.4 Anthropometry, creatinine excretion and biceps muscle nitrogen.

There were no effects of dietary treatment (PRO vs CHO) or training (pre vs post) on: a. weight (PRO=81.95 kg; CHO=81.95 kg); b. percent body fat (PRO=10.10 %; CHO=10.15 %); c. body density (PRO=1.0772 $g \cdot cm^{-1}$; CHO=1.0772 $g \cdot cm^{-1}$); d. lean body mass (PRO=73.70 kg; CHO=73.65 kg); e. urinary creatinine excretion (PRO=10.9 $mmol \cdot d^{-1}$; CHO=11.5 $mmol \cdot d^{-1}$); f. biceps muscle N concentration (PRO=14.86 gN$\cdot$100g D.W.$^{-1}$; CHO=15.00 gN$\cdot$100g D.W.$^{-1}$) (Values in text represent the mean of pre-post values)(Figure 12)(Appendix IV. Table IV 1,2).

Figure 11. Predicted protein intake (PRO_{IN}) for zero nitrogen balance (NBAL). Interpolation was based upon the regression line (NBAL = -119 + 83.1(PRO_{IN}), R^2=0.774, P<0.001) calculated from the NBAL on both diet treatments.

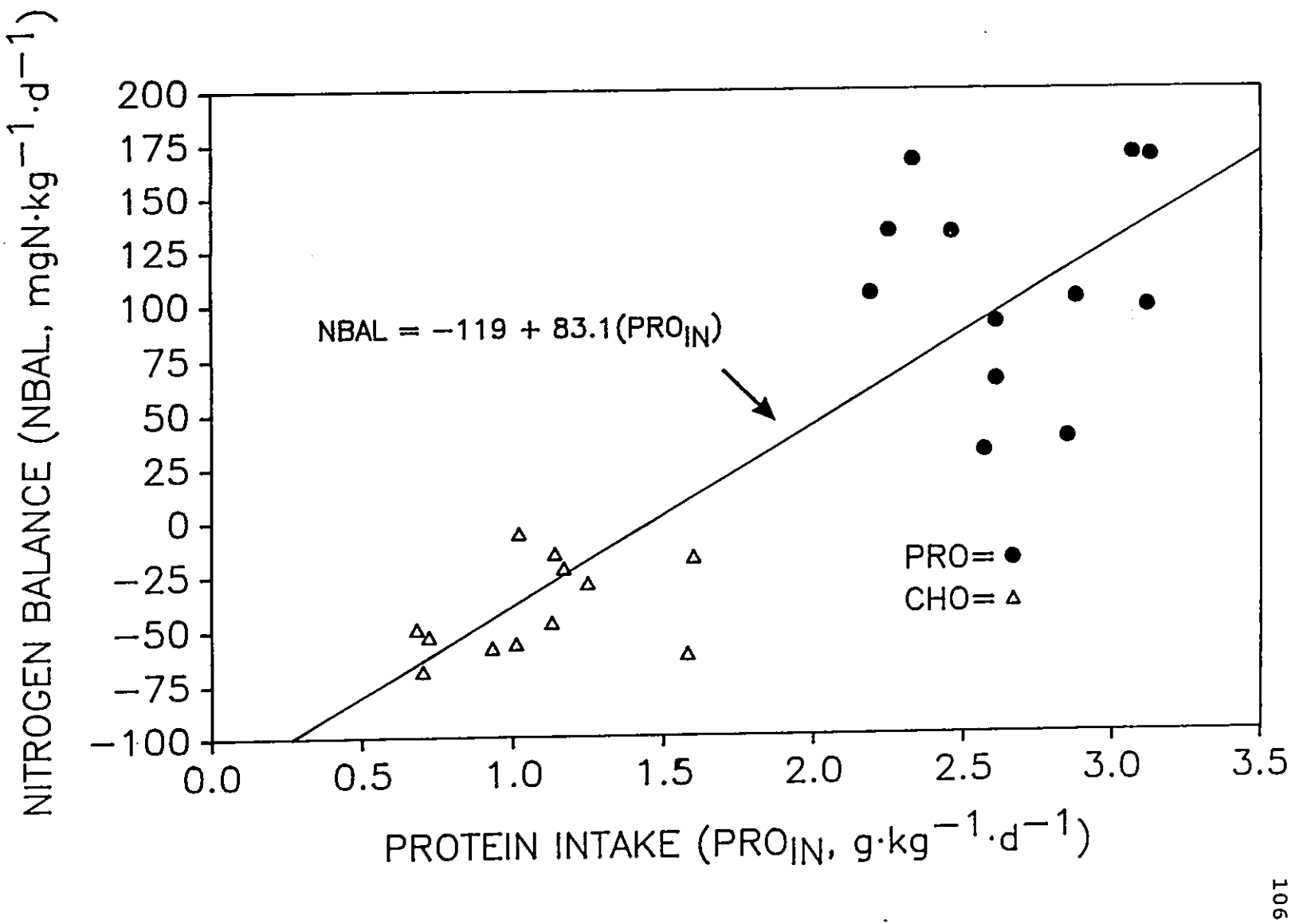

NITROGEN BALANCE (NBAL, mgN·kg^{-1}·d^{-1})
200
175
150
125
100
75
50
25
0
−25
−50
−75
−100
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
NBAL = −119 + 83.1(PRO$_{IN}$)
PRO= ●
CHO= △
PROTEIN INTAKE (PRO$_{IN}$, g·kg^{-1}·d^{-1})

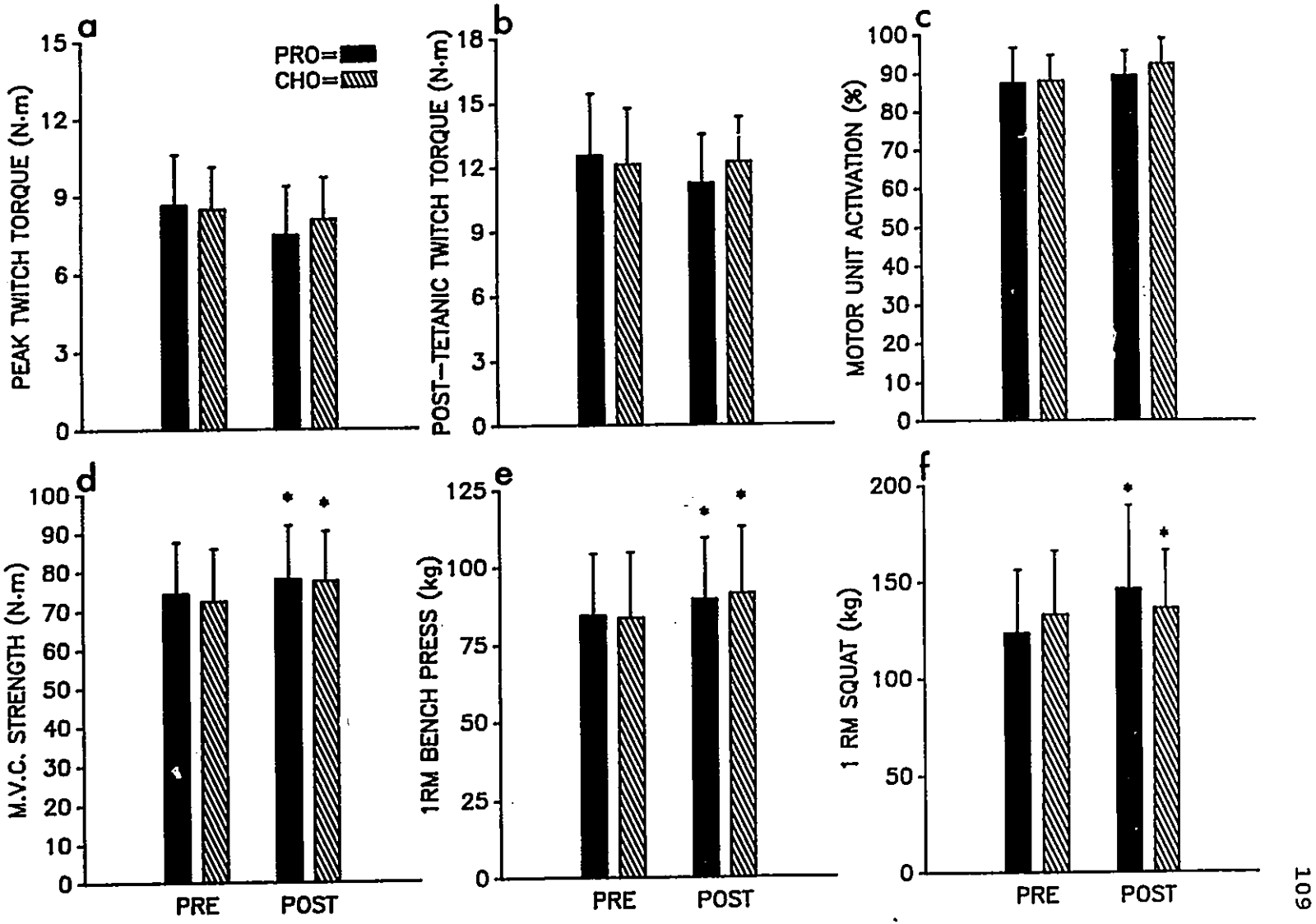
Figure 12. Selected anthropometric measurements and biochemical indices of muscle mass. a. Body weight. b. Percent body fat calculated from density (Brozek et al, 1963). c. Body density (hydrostatic weighing). d. Lean body mass (calculated from a. + b. above). e. Urinary creatinine excretion (mean of each 3 day NBAL period. f. Biceps brachii muscle total nitrogen content (D.W. = dry weight).

a
WEIGHT (kg)
PRO=
CHO=
b
BODY FAT (%)
c
BODY DENSITY (g/cm2)
d
LEAN BODY MASS (kg)
PRE
POST
e
CREATININE (mmol·d−1)
PRO
CHO
f
BICEPS MUSCLE NITROGEN (g·100g D.W.−1)
PRE
POST

3.3.5 Neuromuscular properties and strength data.

There were no effects of diet or training on the neuromuscular properties of the forearm flexors: a. peak twitch torque (PRO=8.05 N·m; CHO=8.25 N·m); b. post-tetanic twitch torque (PRO=11.9 N·m; CHO=12.15 N·m); and c. percent motor unit activation (PRO=88.5 %; CHO=90.4 %)(Values in above text represent mean of pre-post values)(Figure 13). There were no effects of diet treatment upon indices of absolute strength, however, training had a significant ($P<0.05$) effect upon: d. maximal voluntary forearm flexor contraction strength (PRO=74.4→77.9 N·m (↑ 4.7%); CHO 72.4→77.4 N·m (↑ 6.9%)); e. 1 RM bench press strength (PRO=84.6→89.6 kg (↑ 5.9%); CHO=83.7→91.5 kg (↑ 9.3%)); f. 1 RM squat strength (PRO=123.7→146.4 kg (↑ 18.4%); CHO=133.3→136.5 kg (↑ 2.4%))(Figure 13)(Appendix IV, Table IV-3).

Figure 13. Strength measurements. a. Peak evoked twitch torque. b. Peak evoked post-tetanic twitch torque. c. Percent motor unit activation (Belanger and McComas, 1981). d. Maximal voluntary contraction (M.V.C.) strength of dominant arm flexors. e. One repetition maximum (1 RM) bench press strength. f. One repetition maximum (1 RM) leg squat strength. * Significant ($P<0.05$) training effect.


a
PRO=
CHO=
PEAK TWITCH TORQUE (N·m)
15
12
9
6
3
0
b
POST-TETANIC TWITCH TORQUE (N·m)
18
15
12
9
6
3
0
c
MOTOR UNIT ACTIVATION (%)
100
90
80
70
60
50
40
30
20
10
0
d
M.V.C. STRENGTH (N·m)
100
90
80
70
60
50
40
30
20
10
0
PRE
POST
e
1RM BENCH PRESS (kg)
125
100
75
50
25
0
PRE
POST
f
1 RM SQUAT (kg)
200
150
100
50
0
PRE
POST

3.4 Discussion.

The observations of the present study were in agreement with the *a priori* hypotheses: 1. Protein supplementation (total PRO_{IN} = 2.62 $g \cdot kg^{-1} \cdot d^{-1}$) did not result in increased lean body/muscle mass or in strength compared to an isoenergetic supplement of carbohydrate (total PRO_{IN} = 1.35$\cdot g \cdot kg^{-1} \cdot d^{-1}$) each during 1 month of an advanced bodybuilding programme; 2. During the first 2 months of an advanced bodybuilding programme the PRO requirement for zero NBAL was 1.43 $g \cdot kg^{-1} \cdot d^{-1}$, which is 66 and 68% greater than Canadian or U.S. PRO recommendations respectively; and 3. the calculated zero NBAL level of 1.43 $g \cdot kg^{-1} \cdot d^{-1}$ was 63% greater than for the zero NBAL calculated for experienced (habitual) bodybuilders in a previous study completed under similar conditions (Tarnopolsky et al, 1988).

In spite of the general belief among bodybuilders and other resistance athletes that high PRO_{IN} ($\sim$2.3 $g \cdot kg^{-1} \cdot d^{-1}$) are necessary during training (Celejowa et al, 1970; Laritcheva et al, 1978; Faber et al, 1986; Tarnopolsky et al, 1988; van Erp-Baart et al, 1989; Bazzarre et al, 1990), there is a paucity cf investigations that have been designed to address whether this amount of dietary PRO is necessary. The finding of no effect of PRO supplementation upon indices

of lean/muscle accretion and/or strength is in disagreement with the results of several studies (Consolazio et al, 1975; Torun et al, 1977; Marable et al, 1979; Dragan et al, 1985), however, there are important methodological considerations which limit the interpretation of these earlier studies.

Consolazio et al. (1975) reported significantly greater lean body mass (densitometry) and cumulative NBAL in male subjects provided 2.8 g PRO·kg^{-1}·d^{-1} (n=4) compared to a matched group given 1.4 g PRO·kg^{-1}·d^{-1} (n=4) over a 40 day mixed exercise (walking, running, calisthenics, isometrics and cycle ergometry). This study was confounded by the varied forms of exercise (resistance and endurance) for endurance exercise increases amino acid oxidation (Rennie et al, 1981; Wolfe et al, 1982, 1984b; Evans et al, 1983) while resistance exercise does not (Tarnopolsky et al, 1991). Both resistance (Yarasheski et al, 1989; Wong and Booth, 1990a,b; Chesley et al, 1991) and endurance (Carraro et al, 1990) exercise increase MPS after exercise. The additive effect of the two forms of exercise would elevate the PRO requirements of those who perform both endurance and strength exercise. In addition, this study did not use a repeated measures design and the small sample size (n=4/group) limited its statistical power. Another similar study reported greater lean body mass (^{40}K counting) and

NBAL in young men performing isometric exercise (75 min/d, 3 X/wk) for ~5 weeks who received 1.0 $g \cdot kg^{-1} \cdot d^{-1}$ egg and milk PRO (n=4) compared to 7 different subjects who received 0.5 $g \cdot kg^{-1} \cdot d^{-1}$ egg and milk PRO (Torun et al, 1977). Again the study had small subject numbers, did not use a repeated measures design and isometric exercises were used which made it difficult to confirm and equate total workloads between the groups (Torun et al, 1977). It is also not surprising that there were greater LBM gains in the higher PRO group for their total PRO_{IN} was 100% greater than for the low PRO group who were given only about 0.55 g <u>mixed</u> $PRO \cdot kg^{-1} \cdot d^{-1}$ (Torun et al, 1977), which would not only be deficient based upon the results of the present study (1.43 g mixed $PRO \cdot kg^{-1} \cdot d^{-1}$) but would also fall below both the Canadian R.N.I. (Health and Welfare, Canada, 1990) and U.S. R.D.A. (U.S. Food and Nutrition Board, 1989) for PRO.

Marable <u>et al.</u> (1979) also reported greater nitrogen retention (dietary N - urine N) in young men given 300% (n=4) compared to 100% (n=2) of the U.S. R.D.A. for PRO consequent to a 4 week strength training programme. In addition to the low subject numbers and study design concerns expressed above, this study (Marable et al, 1979) was also limited by the fact that the "low" PRO group consumed a PRO_{IN} that was ~68% below the calculated

requirement in the present study and the nitrogen retention would have been overestimated in the higher PRO group because sweat measurements were not made and sweat N excretion increases with N intake (Tarnopolsky et al, 1988). One study examined the effects of increasing the dietary PRO_{IN} in a group of elite weight lifters from habitual PRO_{IN} of 2.2 $g \cdot kg^{-1} \cdot d^{-1}$ to 3.5 $g \cdot kg^{-1} \cdot d^{-1}$ during several months of training and significant increases in muscle strength (↑ 5%), muscle mass (↑ 6%) and lean body mass (↑ 5% (estimated from fat skinfolds)) were observed (Dragan et al, 1985). However, the study may have been confounded due to the fact that the subjects were peaking for competitions, that no information was given with respect to anabolic steroid use, and the subjects and investigators were not "blinded" to the dietary intervention (Dragan et al, 1985).

The present study demonstrated that the PRO_{IN} habitually consumed by bodybuilders/resistance athletes (2.34 $g \cdot kg^{-1} \cdot d^{-1}$) represented a *nutritional overload* (Young and Bier, 1987a). This PRO_{IN} did not result in any positive effects upon objective measures of strength (1 RM bench press and squat, maximal isometric torque, PTT) or lean body/muscle mass (body density, creatinine excretion, muscle N content) compared to a PRO_{IN} close to calculated requirements. These results were obtained during a

relatively short training period (total = 2 months) which did result in a significant training effect (increased voluntary strength). In addition, computerized axial tomography (CAT) scans of the mid-arm and mid-thigh were taken from the subjects in this thesis before and after each dietary treatment, and it was found that training had a significant ($P<0.05$) positive effect on mid-arm muscle area (Lemon et al, 1990). This provided further evidence that the training stimulus was sufficient to induce muscular hypertrophy. A training effect was a necessary pre-requisite to determine whether PRO supplementation was superior to carbohydrate supplementation. This is in agreement with previous work that found no increase in lean body mass (densitometry and creatinine excretion) (Tarnopolsky et al, 1988) or neuromuscular properties (including isometric strength)(Blimkie et al, 1986) in elite bodybuilders consuming 2.77 g $PRO \cdot kg^{-1} \cdot d^{-1}$ compared to 1.05 g $PRO \cdot kg^{-1} \cdot d^{-1}$. This latter study was completed with 6 subjects in a repeated measures design, none of the subjects had used anabolic steroids for at least 2 years, and none were preparing for competition (Tarnopolsky et al, 1988). Another study claimed not to find an effect of PRO supplementation upon lean body mass and strength in men performing weight lifting exercises for 6 weeks, but these

findings are not unexpected given that the total supplement amounted to only an additional 0.03 g PRO·kg^{-1}·d^{-1} (Rasch and Pierson, 1962).

Animal data has demonstrated that MPS and PRO gain plateau and the extra PRO is metabolized through an up-regulated urea cycle (Zaragoza et al, 1987) and excreted as urea when rats are fed PRO in amounts exceeding their requirement (Smith et al, 1982; Zaragoza et al, 1987). In studies of elite bodybuilders (Tarnopolsky et al, 1988) and novice weight-training athletes (Hickson et al, 1988) it has been shown that PRO consumed in excess of requirements is excreted in the urine rather than retained as lean body mass. In humans performing circuit weight training we have recently shown that PRO_{IN} of 2.4 g·kg^{-1}·d^{-1} resulted in an exponential increase in leucine oxidation with no increase in whole body PRO synthesis compared to 1.4 g·kg^{-1}·d^{-1} (Tarnopolsky et al, 1991). This suggested that the mean habitual PRO_{IN} for body builders cited above (2.34 g·kg^{-1}·d^{-1}) represents a *nutritional overload* and would result in an increase in the oxidation of amino acids as fuels rather than their being stored as PRO (Tarnopolsky et al, 1991).

Using linear regression methodology (Tarnopolsky et al, 1988; Meredith et al, 1989; U.S. Food and Nutrition Board, 1989) it was determined that the bodybuilders in this study

required 1.43 g PRO·kg^{-1}·d^{-1} to maintain zero NBAL. This level is 66 and 68 % greater than current Canadian (Health and Welfare, Canada, 1990) and U.S. (U.S. Food and Nutrition Board, 1989) PRO recommendations, respectively. Since the Canadian and U.S. Recommendations for PRO are based upon the mean value for zero NBAL plus 2 standard deviations it would appear appropriate to include 2 standard deviations to the zero NBAL value for the bodybuilders, which resulted in a recommended PRO_{IN} for this group of 1.86 g·kg^{-1}·d^{-1}. However, this recommendation has to be considered given the limitations of a short term nitrogen balance study conducted with only 12 subjects and with the PRO diet providing 87 % more PRO than the calculated zero NBAL. The regression equation generated from the two N intakes being far apart would increase the variance around the PRO_{IN} estimates, thus, it is probably more accurate to compare the estimate of zero NBAL + 1 SD to other PRO_{IN} recommendations. The validity of this is supported by the results from two NBAL studies performed in our laboratory that found the "safe" PRO_{IN} (+ 1 SD) of sedentary individuals (0.89 g·kg^{-1}·d^{-1} (n=6)(Chapter 5); 1.02 g·kg^{-1}·d^{-1} (n=6)(Tarnopolsky et al, 1988) to be very close to national recommendations (U.S. Food and Nutrition Board, 1989 (0.85 g·kg^{-1}·d^{-1}); Health and Welfare, Canada, 1990 (0.86 g·kg^{-1}·d^{-1}).

The recommended PRO_{IN} for novice bodybuilders of 1.62 $g \cdot kg^{-1} \cdot d^{-1}$ is 35 % greater than that recommended for elite bodybuilders studied under nearly identical conditions (Tarnopolsky et al, 1988). The increased requirement for the novice is probably indicative of an increased physiological stress in the early stages of training to which the athlete subsequently adapts and the requirements are not as great (Gontzea et al, 1975). Humans adapt rapidly to a physiological stressor such as unaccustomed muscular contractions for there appears to be no evidence of sarcolemmal disruption (↑ CPK activity) in subjects 2 weeks after the first bout of eccentric exercise (Clarkson and Tremblay, 1988). For bodybuilding exercise there is probably greater myofibrillar PRO turnover (increased breakdown (Dohm et al, 1982; Hickson et al, 1985; Pivarnik et al, 1989) and a greater increase in MPS (Yarasheski et al, 1989; Wong and Booth, 1990a)) in the early stages of training which results in net synthesis and hypertrophy whereas the elite athlete is probably at a plateau with relatively little net muscle accretion. This is apparent in the modest gains observed for indices of strength (↑ 4 %) and muscle fibre diameter (↑ 5.9%) in elite weight lifters over a 2 year period (Hakkinen et al, 1988). In contrast to the elite athlete, the novice bodybuilders had significant

mean (mean of PRO and CHO) increases of 7.9 % for strength indices and a mean increase of 8.8 % for the mid-arm flexor area after only 4 weeks of training (Lemon et al, 1990).

As observed previously (Hegsted, 1975; Oddoye and Margen, 1979; Young et al, 1981a; Tarnopolsky, 1988) there was a marked positive NBAL (+8.9 gN) on the high protein diet that was not accounted for by increases in lean tissue accretion. Over a 4 week period this degree of positive NBAL (+8.9 gN) should have resulted in a net muscle accretion of 7.8 kg (assuming wet muscle tissue is 20 % protein and protein is 16 % N by weight). This increase would have been detectable using the body density method and clearly did not occur. As discussed extensively by others (Hegsted, 1975; Oddoye and Margen, 1979; Young et al, 1981a; Tarnopolsky et al, 1988) this discrepancy may be due to number of factors including: 1. over-estimation of intake and underestimation of losses; 2. slow physiological adaptation to altered protein intakes; 3. true N accretion below the limit of detection of methods to determine accretion (i.e., splanchnic hypertrophy (Lin and Huang, 1982)); and 4. loss of N as molecular N_2. In a well controlled study of 6 sedentary subjects consuming 12 and 36 g N diets each for 50 days in a metabolic ward there was no evidence of unmeasured losses (#1) or long term adaptation

(#2) and NBAL remained at +1.6 g $N \cdot d^{-1}$ after 50 d on the high PRO diet (Oddoye and Margen, 1979).

This controversy is not yet resolved, however, methodological errors (#1) in the present study were not likely, given the lack of difference in creatinine excretion over each of the 3 day NBAL periods, the use of carmine markers to ensure completeness of stool collections, and the measurement of sweat losses. Short term NBAL studies with solid, palatable foods and the use of diet checklists were also employed to maximize compliance with the diet. Since the subjects were not on a metabolic ward compliance could not be verified with 100 % certainty. The lack of day to day variation in urinary urea excretion did provide an objective measure that subjects maintained a fairly constant PRO_{IN} over the NBAL period. It is also not likely that the subjects were still adapting to the diets (#2) for the PRO and energy intakes during the NBAL were based upon each individual's 3 day diet record collected 3.5 weeks into each training session and there was no evidence of a trend in day to day urinary urea excretion over the NBAL period (if subjects were not adapted urea N would be increasing for PRO and decreasing for CHO treatments). As mentioned above, the hydrostatic weighing method would have detected a 7.8 kg increase in lean body mass had it occurred (#3) and no

attempt was made to quantitate molecular N_2 losses (#4).

The positive NBAL on the PRO supplement could not be explained by greater energy intakes for these were nearly identical during both NBAL periods. As well, the contribution of carbohydrate to total energy was greater for the CHO period (60 %) than for the PRO period (41 %) which would tend to have a PRO sparing effect for the CHO and not the PRO treatments. The differences in the carbohydrate level in the diet may have important practical implications for the individual who trains on a regular basis for it has been demonstrated that male body builders had reduced muscular endurance when performing weight-lifting on a hypo-energetic, moderate PRO-low carbohydrate (50 %) diet compared to an isoenergetic, low PRO-high carbohydrate (75 %) diet (Walberg et al, 1988). It is difficult to maintain a high percentage of energy from carbohydrates if PRO_{IN} is excessive which is another reason that strength training athletes should consume adequate (ie. novice = 1.62 g PRO$\cdot kg^{-1}\cdot d^{-1}$; habitual/elite = 0.98 g PRO$\cdot kg^{-1}\cdot d^{-1}$) but not excessive (ie. 2.34 g PRO$\cdot kg^{-1}\cdot d^{-1}$) PRO_{IN}.

In summary there does not appear to be a good explanation for the high NBAL observed on the PRO diet but it does not appear to represent accretion of skeletal muscle mass as described above. Since NBAL experiments measure

only the net balance between WBPS and whole body breakdown accurately over a period of days, and NBAL can be established at a number of different PRO_{IN} (*accommodation* to low intakes), and given the technical and interpretative concerns expressed above, it has been suggested that amino acid turnover studies be used to study the dynamics of PRO metabolism to determine PRO requirements (Young et al, 1981a; Young et al, 1989). For body builders the optimal physiological requirement for dietary PRO would likely be the level at which PRO synthesis plateaus. This approach has been used to determine the PRO requirement of rats (Smith et al, 1982), the amino acid requirement of humans (Young et al, 1981a), and the "plateau" in whole body PRO synthesis was apparent for endurance athletes in a recent study (Meredith et al, 1989).

The mean energy intake of the subjects in this study was 163 $kJ \cdot kg^{-1} \cdot d^{-1}$ which is 31.6 $kJ \cdot kg^{-1} \cdot d^{-1}$ greater than the U.S. R.D.A. level for a 23 year old sedentary male (Pellet, 1990a). Assuming that the bodybuilders in the present study were relatively sedentary at times other than during exercise this would leave 2588 kJ (619 kcal) for physical activity. During similar circuit resistance exercise the energy expenditure is only 915 kJ (219 kcal) (Tarnopolsky et al, 1991) and 1137 kJ (272 kcal) (Pivarnik et al, 1989) per

hour which would be about 1539 kJ for the entire weight training session used by the novice bodybuilders in this study. There is very little post-exercise oxygen consumption after circuit weight training (Tarnopolsky et al, 1991) so the energy consumption in the present study should have been more than adequate as calculated. In addition there was no weight loss over the duration of the study by the subjects.

The habitual energy intakes of males performing similar types of bodybuilding weight training range widely from a low of 105 $kJ \cdot kg^{-1} \cdot d^{-1}$ (Bazzarre et al, 1990) to a high of 251 $kJ \cdot kg^{-1} \cdot d^{-1}$ (Tarnopolsky et al, 1988). The high energy intake in the latter study (Tarnopolsky et al, 1988) may, in part, explain the relatively modest increases in the protein requirements observed for the elite body builders due to the sparing effect of excess energy intake on NBAL (Chiang et al, 1988). A high energy intake *per se* has a positive effect upon PRO synthesis independent of PRO_{IN} (Welle et al, 1989).

In summary, PRO consumed in excess (2.62 $g \cdot kg^{-1} \cdot d^{-1}$) by males performing body building exercise did not result in objective strength or lean mass/muscle gains when compared to a diet supplying 1.35 g $PRO \cdot kg^{-1} \cdot d^{-1}$. The PRO_{IN} for zero NBAL was calculated at 1.43 $g \cdot kg^{-1} \cdot d^{-1}$ which was nearly

identical to the measured, habitual PRO_{IN} of 1.44 $g \cdot kg^{-1} \cdot d^{-1}$ for these athletes given no dietary advice. The recommended PRO_{IN} of 1.62 $g \cdot kg^{-1} \cdot d^{-1}$ for novice body builders is 35 % greater than for elite body builders and is 88 % and 91 % greater than current Canadian and U.S. recommendations respectively.

Chapter 4.
WHOLE BODY LEUCINE METABOLISM DURING AND AFTER RESISTANCE EXERCISE IN FED HUMANS

4.1 Introduction.

Much of the information regarding PRO metabolism during exercise has been derived from NBAL studies (Gontzea et al, 1975; Tarnopolsky et al, 1988; Friedman et al, 1989; Merideth et al, 1989) and urea excretion measurements (Dohm et al, 1982; Lemon et al, 1980; Tarnopolsky et al, 1988; Tarnopolsky et al, 1990). These methods, however, do not provide information about the effects of exercise upon the rates or regulation of amino acid oxidation/PRO synthesis during and after exercise. Several studies have used stable isotope methodology (primarily L-[1-^{13}C]leucine) to investigate the effect of endurance exercise upon amino acid/PRO metabolism (Rennie et al, 1981; Wolfe et al, 1982; Evans et al, 1983; Wolfe et al, 1984b; Meredith et al, 1989). Such studies demonstrated an increase in leucine oxidation during endurance exercise (Rennie et al, 1981; Wolfe et al, 1982; Evans et al, 1983; Wolfe et al, 1984b; Meredith et al, 1989) and several, a reduction in whole body PRO synthesis (WBPS) (Rennie et al, 1981; Wolfe et al, 1982).

There have been no investigations of leucine oxidation

and WBPS during resistance exercise. In addition, few studies have measured leucine oxidation or WBPS for any time period following exercise (Wolfe et al, 1982; Evans et al, 1983; Wolfe et al, 1984b) despite evidence that significant changes occur in both animals (Rogers et al, 1979; Booth and Watson, 1985) and humans (Pivarnik et al, 1989; Devlin et al, 1990).

The primary long term adaptive response to heavy resistance training is an increase in the myofibrillar PRO content of skeletal muscle (MacDougall et al, 1979; MacDougall, 1986); yet little information is available regarding the time course changes in MPS associated with a single resistance exercise bout. However, it is not yet possible to measure MPS in humans during a bout of exercise with the present method of L-[1-^{13}C]leucine incorporation into skeletal muscle (Nair et al, 1988). Because a significant, positive correlation exists between MPS and WBPS (the former accounting for 25-30% of the latter) (Nair et al, 1988), we studied leucine oxidation and WBPS changes during and following an acute resistance exercise bout as an initial attempt to assess changes in MPS.

It has been demonstrated that during exercise there are changes in the background $^{13}CO_2/^{12}CO_2$ breath enrichment (BCGND) and in the bicarbonate retention factor (*c*) (Wolfe

et al, 1984a; Wolfe et al, 1984b; Barstow et al, 1989) which would result in an over-estimation of leucine oxidation and an under-estimation of WBPS during exercise (Wolfe et al, 1984a; Wolfe et al, 1984b). However, these corrections have not always been employed in reported studies (Rennie et al, 1981; Wolfe et al, 1982; Evans et al, 1983). These are the first values of leucine oxidation reported during resistance exercise and they were corrected for measured changes in both BCGND and *c*.

4.2 Methods.

4.2.1 Subjects.

Six healthy males volunteered to participate in the study (Table 1). All were students with at least 3 months recent experience in weight training (two to five times per week). The study was conducted under the approval of the Human Ethics Committee of McMaster University and all subjects provided written, informed consent.

Table 1. *Subject characteristics*

Age, yr	25 ± 3.4
Weight, kg	77.3 ± 7.6
Height, cm	177.3 ± 6.1
Density, $g \cdot cm^{-3}$	1.074 ± 0.012
Body fat, %*	11.1 ± 4.9
Daily Nutrient Intake:	
Protein, $g \cdot kg^{-1} \cdot d^{-1}$	1.4 ± 0.3
Energy, kcal	3067 ± 892

Values are mean ± SD. * Calculated from Brozek et al, 1973.

4.2.2 Experimental protocol.

Subjects completed 3 d food records (including 1 weekend day) from which each individual's mean daily energy and protein intake was calculated using a computerized programme (ANALYZE, McMaster University, Ont.). Based on this information an individual, isoenergetic and isonitrogenous diet was designed for the experimental trials. During the week prior to the experimental trial, each subject had their body density determined by hydrostatic weighing with residual volume measurements made by helium dilution.

The subjects did not perform any strenuous exercise on the day prior to the study. At 0630, following a 12 h overnight fast, the subjects consumed a liquid diet aliquot (Ensure, Ross Laboratories, Montreal, PQ) that was equivalent to 1/26th of each individual's mean energy intake and provided approximately 51 μmol leucine$\cdot h^{-1}$ (see below). The subjects received their respective aliquots every 30 min prior to (2 h), during, and after exercise. Each subject consumed about 60 % of his daily dietary energy intake over the 7 h experiment (Table 1).

At 0830 h the subjects reported to the laboratory, a 22 Ga plastic catheter was inserted into a hand vein, and a baseline "arterialized" blood sample (hot box at 65$\pm$5°C) and

expired gas collection was completed. For isotope infusion, a second catheter was inserted into a vein of the contralateral proximal forearm in a location that was not occluded by arm bending. A priming dose of L-[1-^{13}C]leucine (1 mg·kg^{-1}) and [^{13}C]sodium bicarbonate (0.295 mg·kg^{-1})(both 99% atom %; MSD Isotopes, Pointe Claire, PQ) was administered over 1 min, followed by a constant infusion of L-[1-^{13}C]leucine (1 mg·kg^{-1}·h^{-1}) for 5 h, delivered by a calibrated syringe pump (Harvard Apparatus, Boston, MA). The L-[1-^{13}C]leucine was diluted into sterile saline the day prior to infusion and the [^{13}C]sodium bicarbonate was diluted immediately prior to infusion, both under aseptic conditions, and both were sterilized by microfiltration immediately prior to infusion. Subjects 1-5 received leucine from the same batch, and all subjects received sodium bicarbonate from the same batch.

Blood samples were placed into heparinized, chilled tubes, centrifuged immediately, and the plasma stored at -70°C until analysis. All gas samples (see below) were collected into 150 L meteorological balloons with ventilatory volume determined on the inspired side using an in-line pneumotachometer (Validyne, Northridge, CA). Expired O_2 and CO_2 concentrations were determined within 5 min of collection with an oxygen (Rapox, Bilthoven, Holland)

and carbon dioxide (Hewlett Packard, Avondale, PA) analyzer for subsequent calculations of $\dot{V}O_2$, $\dot{V}CO_2$, and RER. Duplicate gas samples were injected into 10 mL evacuated tubes (Vacutainer; Becton Dickinson, Rutherford, NJ) for subsequent cryogenic extraction of the CO_2 and $^{13}CO_2/^{12}CO_2$ was determined within 48h using an isotope ratio mass spectrometer as described below.

At 90 (t=-30 min before exercise), 105 (t=-15) and 120 (t=0) minutes after the bolus injection, gas and blood samples were taken to ensure an isotopic plateau (pilot data had previously determined this to occur within 60-90 min). Immediately following the t=0 collection, subjects completed three groups of resistance exercises on a Global Gym multi-station training apparatus, with each group consisting of 3 sets of exercises - Group A: bench press, sit-ups, leg press; Group B: latissimus pull-downs, biceps curls, knee extension; Group C: triceps press, military press, leg press. Subjects performed 20 sit-ups and for all other exercises three sets of ten repetitions were performed at 70% of the individual's one repetition maximum (1RM) (determined within one week prior to the study). Each set was performed in approximately 30 s (1.5 s concentric; 1.5 s eccentric), with a 2 min rest between each set. Gas samples were collected for the entire second circuit of 3

sets in each group (approximately 7 min in duration) and blood was slowly withdrawn between 3-4 min (rest) of the respective circuit.

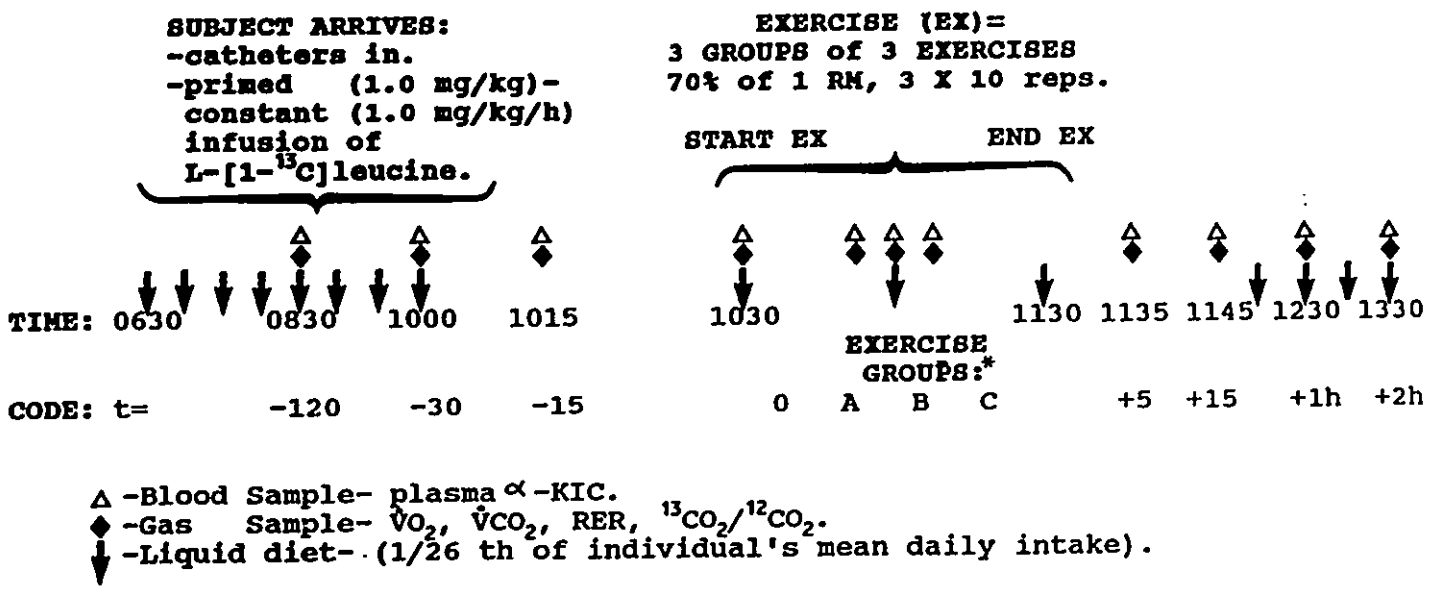
Upon completing the exercise, the subjects lay on a bed (without interruption of the isotope infusion) and gas/blood samples were collected at 5 (t=+65), 15 (t=+75), 60 (t=+120) and 120 (t=+180) min after exercise. (Fig. 14).

4.2.3 Analyses.

a) Breath analysis.

Carbon dioxide from the evacuated tubes was cryogenically extracted and the $^{13}CO_2$ isotopic enrichment of the sample determined using a gas-isotope ratio mass spectrometer (VG Isogas, SIRA 10, Cheshire, England) at m/z=44/45 (Scrimgeour et al, 1988). Our intra-assay coefficient of variation (CV) was 0.46% and the inter-assay CV was 1.03% over a range of sample enrichments from 0.002 to 0.025 atom percent excess (APE). Values are expressed as APE relative to baseline samples.

Figure 14. Study design for the experiment. Six subjects completed the trial with a primed-constant infusion of L-[1-^{13}C]leucine, 4 with a primed-constant infusion of [^{13}C]sodium bicarbonate (to determine c) and 4 with no isotope infusion (to determine background breath $^{13}CO_2/^{12}CO_2$ enrichments). * Each of the 3 exercise groups (A,B,C) consisted of 3 circuits of 3 resistance exercises performed at an intensity of 70 % of each individual's 1 RM (see text).


SUBJECT ARRIVES:
-catheters in.
-primed (1.0 mg/kg)-
constant (1.0 mg/kg/h)
infusion of
L-[1-13C]leucine.
EXERCISE (EX)=
3 GROUPS of 3 EXERCISES
70% of 1 RM, 3 X 10 reps.
START EX
END EX
TIME: 0630 0830 1000 1015 1030 1130 1135 1145 1230 1330
EXERCISE GROUPS:*
CODE: t= -120 -30 -15 0 A B C +5 +15 +1h +2h
△ -Blood Sample- plasma α-KIC.
◆ -Gas Sample- $\dot{V}O_2$, $\dot{V}CO_2$, RER, $^{13}CO_2/^{12}CO_2$.
-Liquid diet- (1/26 th of individual's mean daily intake).

b) Plasma analysis.

The *o*-quinoxalinol-trimethylsilyl derivative of α-keto isocaproic acid (α-KIC) was prepared using a modification of standard procedures (Wolfe et al, 1982). The proteins in 1 mL of plasma were precipitated using absolute ethanol (5 mL X 2) and centrifuged at 10,000 rpm X 10 min. The supernatant was dried under dry air and re-suspended in 1 mL water. To this was added 1 ml 2% *o*-phenylenediamine solution (2% in 4N HCl) and the solution was heated at 100°C X 1 h and cooled. The derivative was extracted in 2.5 mL methylene chloride and centrifuged for 2-3 min at 3,000 rpm. The lower layer was pipetted into a clean tube and the procedure repeated. To this was added 75-100 mg sodium sulfate and the solution was vortexed. The upper layer was transferred to a screw-top tube and evaporated under a gentle stream of dry air. The final derivatization was performed with 75 μL BSTFA + 1% TMCS (Pierce Chemicals, Rockford, IL) heated at 100°C X 30 min.

A VG-Trio-2 GC/MS (VG, Cheshire, England) with a Hewlett Packard Model 5890 GC (Hewlett Packard, USA) were used to determine the α-KIC enrichment in the plasma. The derivatized samples (0.3 μL) were injected onto a 15 m fused silica capillary column (0.25 mm i.d.)(DB5 J.W. Scientific,

Rancho Verda, California) with the oven programme set at an initial temperature of 120°C, ramped to 160°C at a rate of 8°C·min^{-1}, then ramped to 290°C at a rate of 20°C·min^{-1} and held at 290°C for 3 min. Helium was used as the carrier gas (32 cm·sec^{-1}). The column exited directly into the mass spectrometer ion source and electron impact ionization was used (70 eV, trap current 170 μA, source current 1.6 mA).

We monitored the m/z 232.1/233.1 ratios by scanning the instrument over a narrow mass range (228 amu to 238 amu: cycle time 1.35 s (1.25 s scan, 0.05 s interscan)). The abundance of the ^{13}C enriched samples (233.1 amu) α-KIC relative to the ^{12}C (non-enriched) species (232.1 amu) α-KIC was taken from the listing of the mass spectrum acquired at the apex of the chromatographic peak. This method provided an intra-assay CV of 0.77 % for the raw isotope ratios of pooled, enriched plasma samples (n=5) in this study. Thus, we analyzed each subject's results in separate batches.

4.2.4 Calculations.

Whole body leucine kinetics: Leucine flux (Q) was calculated using the reciprocal pool model (from [^{13}C]αKIC values)(Horber et al, 1989), at isotopic plateau, as described by others (Matthews et al, 1980; Wolfe et al, 1982): Q=i(Ei/Ep-1), where; i= L-[1-^{13}C]leucine infusion rate (μmol·kg^{-1}·h^{-1}), Ei=enrichment of the infused leucine

(APE), and Ep=enrichment of the plasma α-KIC (APE) and the term "-1" corrects for the contribution of the infused isotope. The reciprocal pool model assumes that the enrichment of plasma [^{13}C]αKIC, during an infusion of L-[1-^{13}C]leucine, is more indicative of intracellular [^{13}C]leucine enrichment than is plasma [^{13}C]leucine enrichment (Horber et al, 1989).

Leucine oxidation was calculated from the equation (Wolfe et al, 1982):

total leucine oxidation = $[(IECO_2/c)/IE_{\alpha\text{-KIC}}]\cdot\dot{V}CO_2$

where; $IECO_2$ = enrichment of expired CO_2 (APE), $IE_{\alpha\text{-KIC}}$ = enrichment of plasma α-KIC (APE), and $\dot{V}CO_2$ = volume of carbon dioxide evolved ($\mu mol\cdot kg^{-1}\cdot h^{-1}$), and c = bicarbonate retention factor. Because exercise is known to affect the retention of CO_2 in the body (Wolfe et al, 1984a; Barstow et al, 1989), the bicarbonate retention factor (c), was determined for four of the six subjects 1 wk prior to the experiment using the identical experimental design with a primed-continuous infusion of [^{13}C]sodium bicarbonate (prepared within 1 h of infusion to minimize loss as $^{13}CO_2$), as described by Kien (1989): $c = \dot{V}CO_2\cdot(IE\ bicarbonate\ CO_2\cdot F^{-1})$, where; $\dot{V}CO_2$ = volume of carbon dioxide evolved ($\mu mol\cdot kg^{-1}\cdot min^{-1}$), IE bicarbonate CO_2 = isotopic enrichment of expired CO_2 at plateau (corrected for background CO_2 enrichment

changes (see below)), and F = the infusion rate of [^{13}C] sodium bicarbonate (μmol·kg^{-1}·min^{-1}). The individual *c* was used for each of four subjects and the mean of these values was applied to the other two subjects in the calculation of leucine oxidation. In addition, four subjects were studied under identical study conditions (fed state) with no isotope infusion to account for changes in background CO_2 enrichment caused by the protocol (Wolfe et al, 1984a; Barstow et al, 1989), to further correct our leucine oxidation values.

The rate of appearance of endogenous leucine (Ra END LEU) was determined from: total leucine flux (Q) - dietary leucine (I(1-*fo*)) (the *fo* value was estimated at 0.15 from the expected loss of leucine due to absorption from whey and casein hydrolysate (94 % absorption)(Silk et al, 1973; FAO/WHO/UNU, 1985) and for the first pass splanchnic transamination to α-KIC (90 % of amino acids absorbed)(Hoerr et al, 1991)). The Ra end leu was used as an indicator of whole body proteolysis and NOLD was used to estimate whole body protein synthesis (Matthews et al, 1980; Nair et al, 1988) according to the model where: flux (Q)=synthesis (*S*) + oxidation (O) (Waterlow et al, 1978; Matthews et al, 1980), using a tissue leucine content of 590 μmol leucine·g protein^{-1} (approximately 7.8%) (Block and Weiss, 1956; Golden and Waterlow, 1977; Matthews et al, 1980; Wolfe et

al, 1982).

4.2.5 Statistics.

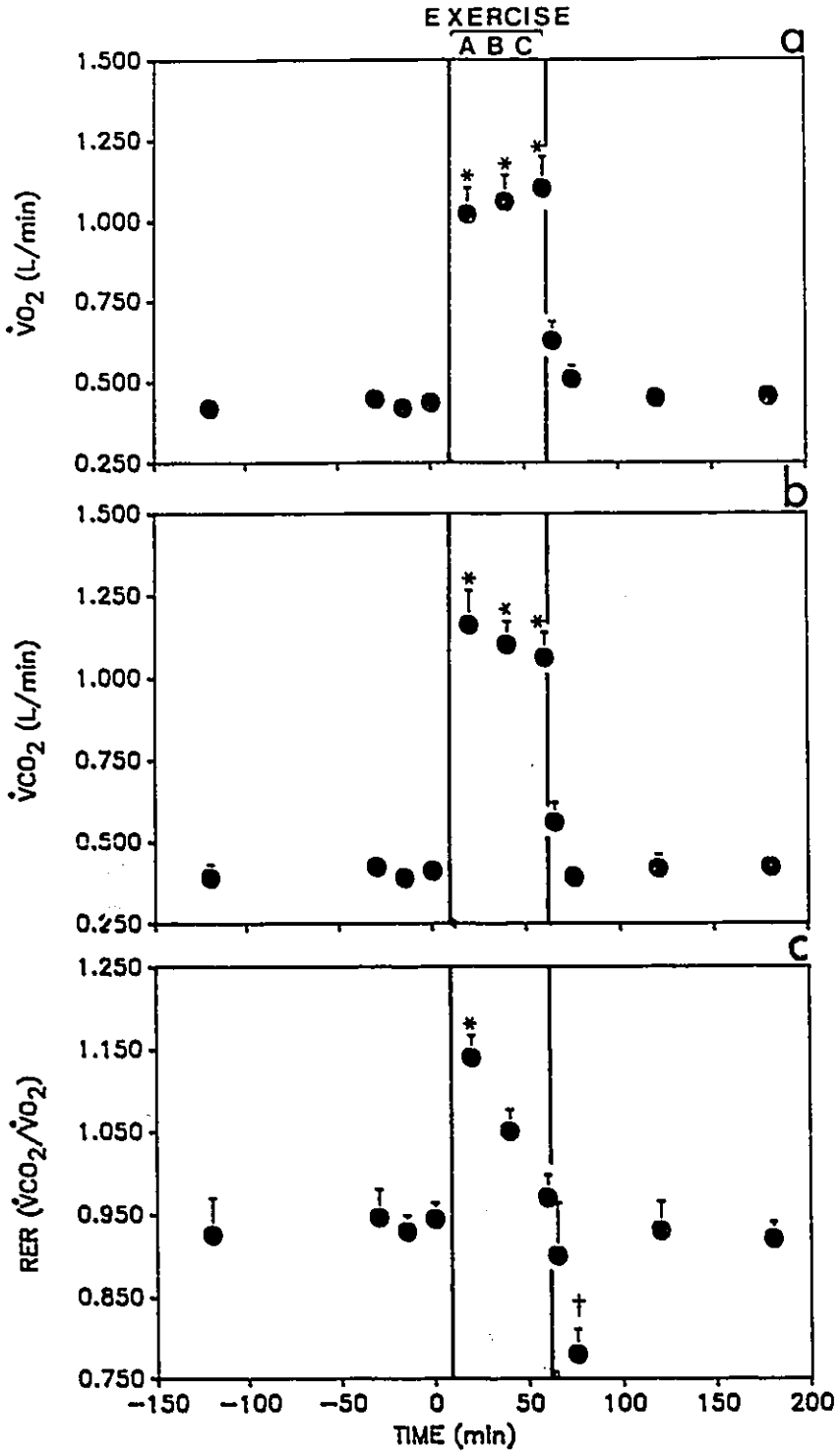
The effect of exercise upon the variables measured was determined using a one-way, repeated measures, analysis of variance (ANOVA) (Minitab, V.7.0, PA, USA). When a significant F-value ($P<0.05$) was observed the location of the pair-wise difference was made with the Tukey *post hoc* analysis (Bruning and Kintz, 1977). Data in tables, figures and text are mean $\pm$ SD.

4.3 Results.

4.3.1 Gas exchange.

There were significant ($P<0.01$) increases for both $\dot{V}O_2$ and $\dot{V}CO_2$ during each exercise group (A, B, and C) relative to pre-exercise values (Fig. 15). The mean RER values were significantly ($P<0.01$) greater during exercise group A relative to resting values and were significantly ($P<0.05$) lower than pre-exercise values by 15 min after exercise (t=+75) (Fig. 15).

Figure 15. Respiratory exchange ratio (RER)(a); volume of carbon dioxide production ($\dot{V}CO_2$)(b); and volume of oxygen production ($\dot{V}O_2$)(c) before, during (exercise groups A,B,C;see text) and after resistance exercise. * $P<0.01$ compared to mean pre-exercise values. † $P<0.05$ compared to mean pre-exercise values.


EXERCISE
A B C
a
b
c
1.500
1.250
1.000
0.750
0.500
0.250
$\dot{V}O_2$ (L/min)
$\dot{V}CO_2$ (L/min)
1.250
1.150
1.050
0.950
0.850
0.750
RER ($\dot{V}CO_2/\dot{V}O_2$)
−150
−100
−50
0
50
100
150
200
TIME (min)

4.3.2 Bicarbonate retention factor and background breath enrichments.

Prior to exercise the mean bicarbonate retention factor (*c*) was 0.83 (n=4) which increased significantly ($P<0.01$) to 1.72 during exercise group A, to 1.46 during exercise group B, and decreased significantly ($P<0.05$) to 0.52 by 15 min after exercise (t=+75) (Fig. 16a). There were no effects of exercise upon the background breath $^{13}CO_2/^{12}CO_2$ isotopic enrichment during exercise (n=4), yet at 1 (t=+120) and 2 (t=+180) h after exercise significant ($P<0.01$) increases in enrichment were observed (Fig. 16b).

4.3.3 Breath and plasma α-KIC isotopic enrichments.

Plateau isotopic enrichments were achieved in both breath CO_2 and plasma α-KIC within 90 minutes of infusion (Fig. 17). A lower breath CO_2 enrichment plateau was achieved during exercise groups A, B, and C and at 5 (t=+65) and 15 (t=+75) min after exercise. This latter plateau was significantly different ($P<0.01$) from that attained before (t=-30, -15, 0), and at 1 (t=+120) and 2 (t=+180) h after exercise (Fig. 17a).

Figure 16. Bicarbonate retention factor (*c*) determined by primed-constant infusion of [^{13}C]sodium bicarbonate (n=4 subjects) (a). Background breath $^{13}CO_2/^{12}CO_2$ isotopic enrichment with no isotope infusion (n=4 subjects). Values are atom percent excess (APE) vs pre-exercise value (b). * $P<0.01$ compared to mean pre-exercise values. † $P<0.05$ compared to mean pre-exercise values.

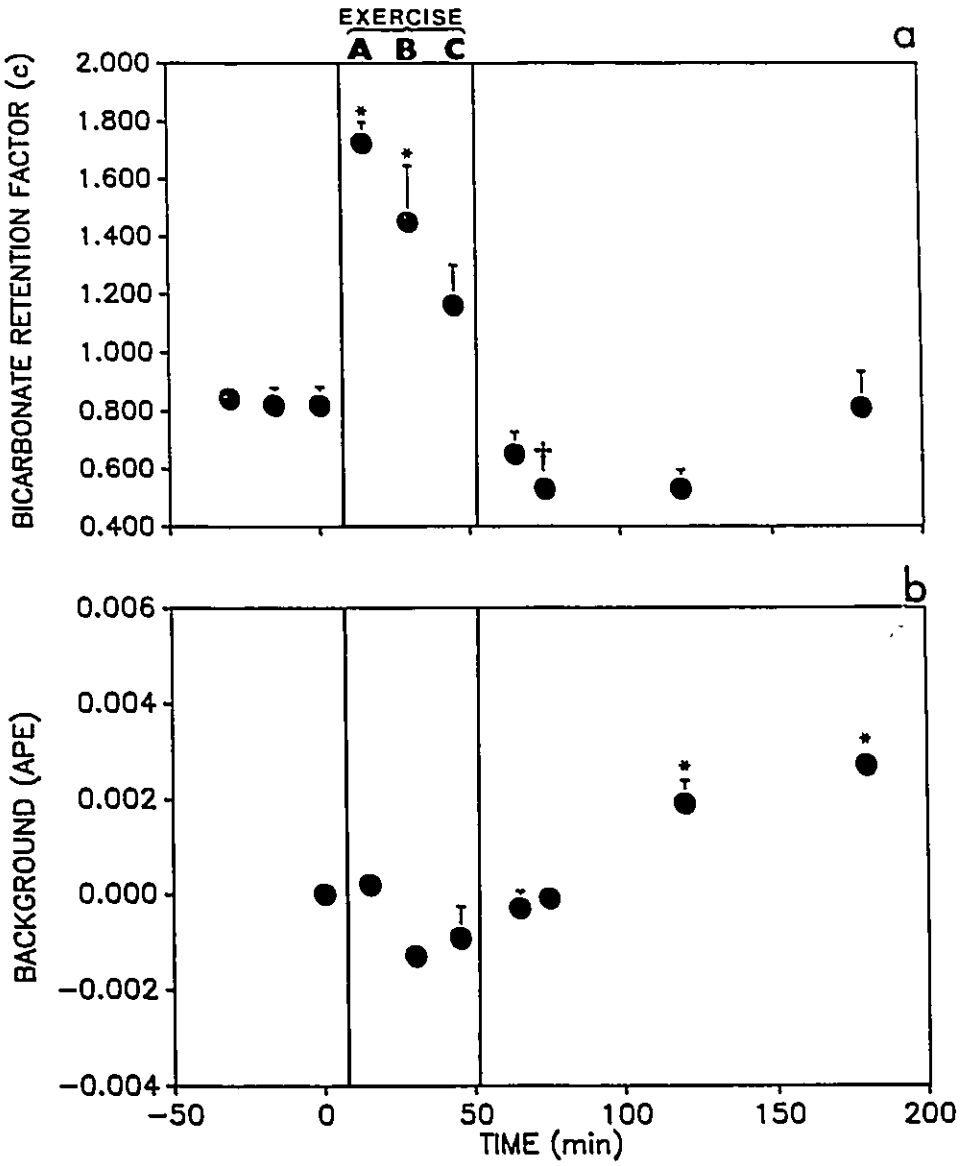
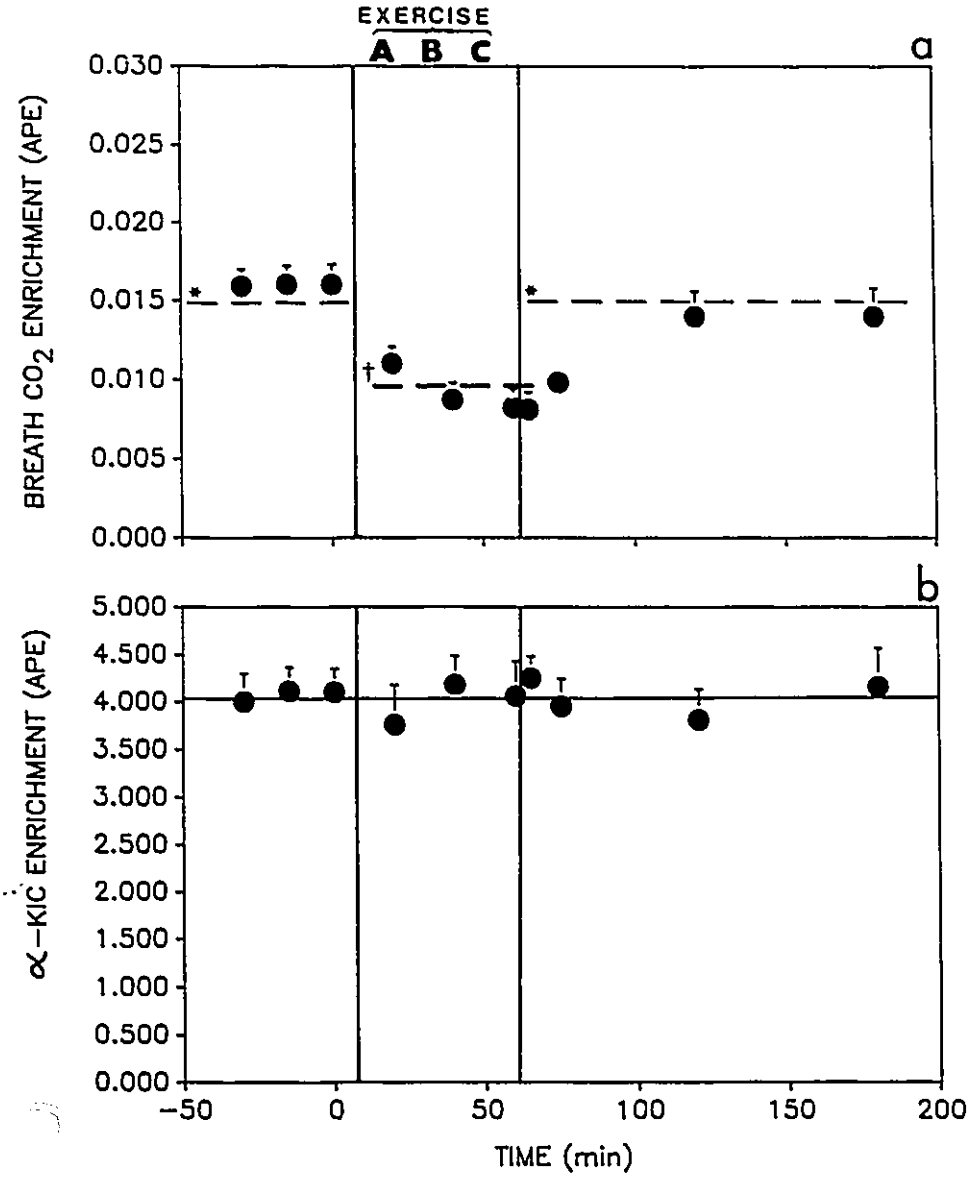

EXERCISE
A B C
a
BICARBONATE RETENTION FACTOR (c)
2.000
1.800
1.600
1.400
1.200
1.000
0.800
0.600
0.400
b
BACKGROUND (APE)
0.006
0.004
0.002
0.000
−0.002
−0.004
−50
0
50
100
150
200
TIME (min)

Figure 17. Breath CO_2 enrichment (corrected for background and c changes)(a) and plasma α-KIC enrichment changes (b) during the L-[1-^{13}C]leucine infusion protocol. All points joined with the thin (*) line are not significantly different from each other but are significantly different ($P<0.01$) from those joined with the thicker line (†).


EXERCISE
A B C
a
b
BREATH CO_2 ENRICHMENT (APE)
α-KIC ENRICHMENT (APE)
0.030
0.025
0.020
0.015
0.010
0.005
0.000
5.000
4.500
4.000
3.500
3.000
2.500
2.000
1.500
1.000
0.500
0.000
−50
0
50
100
150
200
TIME (min)

4.3.4 Calculations of leucine flux and oxidation; estimations of whole body protein synthesis (WBPS) and proteolysis.

We did not find an effect of resistance exercise upon leucine oxidation or WBPS during or for up to 2 h after exercise (Fig. 18). In addition, we did not find that resistance exercise altered leucine flux or the rate of appearance of endogenous leucine (Ra end leu) (Fig.19) (Statistics in Appendix V.1).

4.4 Discussion.

To our knowledge, this study is the first published report of leucine metabolism, bicarbonate kinetics, and background $^{13}CO_2$ enrichments during and after resistance exercise. Resistance exercise did not affect any of the measured indices of leucine metabolism yet there were highly significant effects of exercise upon *c* during exercise and upon the background $^{13}CO_2$ breath enrichments after exercise.

The decrease in background $^{13}CO_2$ enrichment observed at the start of exercise is consistent with the results during endurance exercise (Wolfe et al, 1982; Barstow et al, 1989). The subsequent progressive rise in breath $^{13}CO_2$ enrichment with continued exercise has also been observed (Barstow et al, 1989). However, post-exercise, background breath $^{13}CO_2$ enrichment values have not been previously reported.

Figure 18. Leucine oxidation values (corrected for background enrichment and *c* changes) (a). Whole body protein synthesis (WBPS) values determined from the non-oxidative portion of leucine flux (b).

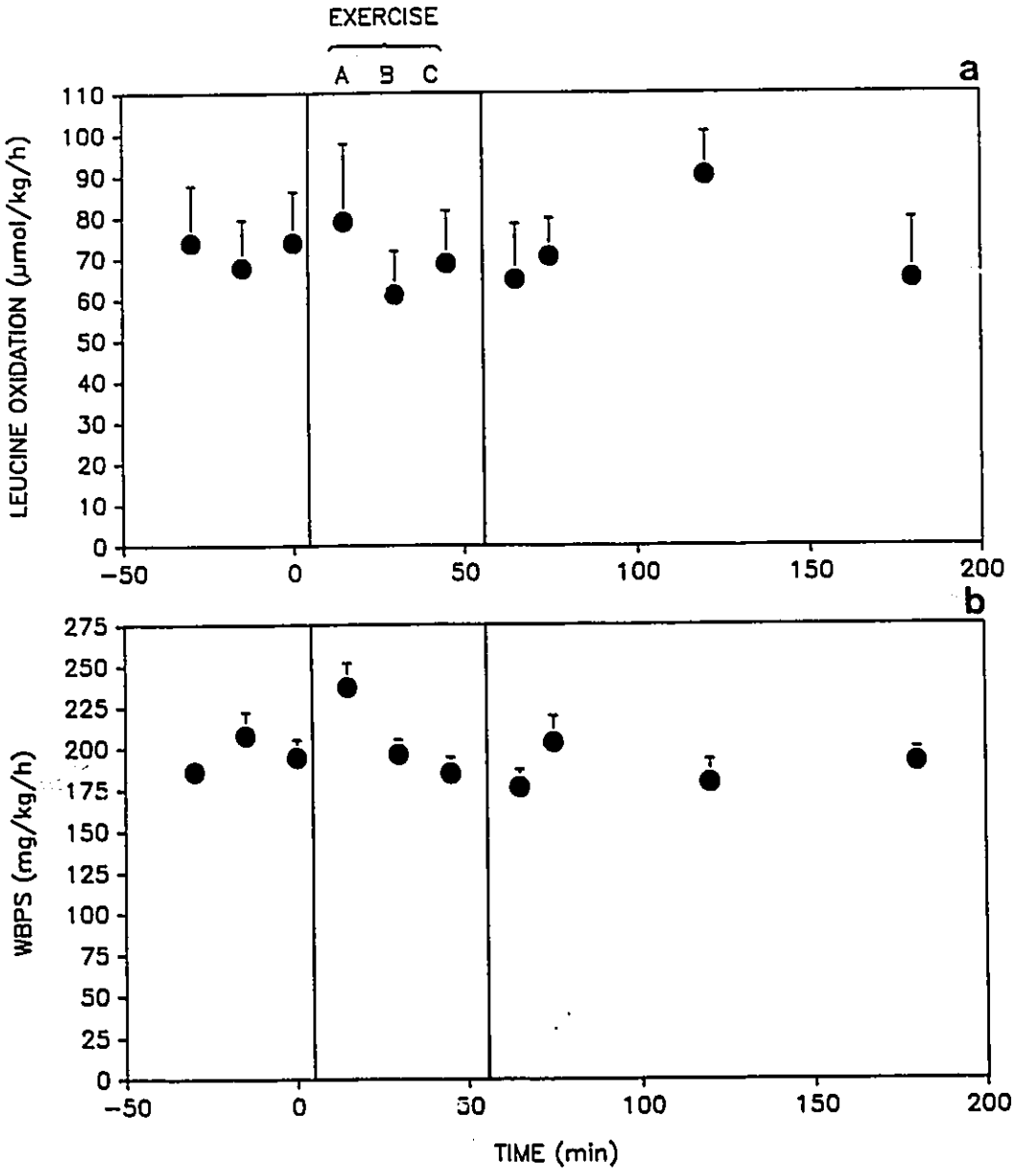
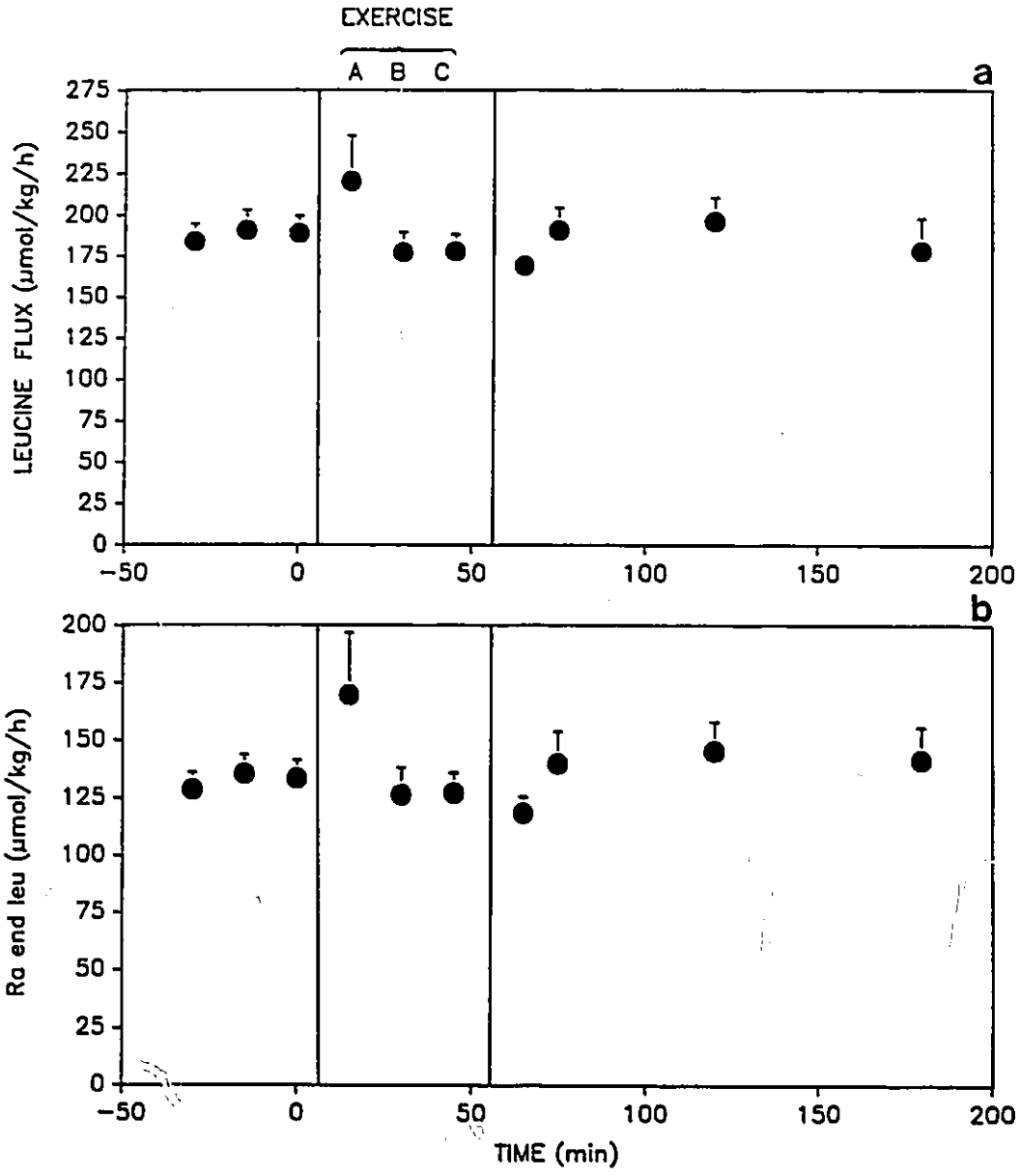

EXERCISE
A B C
a
b
LEUCINE OXIDATION (µmol/kg/h)
WBPS (mg/kg/h)
TIME (min)
110
100
90
80
70
60
50
40
30
20
10
0
275
250
225
200
175
150
125
100
75
50
25
0
−50
0
50
100
150
200

Figure 19. Leucine flux (a) and the rate of appearance of endogenous leucine (an indicator of proteolysis) (b).


EXERCISE
A B C
a
b
LEUCINE FLUX (µmol/kg/h)
Ra end leu (µmol/kg/h)
TIME (min)
275
250
225
200
175
150
125
100
75
50
25
0
−50
0
50
100
150
200

The breath $^{13}CO_2$ enrichment adjusts much more slowly during exercise than do changes in $\dot{V}O_2$ and $\dot{V}CO_2$ (Barstow et al, 1989), which likely reflects the delay for a $^{13}CO_2$ molecule to pass through one or all of the bicarbonate pools (Irving et al, 1983). Thus, the increased $^{13}CO_2$ enrichment at 1 and 2 hours post exercise may indicate an increased carbohydrate oxidation (greater ^{13}C enrichment vs fats (Schoeller et al, 1980)) during and not after exercise (Barstow et al, 1989). In addition, the subjects were continuously fed a defined formula diet with both corn starch and corn oil (Ensure, Ross Laboratories, Montreal, PQ), which would increase the breath background $^{13}CO_2/^{12}CO_2$ enrichment (Schoeller, 1980).

At rest, the mean bicarbonate retention factor was 0.83, which is consistent with the value of 0.82 found in a recent study of fed, healthy, young male subjects (Hoerr et al, 1989). It is also very close to the accepted value of 0.81 that has commonly been used to calculate leucine oxidation in studies where *c* was not determined directly (Motil et al, 1981; Rennie et al, 1981; Wolfe et al, 1982). The finding of a large increase in *c* at the onset of exercise with a subsequent gradual decrease is consistent with the results obtained by Wolfe et al. (1984a) during endurance exercise. We considered this increase to be

indicative of an increased flux of $^{13}CO_2$ through body bicarbonate pools consequent to exercise (Barstow et al, 1987; Barstow et al, 1989) rather than an indication of increased oxidation of carbohydrates as suggested by others (Wolfe et al, 1984a). Further support for our interpretation comes from the fact that there was a strong correlation ($r = 0.91$) between the RER and c (Appendix V.2) and it is known that RER does increase at the onset of exercise consequent to an increased CO_2 flux (so called "blowing off" of CO_2). If increased c values were indicative of increased carbohydrate oxidation it would be expected that breath $^{13}CO_2$ enrichment would also increase at the same time, which did not occur.

The significant effects of exercise upon background enrichment and c clearly demonstrate the need to perform preliminary studies to quantitate these variables in any study using stable isotopes in order to determine substrate oxidation during an altered metabolic state such as exercise (Wolfe et al, 1984a).

Contrary to previous studies involving endurance exercise (Rennie et al, 1981; Wolfe et al, 1982; Evans et al, 1983; Wolfe et al, 1984b) leucine oxidation was not increased during resistance exercise. The calculation of leucine oxidation in the present study has taken into

account measured changes in background breath isotopic enrichment and in the bicarbonate retention factor. Studies which have neglected to measure the effects of the study protocol upon these variables and to correct the oxidation values may have overestimated leucine oxidation during (Rennie et al, 1981; Wolfe et al, 1982; Evans et al, 1983) and under-estimated it following exercise. If changes in *c* were not determined in the present study, the leucine oxidation values would have been over-estimated by 107 % and 76 % during exercise groups A and B, respectively, and would have been under-estimated by 37 % at 15 min after exercise. This would also lead to a bias in favour of underestimating WBPS during and overestimating WBPS after exercise because WBPS is estimated from the non-oxidative portion of leucine disposal (Matthews et al, 1980; Wolfe et al, 1982).

During the infusion of L-[1-^{13}C]leucine, our pre-exercise, corrected (for background and *c* changes) breath CO_2 enrichments had reached an isotopic plateau which decreased to a new plateau during exercise groups A, B, C and at time +65 and +75 min. These results were similar to those reported by Wolfe et al. (1982) in subjects performing endurance exercise at about 30% $\dot{V}O_{2max}$. We also demonstrated an isotopic plateau in α-KIC enrichment for the duration of the experiment, which is another of the prerequisites to

using the steady-state equations (described above in Results) for the calculation of leucine oxidation (Wolfe et al, 1982).

The calculation of total leucine oxidation from plasma α-KIC enrichment (reciprocal pool model (Horber et al, 1989)) is superior to values obtained using plasma leucine enrichment, for leucine oxidation values during exercise are under-estimated when derived from plasma leucine enrichment (Wolfe et al, 1982). It has also been demonstrated that the enrichment of plasma α-KIC is more indicative of intracellular leucine enrichment than is plasma leucine enrichment (Horber et al, 1989).

Using L-[1-^{13}C]leucine, it has been found that endurance exercise at approximately 30% $\dot{V}O_{2max}$ resulted in a 371% increase in total leucine oxidation (Wolfe et al, 1982). In a later publication this same group (Wolfe et al, 1984b) determined the *c* value for a group of subjects under identical exercise conditions and found that, when applied to their original results (Wolfe et al, 1982), the leucine oxidation values were reduced by 36 % yet the increased oxidation values were still highly significant ($P<0.001$) (Wolfe et al, 1984b).

Although we did not measure $\dot{V}O_{2max}$ in this study, the peak mean value attained by the subjects for all 3 exercise

Groups combined (1.12 $l \cdot min^{-1}$) is similar to that obtained in another study of circuit resistance training of about the same intensity (Pivarnik et al, 1989), and would represent about 30-35% $\dot{V}O_{2max}$. The intermittent nature of the exercise protocol in this study (30 s exercise:2 min rest) may have contributed to the differences in leucine oxidation that we observed compared to endurance exercise performed at a similar oxygen uptake (Wolfe et al, 1982). During endurance exercise the activity of the BCKAD enzyme increases (Kasperek, 1989; Wagenmakers et al, 1989), increasing leucine oxidation, and this enzyme activity is inversely correlated with adenosine triphosphate (ATP) levels and energy charge (ATP/ADP + P_i)(Kasperek, 1989). The 2 min rest period in the present study would have allowed for a repletion of the high energy phosphate pool, which would decrease BCKAD activity and subsequently, decrease leucine oxidation.

In addition, we have previously demonstrated, using nitrogen balance techniques, that endurance athletes require about 67% more dietary PRO yet resistance athletes require only 12% more PRO than sedentary controls (Tarnopolsky et al, 1988). An increased UN excretion for male endurance athletes (Tarnopolsky et al, 1988; Tarnopolsky et al, 1990) indirectly supports the hypothesis that increased amino acid

oxidation is responsible for the increased PRO requirements of this group, while the small increase in requirements for resistance athletes likely results from increased muscle PRO accretion (MacDougall et al, 1979; MacDougall, 1986) and not increased amino acid oxidation (Tarnopolsky et al, 1988). The higher state of training of our subjects (Tarnopolsky et al, 1988) compared to those of other studies (Rennie et al, 1981; Wolfe et al, 1982) may also have contributed to our finding no increase in leucine oxidation for the progressive improvement in NBAL demonstrated for subjects during training (Gontzea et al, 1975) may involve an adaptive reduction in leucine oxidation. Finally, our subjects were fed a regular, defined formula, diet whereas other studies have examined subjects in the fasted state (Wolfe et al, 1982; Wolfe et al, 1984b) and it is known that oral feeding (Motil et al, 1981) increases leucine flux and oxidation. Therefore, feeding *per se* may have had a predominant effect upon leucine oxidation that masked an effect of exercise. For up to 2 h after exercise, we found no changes in leucine oxidation; however, leucine oxidation is decreased between 2-3 h after endurance exercise (Devlin et al, 1990). It has been proposed that the decrease in leucine oxidation after endurance exercise is due to increased levels of free fatty acids and/or ß-hydroxybutyrate (Devlin et al, 1990), neither

of which were likely to be elevated to the same level by the present study protocol (Keul et al, 1978).

In exercising animals, MPS is reduced during, and increased at about 1 to 2 h after exercise (Rogers et al, 1979; Booth and Watson, 1985). Because resistance exercise results in increased muscle mass (MacDougall et al, 1979; MacDougall, 1986), it would have been desirable to directly measure MPS during and after the exercise bout. However, the present method of determining MPS from muscle biopsy samples using a primed-constant infusion of L-[1-^{13}C]leucine requires at least 4 h of constant isotope infusion to determine MPS with precision (Nair et al, 1988). Clearly, this method would not be applicable during exercise due to the time requirement. In a recent study in our laboratory, we have found that MPS is increased by about 80 % both at 4 h and 24 h after resistance exercise in exercise compared to control arms (Chesley et al, 1991).

The fact that we did not find an effect of exercise upon WBPS may relate to one or several factors: First, since MPS represents only 25-30% of WBPS (Nair et al, 1988), changes in MPS consequent to exercise may have gone undetected; second, WBPS may have increased at some time point beyond 2 h post-exercise for it is known that satellite cell proliferation occurs at about 2-3 d post

exercise (Darr and Schultz, 1987); third, the measurement of WBPS during exercise may not be accurate based upon different protein breakdown and WBPS results obtained using [^{13}C]lysine vs L-[1-^{13}C]leucine as tracers (Wolfe et al, 1984b); and fourth, MPS for exercised and non-exercised muscle and splanchnic protein synthesis may each change in opposite directions after exercise (Rennie et al, 1981; Devlin et al, 1990), which may render WBPS unchanged. We now have evidence that MPS is elevated at 4 h (↑ 43 %) and 24 h (↑ 80 %) after resistance exercise (Chesley et al, 1991). Therefore, it is likely that an increase in MPS of this magnitude would be apparent in WBPS measurements if PRO synthesis in non-muscle tissues remained constant. In sedentary men given testosterone for 3 months, an increase in lean body mass was associated with a 27 % increase in MPS and no change in WBPS (Griggs et al, 1989), which suggested a reduction in PRO synthesis in non-muscle tissues.

Further studies are needed to determine the effects of resistance exercise upon WBPS beyond 2 h post-exercise and multiple tracer studies need to be employed to determine the validity of using L-[1-^{13}C]leucine to measure WBPS after exercise. Any study using stable isotopes to measure substrate oxidation during exercise must quantitate the effects of the protocol upon BCGND and *c*.

Chapter 5.

A KINETIC AND NITROGEN BALANCE APPROACH TO THE DETERMINATION OF PROTEIN REQUIREMENTS FOR RESISTANCE ATHLETES

5.1 Introduction.

Nitrogen balance (NBAL) methodology has traditionally been used to establish the PRO requirements of both sedentary (Garza et al, 1973; Pellet, 1990b) and athletic populations (Consolazio et al, 1975; Torun et al, 1977; Tarnopolsky et al, 1988). However, there have been a number of concerns about the interpretation of NBAL studies (Hegsted, 1975; Young et al, 1981a; Young and Bier, 1987a; Young et al, 1989). Two of the more disturbing issues with respect to the NBAL studies are: 1. the ability to establish NBAL at PRO_{IN} that are arguably sub-optimal (*accomodation*), and 2. the well documented positive NBAL apparent at very high nitrogen intakes which does not result in the expected lean body mass accretion. These two issues are of particular importance for the athlete involved in strength/resistance exercise. A PRO_{IN} that is too low may result in sub-optimal strength/lean mass accretion in spite of maintaining a positive NBAL (*accomodation*), while one that is excessively high (*nutrient overload*) may be an

expensive waste at best and at worst may expose the athlete to PRO_{IN} that may potentially have negative long-term health consequences (Brenner et al, 1982; Zaragoza et al, 1987).

The NBAL method can only provide information on the net balance of all PRO synthetic and catabolic process in the body and not the quantitation of these various processes. This limitation, in combination with the interpretive concerns outlined above, has led to an interest in using stable isotope methods to determine amino acid (Young and Bier, 1987a; Young et al, 1987b; Millward and Rivers, 1989; Young et al, 1989) and PRO requirements (Meredith et al, 1989). Using stable isotope methods it has been suggested that the determination of the PRO_{IN} at which amino acid oxidation exponentially increased and/or the intake at which PRO synthesis plateaued would be a more "physiological" measure of PRO requirements than NBAL methods (Young et al, 1981a; Young, 1981b). In rats it has been demonstrated that there is an upper limit of PRO_{IN}, above which, PRO synthesis plateaued (Smith et al, 1982; Zaragoza et al, 1987). In addition, it has been demonstrated that excessive PRO_{IN} in male resistance athletes did not result in increased lean body mass/muscle accretion (synthesis plateau) when compared to an adequate PRO_{IN} (Tarnopolsky et al, 1988).

Urea excretion has been shown to increase in response to an elevated dietary PRO_{IN} (Hickson et al, 1988; Tarnopolsky et al, 1988), suggesting that amino acid oxidation was increased. It is also known that the activity of branched chain keto-acid dehydrogenase (BCKAD; Aftring et al, 1985; Block et al, 1985)) and liver urea cycle enzymes (Zaragoza et al, 1987) are increased in response to a high PRO_{IN}. This would facilitate the oxidation (BCKAD) and excretion (urea cycle) of the increased amino group delivered to the liver. These observations suggest that PRO consumed in excess of need is metabolized and/or excreted and is not stored as lean body/muscle mass. Therefore, the point at which amino acid oxidation and/or urea production increases disproportionately to additional PRO_{IN} may help to further define physiological PRO requirements (Young et al, 1981a; Young, 1981b).

We and others have previously argued that the PRO requirements for resistance athletes (body builders) are greater than for sedentary individuals (Consolazio et al, 1975; Torun et al, 1977; Tarnopolsky et al, 1988). The increased requirement for these athletes is likely due to a chronic increase in MPS rates (Wong and Booth, 1990a; Yarasheski et al, 1990; Chesley et al, 1991) and subsequent muscle accretion (MacDougall et al, 1982; MacDougall, 1986)

consequent to resistance training. However, we have also argued, using the NBAL approach, that the average habitual PRO_{IN} of body-builders (2.34 ± 0.51 $g \cdot kg^{-1} \cdot d^{-1}$) is excessive and does not result in greater strength/muscle mass gains compared to a recommended PRO_{IN} of about 0.98 $g \cdot kg^{-1} \cdot d^{-1}$ for habitual body-builders (Tarnopolsky et al, 1988) or a maximum of 1.62 $g \cdot kg^{-1} \cdot d^{-1}$ for novice body-builders performing very strenuous training (Tarnopolsky et al, 1990).

The purpose of the present study was to determine the PRO requirements for both sedentary and resistance-trained (body-builders) men by examining NBAL and leucine turnover (oxidation, WBPS, flux, protein breakdown) in response to three levels of dietary PRO_{IN} (low PRO (LP) = 0.86 $g \cdot kg^{-1} \cdot d^{-1}$; moderate PRO (MP) = 1.40 $g \cdot kg^{-1} \cdot d^{-1}$; high PRO (HP) = 2.4 $g \cdot kg^{-1} \cdot d^{-1}$).

The *a priori* hypotheses of the present study were that: 1. WBPS rates would be greater for body builders (BB) than for sedentary controls (S); 2. WBPS would be decreased for the BB group on a diet supplying PRO at the Canadian R.N.I. for PRO (LP)(*accomodation*), increased at a PRO_{IN} previously recommended intake for BB (MP)(*adaptation*)(Tarnopolsky et al, 1988), and would not increase any further (plateau) on the excessive PRO_{IN} (HP)(*nutrient overload*), while the WBPS

would remain constant for the S group for they would already be at or above their physiological PRO requirement (*adaptation*) on the LP diet; 3. Leucine oxidation would increase significantly for BB from the MP to HP diets while it would increase significantly for the S group fom LP to MP and HP diets; and 4. The NBAL determined PRO requirements for BB would be greater than the Canadian R.N.I. and greater than for the S group and these requirements would be close to that predicted using the leucine kinetic approach.

5.2 Methods.

5.2.1 Subjects.

Two groups of young males volunteered for the study and informed written consent was obtained after subjects were advised of the risks and benefits of participating in accordance with the McMaster University Research Advisory Committee. One group (n=7) habitually (>4 $d \cdot wk^{-1}$) performed resistance-type exercise (>60% circuit weight training) for at least 2 months prior to the study and were designated as "body builders" (BB) due to the nature of their training. A second group (n=6) consisted of sedentary (S) age-matched controls. All subjects were in good health as determined by medical history and semi-quantitative urinalysis (pH, leukocytes, protein, glucose, ketones, blood and hemoglobin (Chemstrip 5L, Boehringer Mannheim, Laval, PQ)). The

characteristics of each group are given in Table 5.

Table 5. *Physical characteristics of the subjects*

	Body Builders (BB) (n=7)	Sed. Controls (S) (n=6)	P*
Age, yr	21.6±1.5	24.5±3.6	NS
Weight, kg	85.4±7.3	84.9±11.4	NS
Height, cm	179.7±7.9	178.9±8.1	NS
Body fat, %	9.8±2.7	20.8±2.3	< 0.001
Lean mass, kg	77.2±6.5	66.9±7.5	< 0.01
Physical activity:			
$h \cdot wk^{-1}$	9.7±2.8	0.2±0.1	< 0.001
length, mo	6.6±3.7	NA	NA
strength, N·m	92.9±7.5	68.2±15.0	< 0.001

Values are mean ± SD. * P=Between group comparisons. Mean value for all diet periods. Number of months of consistent weight training immediately prior to the study. Isometric, dominant arm flexor strength.

5.2.2 Experimental protocol.

All subjects participated in 3 different experiments each of 13 days duration with a mean 8 (range=2-30 d) day *ad libitum* washout diet period. During each experimental period the subjects were randomly (counter balanced) assigned to dietary PRO at one of 3 levels: LP-a PRO_{IN} considered low for BB athletes (Tarnopolsky et al, 1988) and equal to the Canadian Recommended Nutrient Intake for PRO ($0.86\ g \cdot kg^{-1} \cdot d^{-1}$) (Dept. of National Health and Welfare, Ottawa, 1990); MP-a moderate PRO_{IN} considered adequate for BB athletes ($1.4\ g \cdot kg^{-1} \cdot d^{-1}$) (Tarnopolsky et al, 1988); and HP-a high intake considered to be a *nutrient overload* for BB

athletes (2.4 g PRO·kg^{-1}·d^{-1}) (Tarnopolsky et al, 1988; Tarnopolsky et al, 1990). The diets provided energy to match each individual's habitual intake as determined from calculated analysis of 4 day, weighed food records (N-Squared Nutritionist III, Silverton, OR) collected immediately prior to the study.

All subjects completed the three 13-day experimental periods. Each 13 day period began with a 6 day phase when subjects were instructed to follow provided food exchanges in order to modify their habitual diet to match the PRO and energy intake of the given diet period. They then consumed a 4 day meat-free diet from a checklist of foods that was identical in composition to that given in the final 3 day NBAL period. During the checklist period subjects were given scales, measuring cups and ~30% of their energy and PRO was provided as a defined formula and supplement (see below). During the 4 day checklist period it was determined from the food records that the subjects consumed 99.5±2.2% of the required PRO_{IN}.

During the final 3 day NBAL period food was provided in a pre-packaged form with each item weighed to ±0.05g on a digital scale (OHAUS, E400D, Florham Park, NJ). Each diet consisted of a combination of 3 major food categories: 1) defined formula diet (Nutren, Clintec Nutrition,

Mississauga, Ont) supplying between 25-50% of the total energy intake (E_{IN}); 2) supplement of either glucose polymers (Polycose, Ross Laboratories, Columbus, OH) (0-30% of E_{IN}) or whey PRO hydrolysate (Ross Laboratories, Columbus, OH) (0-32% of E_{IN}); and 3) miscellaneous foods (spaghetti, jam, whole wheat bread, cookies, margarine, apple juice, lettuce, corn flake cereal, 2% milk, peanut butter) (35-65% of E_{IN}). Subjects were instructed to adhere strictly to each diet and were permitted to consume water *ad libitum* and could have up to 3 cups of coffee and/or 750 ml of diet pop per day (except on the morning of the final test day). Subjects recorded consumption of all foods and liquids and reported compliance was >98%. Compliance was maximized by having the subjects check off all foods immediately after consumption and by having them report to the testing centre at least twice over the NBAL period to deliver urine samples.

Group S performed no physical activity during each 13 day diet treatment (except one S subject who jogged 3 times per week for 20-30 min during the adaption phases) and all S subjects remained sedentary during each NBAL period. Group BB performed their habitual activity during the adaptation phases (10 d, 60 % weights/40 % rugby/rowing). During day 1 of the NBAL period group BB performed their habitual weight

routine and kept a training log to minimize inter-test variability. On day 2 of the NBAL period BB subjects performed a standardized, whole-body, circuit weight routine on a multi-station apparatus (Global Gym, Weston, Ont). The routine has recently been described in detail (Tarnopolsky et al, 1991; Chapter 4) and consisted of 3 groups of 3 exercises performed at 75% of each individuals one repetition maximum (3 sets of 10 repetitions). The routine was completed in about 1 h and subjects finished 23.0 ± 2.1 h prior to the mid-point of the leucine infusion protocol (see below) on NBAL day 3.

On day 3 of NBAL each subject reported to the testing centre at 0830 in the fed state (due to scheduling problems one subject in each group was started at 1630 under all 3 diet treatments). A portion of defined formula with or without glucose polymers or PRO powder was consumed at 30 min intervals 2 h prior to the start of the infusion protocol (see below) and continued for 4.5 h. The total energy and PRO consumed over the period was equivalent to 50% of each individual's daily PRO and energy intake during the given diet allocation.

5.2.3 Infusion protocol.

Subjects rested in a supine position at an ambient temperature of 23-25°C for 10 min prior to obtaining a 5 min

gas sample into a 150 L meteorological balloon. Over the 5 min period the subject's VCO_2, VO_2, and RER were determined from at least 9 consecutive 20 s on-line measurements. VCO_2, VO_2, and RER were determined with a computerized acquisition system (Alkin Systems, Hamilton, Ont) interfaced with: a pneumotachometer (Validyne, Northridge, CA); an oxygen analyzer (Rapox, Bilthoven, Netherlands); and a carbon dioxide analyzer (Hewlett-Packard, Palo Alta, CA). The system was calibrated with a known O_2 and CO_2 gas mixture prior to each measurement. The intra-test coefficient of variation (CV) (39 X 4 measures every 30 min) of this system was 7.1 % and the inter-test CV (13 subjects X 3 trials separated by ~ 20 d) was 7.9 % for the determination of VCO_2. Two gas samples were injected into 20 ml evacuated tubes (Vacutainer, Rutherford, NJ) for subsequent determination of background breath $^{13}CO_2/^{12}CO_2$ ratios as described below.

A 22 Ga plastic catheter was inserted into a dorsal hand vein and an arterialized (60±5°C, hot box) sample was obtained for subsequent determination of plasma [^{13}C]-α ketoisocaproic acid (α-KIC) enrichment and creatine phosphokinase (CPK) activity (see below). A 20 Ga plastic catheter was inserted in a retrograde manner into a contralateral, proximal forearm vein for isotope infusion.

Priming doses of L-[1-^{13}C]leucine (1 mg·kg^{-1}) and [^{13}C]$NaHCO_3$ (0.295 mg·kg^{-1}) (both 99% ^{13}C- MSD Isotopes, Pointe Claire, PQ) were mixed immediately prior to administration and infused over 1 min to prime the respective pools (Allsop et al, 1978). This was immediately followed by a continuous infusion of L-[1-^{13}C]leucine (1 mg·kg^{-1}·h^{-1}) for 3.5 h, delivered by a calibrated syringe pump (Harvard Apparatus, Boston, MA). All isotopes were from the same batch and leucine was diluted to ~15 mg·mL^{-1} with sterile, normal saline and micro-filtered (0.2 μm, Gelman Sciences, AnnArbor, MI) immediately prior to infusion.

After 2 h (to establish isotopic plateau (Tarnopolsky et al, 1991a)) gas and blood samples were obtained every 30 min for 90 min (t=0,30,60,90). At isotopic plateau the CV of the plasma α-KIC enrichment was 6.4 % and that for the breath enrichment was 3.8 % over the duration of the experiment. All blood samples were collected into heparinized tubes, centrifuged immediately, and stored at -70°C for subsequent determination of [^{13}C]-αKIC enrichment. All gas samples were stored at room temperature and the CO_2 was cryogenically trapped within 7 days for subsequent determination of breath $^{13}CO_2/^{12}CO_2$ enrichment using a gas-isotope ratio mass spectrometer (VG Isogas, SIRA 10, Cheshire, England) at m/z=44/45 (Scrimgeour et al, 1988).

Our intra-assay CV was 0.89 % and the inter-assay CV was 1.25 % over a range of sample enrichments from 0.002 to 0.025 atom percent excess (APE)(Appendix I.2).

5.2.4 Strength measurements.

Following the isotope infusion, the isokinetic elbow flexor muscle strength was determined in each subject's dominant arm, at $0°\cdot s^{-1}$, $30°\cdot s^{-1}$ and $180°\cdot s^{-1}$ using an isokinetic dynamometer (Cybex II, Ronkonkona, NY).

5.2.5 Lean body mass.

Lean body mass (LBM) was determined using the hydrostatic weighing technique and residual lung volume was determined using a helium dilution method (W.E. Collins, Braintree, MA)). The percent body fat was estimated from the equation of Brozek (1967). The subjects were weighed in the same shorts and T-shirt (measured weight of 300 g) each time on an electronic scale accurate to ± 10 g (Mott Scales, Brantford, Ont.). The CV for determining LBM with this method was 1.3 % for a total of 13 subjects tested >13 d apart with 3 determinations for each subject.

5.2.6 Nitrogen balance.

During the 3 d NBAL period, daily urine samples were collected into 4 L containers pre-treated with 5 mL of glacial acetic acid. The collected urine was kept at <4°C by the subjects and delivered to the testing centre within

48 h. After volume determination, aliquots were taken and stored at -70°C for subsequent determination of total nitrogen (TN), urea nitrogen (UN) and creatinine. Compliance was determined by creatinine excretion and successful adaptation to each diet was confirmed by daily UN measurements (Appendix VI,D).

Fecal samples, collected between carmine markers, were kept frozen by the subjects and were delivered to the testing centre within 48 h of completion of the period. Each collection was weighed, diluted with distilled water (~50 %), homogenized, lyophilized, ground and stored for TN analysis.

Exercise and resting sweat N losses at different protein intakes were estimated for BB from the mean data of several recent studies (Tarnopolsky et al, 1988; Tarnopolsky et al, 1990). Resting sweat N losses were estimated for group S from recent data (Tarnopolsky et al, 1988). Miscellaneous N losses (semen, tooth brush, toilet paper, plate losses, hair, blood sampling, N_2 gas) were estimated at 140 mg $N \cdot d^{-1}$ per subject in both groups (Calloway et al, 1971).

Three diets (~3000 kcal) representative of each of the 3 protein intakes were prepared from the identical foods that were consumed during the study period. Twenty percent

of each dietary component was homogenized for 10 min, lyophilized, ground and analyzed for TN and gross energy content (Appendix II,A1,2).

Apparent NBAL was calculated as the difference between N_{IN} (diet) - N excretion (urine + feces + sweat + misc.).

5.2.7 Biochemical analyses.

TN content of the diet, urine and fecal samples was determined using the micro-Kjeldahl technique (Assoc. of Official Analytical Chemists, 1965). The intra-assay CV's for diets, urines and feces were 4.4 %, 5.8 %, and 3.8 % respectively while the inter-assay CV's were 9.2 %, 1.1 %, and 5.0 % respectively. The ratio of the measured:calculated N was 1.06, 0.98 and 0.99 for the LP, MP and HP diets respectively (assuming mixed proteins are 16% N by weight).

The gross energy content of each diet was determined by adiabatic bomb calorimetry (Parr Instruments, Moline, IL). To convert from metabolizable energy (diet calculations) to gross energy the % metabolizable energy contribution of carbohydrate, fat and protein were multiplied by 1.00, 1.03 and 1.43 (Merrill and Watt, 1973; Buskirk and Mendez, 1980). The intra-assay CV of the bomb calorimeter was 1.6 %. The ratio of measured:calculated gross energy content of each of LP, MP and HP diets was

0.99, 0.95 and 1.08 respectively. All N and energy data given in tables and figures are expressed as the measured values (Appendix II.1 + II.2).

Urine UN was determined using the urease-phenol method (Kit #640, Sigma, St. Louis, MO). The intra and inter-assay CV's were 4.0 % and 7.6 % respectively. Creatinine was determined using a colorimetric picric acid method (Kit #555, Sigma, St.Louis, MO). The intra and inter-assay CV's were 3.0 % and 9.9 % respectively.

Plasma [^{13}C]α-KIC enrichment in *O*-trimethylsilyl quinoxalinol derivatives was determined by capillary gas chromatography-mass spectroscopy (GC/MS(Hewlett Packard 5890 GC (Avondale, PA), VG trio-2 MS (Cheshire, England)) using electron impact ionization (m/z=232.1/233.1) as described previously (Tarnopolsky et al, 1991; Chapter 2). The *O*-trimethylsilyl quinoxalinol derivative was prepared using a modification of standard procedures (Wolfe, 1984c; Shangraw et al, 1988) as previously described (Tarnopolsky et al, 1991a). The intra- and inter-assay CV's were 0.81 % and 0.42 % respectively (Appendix I,1).

5.2.8 Calculations.

Whole body leucine kinetics- Total leucine flux (Q) was calculated using the reciprocal pool model (from [^{13}C]α-KIC values)(Horber et al, 1989), at isotopic plateau: Q=i(Ei/Ep-

1), where, i= L-[1-^{13}C]leucine infusion rate (μmol·kg^{-1}·h^{-1}), Ei= enrichment of the infused leucine (% ^{13}C), and Ep= enrichment of the plasma α-KIC (APE) and the term "-1" corrects for the contribution of the infused isotope to Q. The reciprocal pool model assumes that the enrichment of plasma [^{13}C]α-KIC, during an infusion of L-[1-^{13}C]leucine, is more indicative of intracellular [^{13}C]leucine enrichment than is plasma [^{13}C]leucine enrichment given that transamination only occurs intracellularly (Horber et al, 1989). Leucine oxidation was calculated from the equation (Wolfe et al, 1982): Total leucine oxidation = $[(IECO_2/c)/IE_{\alpha\text{-}KIC}]\cdot\dot{V}CO_2$, where, $IECO_2$ = enrichment of expired CO_2 (APE), $IE_{\alpha KIC}$ = enrichment of plasma [^{13}C]α-KIC (APE), $\dot{V}CO_2$ = volume of expired carbon dioxide (μmol·kg^{-1}·h^{-1}), and c = fed state bicarbonate retention factor (0.83 (Irving et al, 1983; Hoerr et al, 1989; Tarnopolsky et al, 1991)). The leucine oxidation values were corrected for changes in background breath $^{13}CO_2/\ ^{12}CO_2$ enrichment due to the dietary intervention (Schoeller et al, 1980; Wolfe et al, 1984a) by studying 2 subjects under LP and HP diet conditions with no tracer infusion. The rate of appearance of endogenous leucine (Ra end leu) was determined from: total leucine flux (Q) - dietary leucine intake (I(1 - fo)). The I value was derived from the expected rate of appearance of

exogenous α-KIC into the hepatic vein following an oral feeding with casein and whey PRO (85 % of dietary leucine; I(0.85))(Silk et al, 1973; FAO/WHO/UNU, 1985; Hoerr et al, 1991). Therefore, the Ra end leu was used as an indicator of whole body PRO breakdown and non-oxidative leucine disposal (NOLD) was used to estimate WBPS (Matthews et al, 1980; Schwenk et al, 1987; Nair et al, 1988) according to the model where flux (Q) = synthesis (S) + oxidation (O) = dietary intake (I) + PRO breakdown (B) (Matthews et al, 1980), using an average tissue leucine content of 590 μmol leucine·g PRO $^{-1}$ (Wolfe et al, 1982). The validity of using NOLD as an indicator of WBPS has been established in rats using a PRO synthesis inhibitor (Schwenk et al, 1987).

5.2.9 Statistical Analyses.

The physical characteristics of the subjects were compared using an independent *t* test. All other data was analyzed using a 2-way between-within split-plot analysis of variance (ANOVA) with diet (LP vs MP vs HP) as the within-subject variable and group (BB vs S) as the between-subject variable. When a significant within group F ratio was obtained the location of pair-wise differences was performed with the Tukey *post-hoc* test. A $P \leq 0.05$ was taken to indicate significance. All data in tables and figures are mean ± SD.

5.3 Results.

5.3.1 Dietary analysis.

The data for the habitual intakes of subjects and for the intakes during each diet phase are given in Table 6. There were no within-subject differences in total energy intake but, as expected, the energy intake for BB was significantly ($P< 0.01$) greater that for S. BB habitually consumed more PRO than did S ($P< 0.05$). There were no between-subject differences in PRO_{IN} during the experimental protocol. Within the BB group the habitual PRO_{IN} was not different from the MP diet but was greater ($P< 0.05$) than for LP and less ($P< 0.05$) than for HP. Within the S group the habitual PRO_{IN} was significantly different ($P< 0.05$) from all of the diet treatments. There were no significant between-subject differences for the % energy intake of PRO, CHO, and FAT. The % energy intake from PRO increased significantly ($P< 0.05$) and the % energy intake from carbohydrate decreased significantly ($P< 0.05$) for both groups from LP to MP to HP diets (Table 6).

TABLE 6. *Habitual and treatment dietary characteristics*

		Body Builders (BB) (n=7)	Sed. Controls (S) (n=6)	P*
Energy intake ($kcal \cdot kg^{-1} \cdot d^{-1}$)	HAB	43.0±9.4	30.0±4.6	< 0.01
	LP	42.1±8.7	31.6±5.4	< 0.01
	MP	43.7±9.0	31.3±3.9	< 0.01
	HP	43.6±9.2	34.0±2.7	< 0.01
Protein intake ($g \cdot kg^{-1} \cdot d^{-1}$)	HAB	$1.77 \pm 0.38^{a\dagger}$	$1.21 \pm 0.29^{a\dagger}$	< 0.05
	LP	0.89 ± 0.02^{b}	0.90 ± 0.02^{b}	NS
	MP	1.42 ± 0.08^{a}	1.41 ± 0.04^{c}	NS
	HP	2.32 ± 0.09^{c}	2.37 ± 0.03^{d}	NS
% Energy intake (PRO,CHO,FAT,EtOH)	HAB	(16,49,32,3)	(16,48,34,2)	NS
	LP	(08,65,27,0)	(11,66,23,0)	NS
	MP	(14,60,27,0)	(19,56,25,0)	NS
	HP	(22,48,30,0)	(28,42,30,0)	NS

Values are mean ± SD. HAB=habitual intake; LP=low protein trial; MP=medium protein trial; HP=high protein trial. * Between group P value. † Within group comparisons; values within each group with different letters are significantly ($P<0.05$) different from each other.

5.3.2 Lean body mass, CPK activity, and strength.

There were no within-subject effects (diet) on either lean body mass or urinary creatinine excretion for either group (Table 7). The CV for creatinine excretion over the diet treatments was 4.3 % for BB and 2.9 % for S. For both lean body mass and urinary creatinine excretion values for the BB group were significantly greater than S ($P< 0.01$). This was expected for urinary creatinine has been frequently used as a measurement of muscle mass in humans (Heymsfield et al, 1983). BB were significantly stronger than S on all diet treatments and for all speeds but there were no within-subject (diet) effects for either group in isokinetic strength (Table 7). BB had significantly ($P< 0.01$) greater plasma CPK activity than for S and there were no within-subject (diet) effects (Table 7). This provided some evidence that the BB group kept training fairly constant and that S did not do any strenuous activity.

TABLE 7. *Effects of dietary treatment and activity on lean body mass, urinary creatinine excretion, isokinetic strength, and plasma CPK activity.*

		Body Builders (BB) (n=7)	Sed. Controls (S) (n=6)	P*
Lean body mass (kg)	LP	77.3±7.1	66.6±7.9	< 0.01
	MP	77.7±6.6	67.3±8.4	< 0.01
	HP	76.6±6.7	67.9±8.5	< 0.01
Creatinine excretion ($mmol \cdot d^{-1}$)				
	LP	14.0±3.6	10.7±2.4	< 0.01
	MP	14.6±3.8	11.3±3.4	< 0.01
	HP	13.4±2.4	11.2±2.2	< 0.01
Isokinetic strength: (arm flexors)				
$0° \cdot sec^{-1}$	LP	92.7±6.1	65.5±15.4	< 0.01
	MP	95.9±6.0	64.0±15.6	< 0.01
	HP	90.0±9.7	75.1±14.2	< 0.05
$30° \cdot sec^{-1}$†	LP	81.4±10.5	54.8±15.6	< 0.01
	MP	76.6±11.2	53.0±15.0	< 0.05
	HP	74.1±7.4	55.5±10.6	< 0.01
$180° \cdot sec^{-1}$†	LP	55.3±4.6	39.3±12.9	< 0.05
	MP	58.6±7.2	41.5±7.7	< 0.05
	HP	55.9±7.1	43.3±6.1	< 0.05
CPK activity ($U \cdot l^{-1}$)	LP	163±124	60±50	< 0.01
	MP	163±99	57±41	< 0.01
	HP	186±132	66±51	< 0.01

Values are mean ± SD. * P=between group comparisons (there were no significant within group (diet effect) differences for any of these variables). †Isokinetic strength decreased significantly ($P<0.05$) for both groups as speed increased.

5.3.3 Nitrogen balance.

The mean NBAL was negative for BB on the LP diet (5 of 7 subjects negative) and was positive for S (Table 8). Nitrogen intake, urinary N excretion, total N excretion and NBAL increased significantly ($P< 0.01$) from LP to MP to HP diets for both groups with no between-subject differences (Table 8). For BB there was no effect of N intake (ie. diet) on fecal N loss but for S fecal N losses were greater for the HP diet vs MP and LP diets ($P< 0.05$)(Table 8). Adaptation to the N intake during each of the NBAL periods was confirmed by the lack of a significant day to day change (positive or negative slope) in urinary urea N excretion. Urinary urea N excretion was significantly ($P< 0.01$) greater for HP vs MP vs LP diets (Figure 20). Completeness of the 3 day pooled urine sample between diet periods was confirmed by the low CV's for urinary creatinine excretion of 4.2 % and 2.9 % for BB and S respectively.

The PRO_{IN} to achieve NBAL was interpolated from multiple regression analysis of N intake ($g \cdot kg^{-1} \cdot d^{-1}$) vs NBAL ($mg\ N \cdot kg^{-1} \cdot d^{-1}$) for both groups (BB: $NBAL=-71.6 + 50.9(PRO_{IN})$, $SD=9.99$, $R^2=0.58$; S: $NBAL=-16.8 + 24.3(PRO_{IN})$, $SD=5.47$, $R^2=0.55$)(Figure 21) (Appendix VI.1).

TABLE 8. *Nitrogen balance summary*

	N Intake, ($g \cdot d^{-1}$)	N Excretion, $g \cdot d^{-1}$				
		Urine	Feces	Sweat + misc.*	Total	Balance
BBLP	$12.2 \pm 1.0^{a\dagger}$	11.0 ± 2.4^{a}	2.0 ± 0.5^{a}	1.64	14.6 ± 2.6^{a}	-2.4 ± 3.1^{a}
BBMP	19.9 ± 2.4^{b}	15.3 ± 2.4^{b}	2.1 ± 0.5^{a}	1.87	19.2 ± 2.3^{b}	$+0.7 \pm 1.5^{b}$
BBHP	31.9 ± 3.2^{c}	23.5 ± 3.1^{c}	2.6 ± 0.2^{a}	1.99	28.1 ± 3.2^{c}	$+3.8 \pm 2.3^{c}$
SLP	12.2 ± 1.6^{a}	9.8 ± 1.3^{a}	1.5 ± 0.1^{a}	0.49	11.8 ± 1.3^{a}	$+0.4 \pm 1.2^{a}$
SMP	19.6 ± 2.9^{b}	15.3 ± 2.5^{b}	1.7 ± 0.2^{a}	0.87	17.9 ± 2.6^{b}	$+1.7 \pm 1.2^{b}$
SHP	32.2 ± 4.3^{c}	25.0 ± 4.2^{c}	2.3 ± 1.4^{b}	1.42	28.8 ± 4.2^{c}	$+3.4 \pm 1.4^{c}$

Values are mean ± SD. * Sweat and miscellaneous values were obtained from references (Calloway, 1973; Tarnopolsky, 1988; Tarnopolsky, 1990). † Within group comparisons; values within groups with different letters are significantly ($P < 0.01$) different from each other.

Figure 20. Effect of dietary intervention and habitual activity (subject group) on daily urinary urea nitrogen excretion during the NBAL periods. BB= bodybuilders; S= sedentary controls; LP= low protein diet; MP= medium protein diet; HP= high protein diet. * Urinary urea nitrogen excretion increased significantly ($P < 0.01$) from LP to MP to HP diets. There were no significant day to day changes in urinary urea excretion, indicating dietary adaptation.

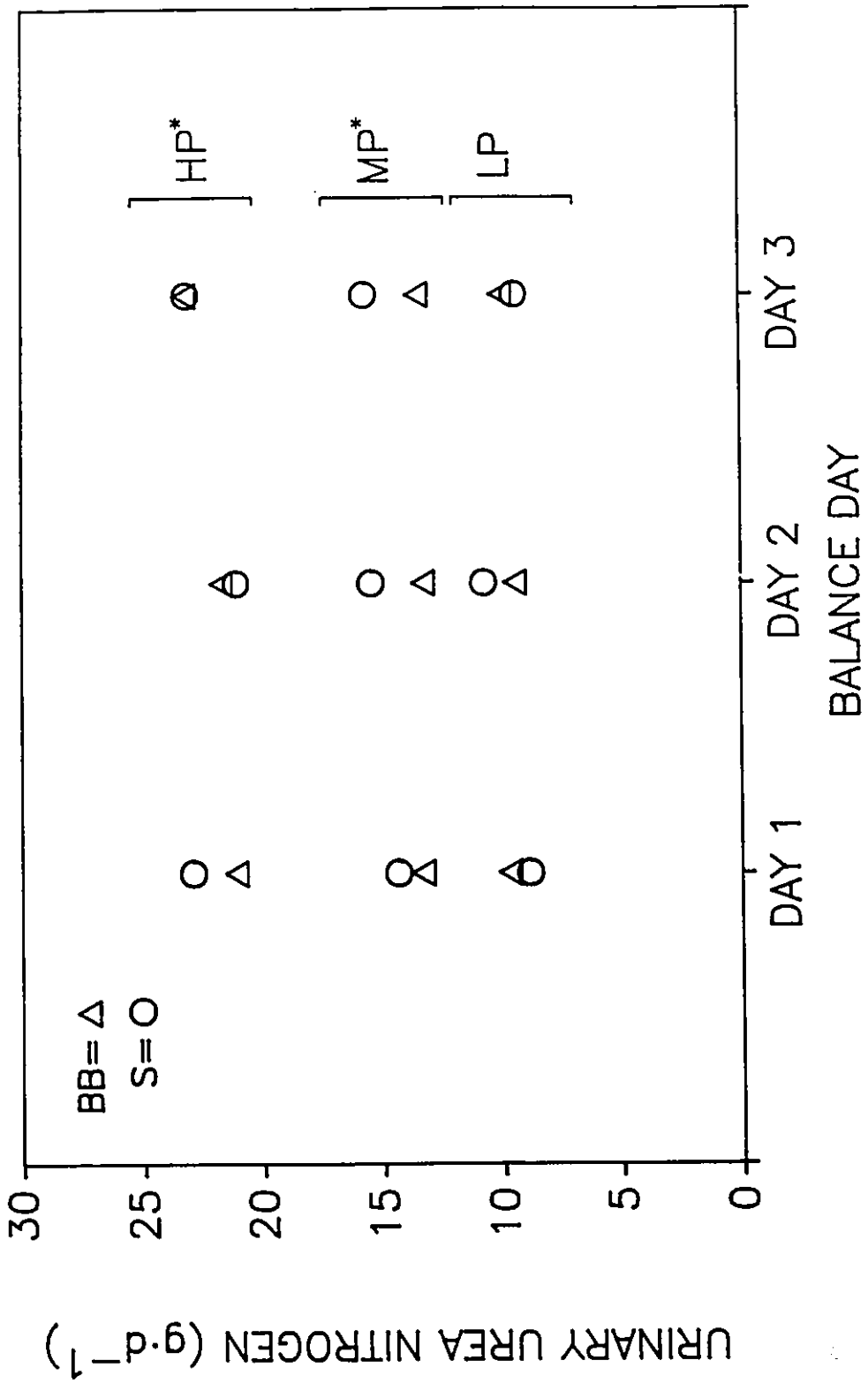
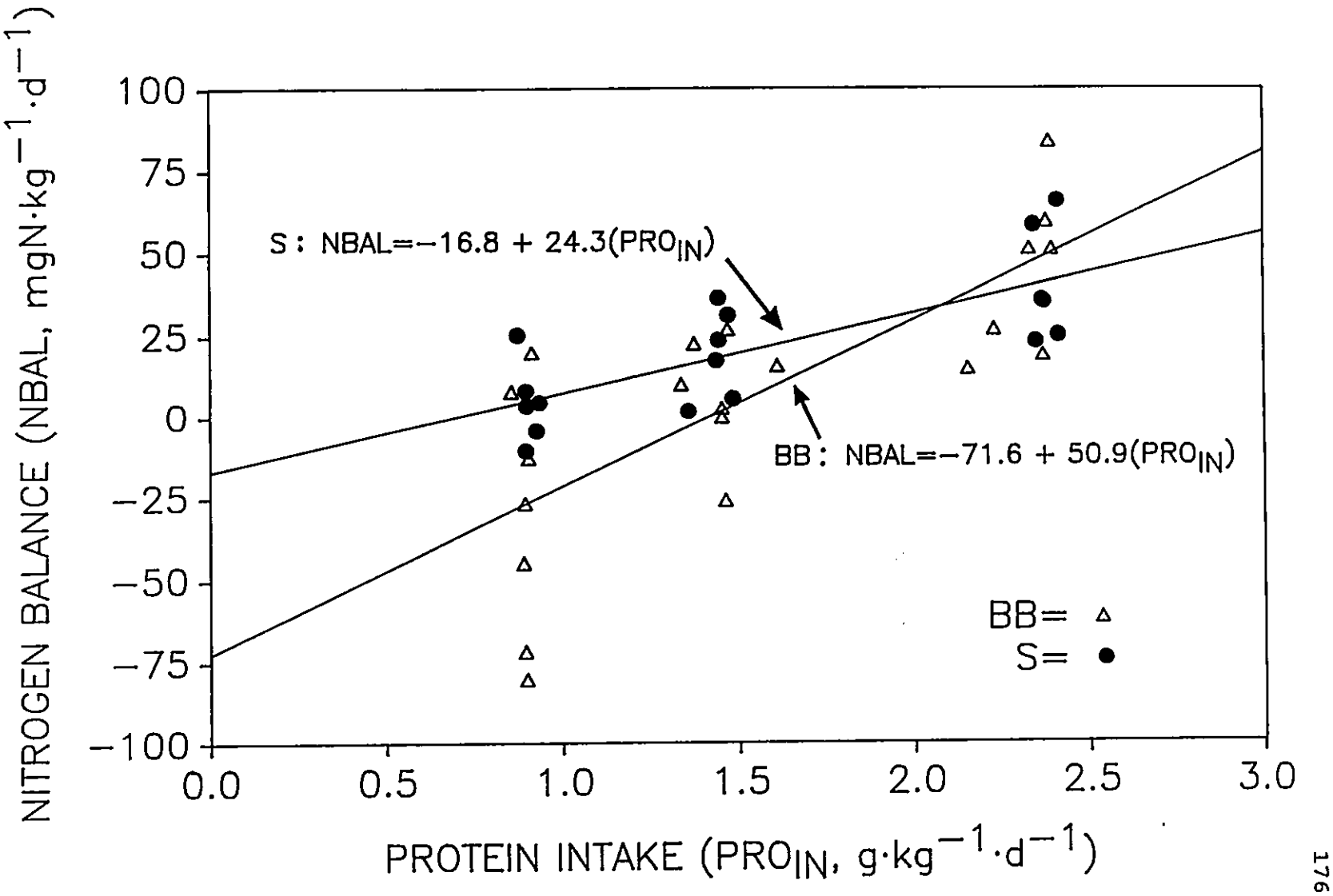

URINARY UREA NITROGEN (g·d⁻¹)
30
25
20
15
10
5
0
BB= Δ
S= O
HP*
MP*
LP
DAY 1
DAY 2
DAY 3
BALANCE DAY

Figure 21. Plot of protein intake vs. nitrogen balance for both groups. The predicted protein intake for 0 NBAL was interpolated from the generated regression equations. See legend of Fig. 20. for definitions and text for calculated protein intakes.


NITROGEN BALANCE (NBAL, mgN·kg^{-1}·d^{-1})
100
75
50
25
0
−25
−50
−75
−100
0.0
0.5
1.0
1.5
2.0
2.5
3.0
S : NBAL=−16.8 + 24.3(PRO$_{IN}$)
BB : NBAL=−71.6 + 50.9(PRO$_{IN}$)
BB=
S=
PROTEIN INTAKE (PRO$_{IN}$, g·kg^{-1}·d^{-1})

The PRO_{IN} to achieve zero NBAL was 1.41 $g \cdot kg^{-1} \cdot d^{-1}$ for BB and 0.69 $g \cdot kg^{-1} \cdot d^{-1}$ for S (↑ 104 %). With a safety margin of +1 SD the requirements were calculated to be 1.76 and 0.89 g $PRO \cdot kg^{-1} \cdot d^{-1}$ for BB and S respectively (increased 98 %).

5.3.4 Leucine turnover.

WBPS was significantly greater for group BB vs S ($P< 0.05$). There was a significant interaction for WBPS between the groups ($P< 0.05$). Post-hoc analysis revealed that there was a significant increase ($P< 0.01$) in WBPS for BB from LP to both MP and HP with no significant difference between MP and HP diets (Figure 22, a). There were no between-subject differences for leucine oxidation and both groups demonstrated significantly ($P< 0.001$) increased oxidation for the HP diet vs both MP and LP (Figure 22, b). There was a significant correlation between leucine oxidation and urinary urea excretion ($r=0.965$, $P< 0.001$).

Total leucine flux was significantly ($P< 0.001$) greater for BB than S (between-subject effect). Total flux for BB was significantly greater for HP vs MP and LP ($P< 0.01$) and for MP vs LP ($P< 0.05$). Total leucine flux for S was significantly greater for HP vs MP and LP ($P< 0.05$)(Figure 23, a). Protein breakdown was significantly ($P<0.01$) greater for BB vs S (between-subject effect)(Figure 23, b). (See Appendix VI.3 for ANOVA tables).

Figure 22. a. Whole body protein synthesis (WBPS): * For BB, WBPS increased significantly on diets MP and HP vs diet LP. † WBPS was significantly ($P< 0.05$) greater for BB than for SED. b. Leucine oxidation: * Leucine oxidation was significantly ($P<0.01$) greater for both groups on HP diet vs MP and LP diets. There were no between group differences. See legend of Fig. 20. for definitions.

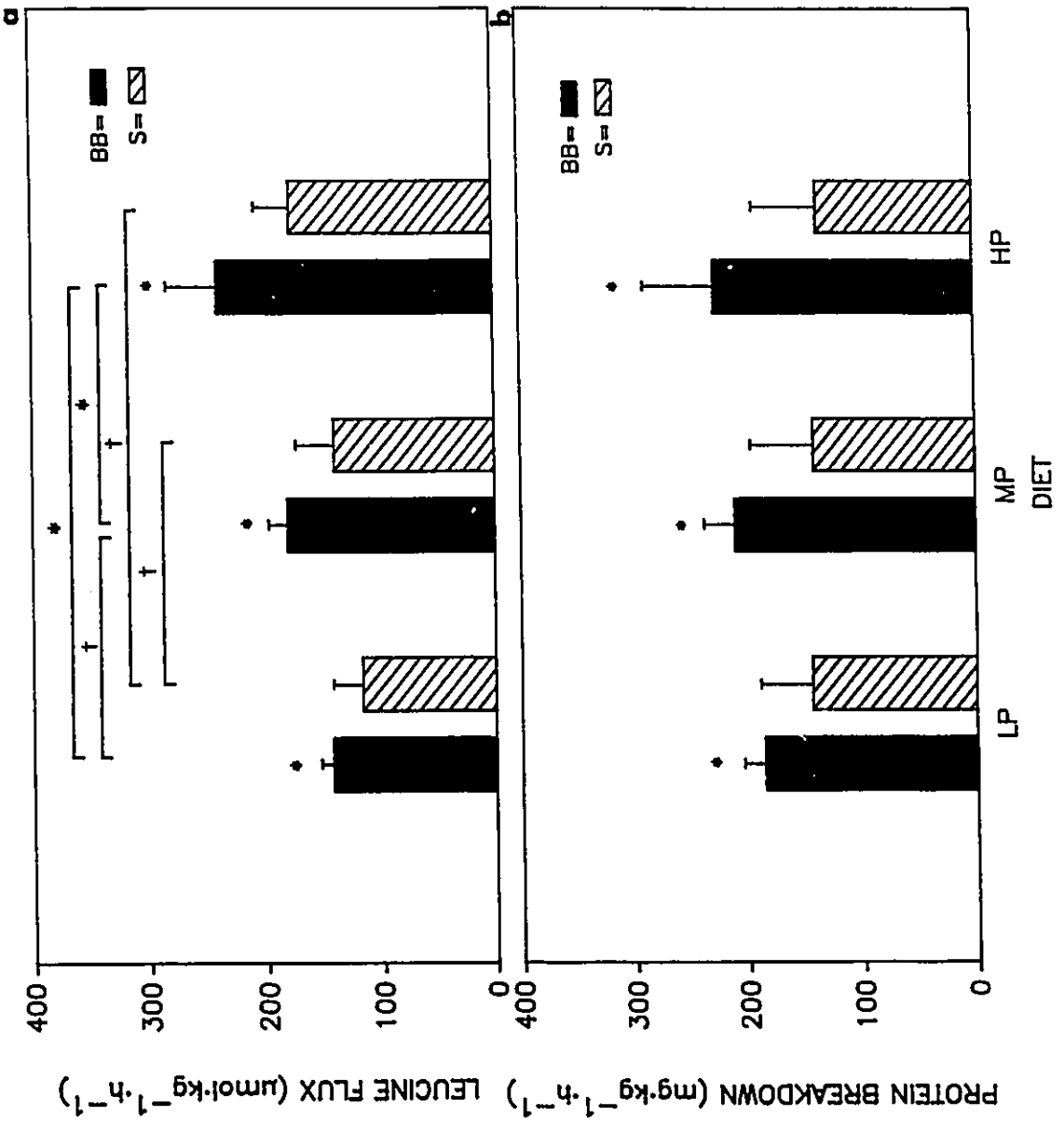
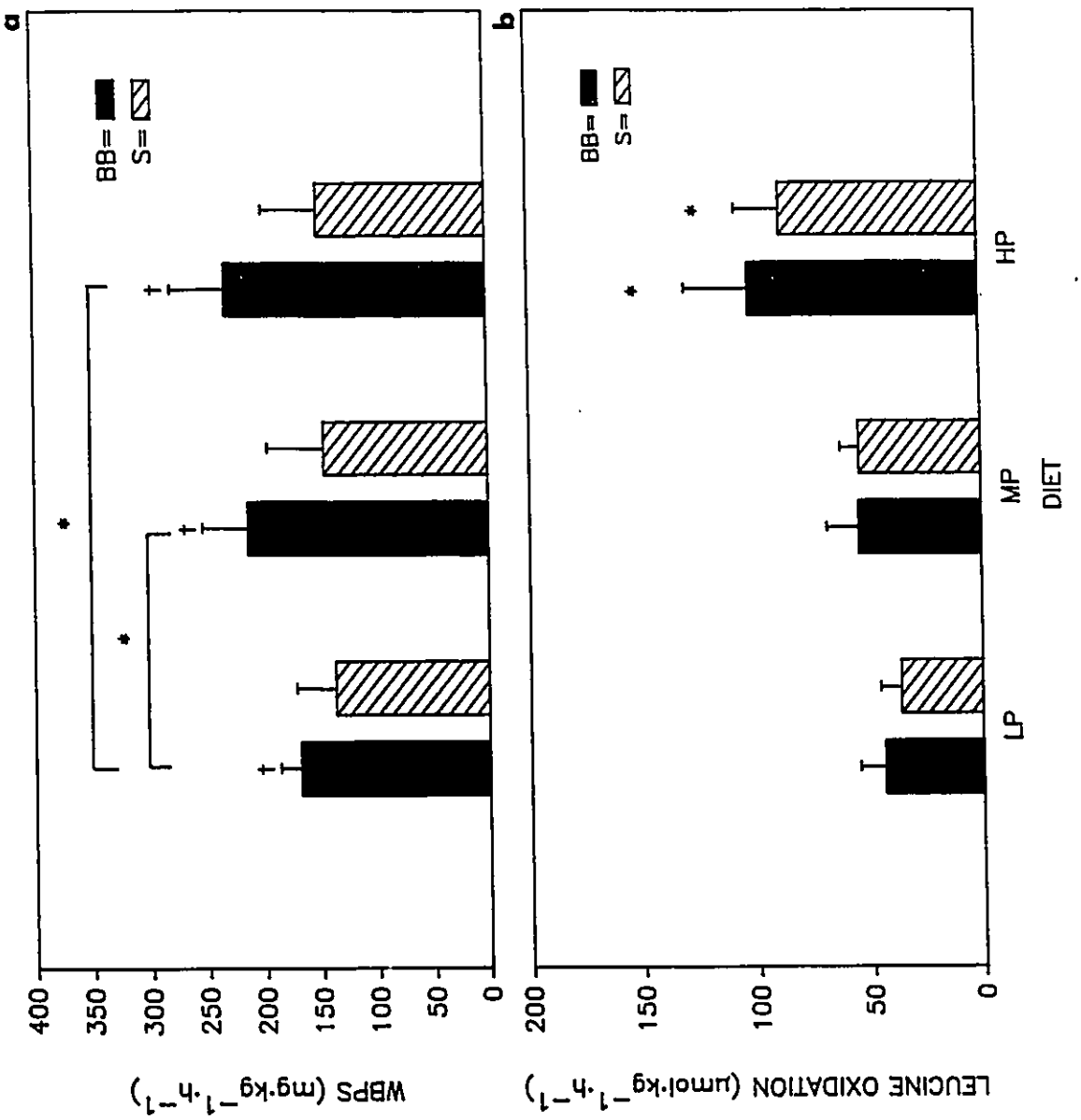

a
b
BB=
S=
400
300
200
100
0
LP
MP
HP
DIET
LEUCINE FLUX (μmol·kg⁻¹·h⁻¹)
PROTEIN BREAKDOWN (mg·kg⁻¹·h⁻¹)

Figure 23. a. Total leucine flux: * Flux was significantly (P< 0.01) greater for BB vs S. For S, flux was significantly († , P< 0.05) greater on MP and HP diets vs LP diet. For BB, flux was significantly († , P< 0.05) greater for MP diet vs LP and was significantly (*, P< 0.01) greater for HP diet vs both MP and LP diets. b. Protein breakdown: * PRO breakdown (Ra end leu) was significantly (P< 0.01) greater for BB vs S. See legend of Fig. 20. for definitions.


a
b
BB=
S=
WBPS (mg·kg⁻¹·h⁻¹)
LEUCINE OXIDATION (μmol·kg⁻¹·h⁻¹)
0
50
100
150
200
250
300
350
400
LP
MP
HP
DIET

5.4 Discussion.

The finding of a lower rate of WBPS for BB on the LP diet compared to MP and HP diets suggested that a PRO_{IN} of 0.89 $g \cdot kg^{-1} \cdot d^{-1}$ was inadequate for young men performing resistance exercise. In addition, all of the BB subjects were in negative NBAL on the LP diet which confirmed that this level of PRO_{IN} was sub-optimal during this type of training. The PRO_{IN} for the LP diet was very close to the Canadian R.N.I. for PRO of 0.86 $g \cdot kg^{-1} \cdot d^{-1}$ for men 19 years of age or older (Health and Welfare, Canada, 1990). This suggests that the Canadian R.N.I. for PRO may not be an optimal or "safe" PRO_{IN} in young men habitually performing resistance exercise.

Since MPS represents about 25-30 % of WBPS (Nair et al, 1988), the level of PRO provided by the LP diet may have resulted in an *accommodated* state with a reduction in MPS and hence in the capacity of these athletes to achieve optimal muscle mass/strength gains in the long-term. However, MPS was not measured directly in this study and the reduction in WBPS may have come from tissues other than exercised muscle (Devlin et al, 1990). In the future, studies of MPS (Nair et al, 1988; Carraro et al, 1990b; Devlin et al, 1990), hepatic PRO synthesis (ie. albumin, fibronectin, fibrinogen (Carraro et al, 1990a)) and PRO

synthesis in other tissues should be combined with simultaneously acquired WBPS data to determine the contribution of muscle, hepatic and other tissues to the observed reduction in WBPS for the BB athletes on the LP diet. During endurance exercise there is an increase in the fibronectin synthetic rate, and no change in fibrinogen or albumin synthesis while after exercise fibrinogen synthesis is increased with no change in albumin or fibronectin synthesis rates (Carraro et al, 1990a). Clearly, it will be very difficult to gain a comprehensive picture of the rates of PRO synthesis in many tissues after a dietary or exercise intervention, and the simultaneous determination of WBPS and MPS will be the most feasible study at this time.

Neither the quantitative measures of lean body mass nor arm muscle strength data showed reductions consequent to the 13 days on the LP diet. This may indicate either a preservation of muscle mass at the expense of other body PRO pools or an inability to detect LBM changes over the 13 day period with the methods employed. It is likely that both explanations are plausible. Firstly, it is known that rat skeletal muscle can hypertrophy under the extreme conditions of starvation, diabetes and hypophysectomy in response to tenotomy of synergist muscles (hypertrophy model)(Goldberg and Goodman, 1969; Goldberg et al, 1975). Thus muscle

contraction *per se* may have a sparing effect on skeletal muscle at the expense of other tissues during periods of PRO deprivation. Secondly, from the NBAL data, it would be predicted that the BB group would have lost 1.13 kg of LBM (vs MP diet) but the measured LBM decreased by 0.4 kg, leaving 0.73 kg unaccounted for. The CV for the determination of LBM in the present study was 1.3 % and with a mean LBM of 77.5 kg the discrepancy of 0.73 kg could easily be accounted for by variation in the LBM determination alone.

Assuming that muscle mass was maintained on the LP diet, the reduction in WBPS *per se* is of concern for in the long term a negative impact on LBM may become apparent and reductions in WBPS are characteristic of short-term starvation at rest (Waterlow, 1986; Hoffer et al, 1990) and during exercise (Knapik et al, 1991). It has been suggested that reductions in WBPS and amino acid flux may have a negative impact on health status due to reductions in lymphocyte amino acid flux (Ardawi and Newsholme, 1983; Newsholme et al, 1985) and due to a reduction in the availability of metabolic intermediates for biosynthetic pathways (Newsholme et al, 1985; Young and Bier, 1987a; Millward and Rivers, 1989). A long-term reduction in the hepatic production of plasma PRO (Garza et al, 1977;

Carraro, 1990a) would not be advantageous to a human.

The BB group did not show an increase in WBPS from the MP to HP diets which suggested that the MP diet (1.41 g PRO·kg^{-1}·d^{-1}) was already close to the optimal physiological requirement (*adaptation*) while the HP diet (2.32 g protein·kg^{-1}·d^{-1}) represented a *nutrient overload* (Young and Bier, 1987a). The fact that the S group did not show a significant increase in WBPS with increasing PRO_{IN} suggested that the LP diet (0.90 g PRO·kg^{-1}·d^{-1}) was at or above their physiological requirement. This is an expected finding since the Canadian R.N.I. for PRO is 0.86 g·kg^{-1}·d^{-1} (Health and Welfare, Canada, 1990). In fed, sedentary, males it has been demonstrated that WBPS increased with PRO_{IN} (Motil et al, 1981). However, PRO_{IN} of 0.1, 0.6, and 1.5 g PRO·kg^{-1}·d^{-1} were used in that study and the 0.6 g·kg^{-1} ·d^{-1} level may have been marginal and resulted in an *accommodated* state with a reduction in WBPS in spite of a positive NBAL (clearly the 0.1 g·kg^{-1}·d^{-1} diet was deficient in PRO)(Motil et al, 1981). The results of the present study and the aforementioned one (Motil et al, 1981), support the prediction that a PRO_{IN} at about the Canadian R.N.I. (0.86 g·kg^{-1}·d^{-1}, (Health and Welfare, Canada, 1990)) is appropriate for sedentary men greater than 19 years old.

The finding of a significantly greater WBPS for BB vs S (+ 42 %) was expected for it has been well documented that people who engage in heavy resistance training have an increased muscle mass (MacDougall et al, 1982; MacDougall, 1986; Tarnopolsky et al, 1988) due to an increased MPS (Carraro et al, 1990b; Wong et al, 1990, Yarasheski et al, 1990; Chesley, 1991). The increased WBPS in BB is likely to be a chronic phenomenon because the magnitude of increase is present both during and for 2 hours after circuit weight training exercise (Tarnopolsky et al, 1991) in addition to the elevated WBPS 24 h after exercise as demonstrated in the current study. Weight lifting exercise has been shown to increase MPS by 85 % in exercised compared to rested muscle for at least 24 h after exercise (Chesley et al, 1991). Assuming that MPS represents about 30 % of WBPS (Nair et al, 1988), 72 % of the increased WBPS for the BB group compared to the S group could be accounted for by an increased MPS. Therefore, some of the increase in WBPS for BB must have come from tissues other than skeletal muscle. It has also been shown that endurance athletes have greater rates of leucine flux and WBPS than sedentary controls (Lamont et al, 1990) and endurance exercise increases the fibrinogen synthetic rates (Carraro et al, 1990a). This may explain some of the increases in WBPS for BB because they also

engaged in activities with some endurance component (rugby/rowing) 40 % of the time.

In addition to increased WBPS, a significant increase in PRO breakdown was observed in the BB compared to the S group. This finding was expected because hypertrophy of skeletal muscle is associated with simultaneous increases in both PRO synthesis and PRO breakdown, with synthesis increasing to a greater degree (Laurent and Millward, 1980; Goldspink et al, 1982). In the present study the net PRO balance (WBPS-PRO breakdown) for the BB group was -16.24 $mg \cdot kg^{-1} \cdot d^{-1}$ for LP diet, +3.71 $mg \cdot kg^{-1} \cdot d^{-1}$ for MP diet, and +5.54 $mg \cdot kg^{-1} \cdot d^{-1}$ for HP diet. These results support the interpretation that the LP diet was deficient in PRO and the MP diet was close to providing a sufficient amount of PRO. These balances are however, only semi-quantitative for the WBPS data was based upon the non-oxidative portion of total leucine disposal (NOLD) which has been shown to be a predictive, though not a quantitative measurement of WBPS (Schwenk et al, 1987a). In addition, the breakdown values were determined from total leucine flux assuming a constant and fixed rate of appearance of exogenous leucine based upon published data (Hoerr et al, 1991). These measurements would provide directional changes which were in agreement with both the WBPS and NBAL data of the study.

Simultaneous increases in PRO synthesis and breakdown would appear to attenuate net increases in synthesis, but it has been suggested that the two processes may be obligatorily coupled (Laurent and Millward, 1980; Goldspink et al, 1982). No studies have measured MPS and breakdown simultaneously in human subjects during weight training but independent studies have found increased urinary 3-methylhistidine excretion (an indicator of myofibrillar proteolysis (Sjolin et al, 1989)); (Dohm et al, 1982; Pivarnik et al, 1988) and MPS (Yarasheski et al, 1990; Chesley et al, 1991) consequent to weight lifting. The increased flux through the pathways of synthesis and breakdown would provide greater amino acid availability for a variety of biosynthetic pathways which would give greater sensitivity of control that may be of physiological advantage to the athlete during accretion of muscle mass (Newsholme et al, 1985; Young and Bier, 1987a; Millward and Rivers, 1989).

In support of the fact that the HP diet was a *nutrient overload* was the finding of a significant increase in leucine oxidation for both groups on the HP diet vs LP or MP diets. This suggested that PRO consumed in excess was oxidized as energy rather than stored as lean tissue and that the point of inflection in oxidation may be useful in

helping to determine PRO requirements (Young et al, 1981a; Young, 1981b). For the BB group it appeared that the inflection point was at some dietary PRO_{IN} above that provided by the MP diet, while for the S group an inflection towards exponential increase in oxidation was more difficult to determine. However, the HP diet resulted in a large increase in oxidation as observed for BB. A very high correlation was found between leucine oxidation and urinary urea excretion (r=0.97), which would be expected since leucine oxidation is preceded by intra-cellular transamination with subsequent hepatic ureagenesis. Wolfe et al. (1984b) have reported that during very light endurance (~30 % $\dot{V}O_{2max}$) exercise, stable isotope measurements of leucine oxidation and ureagenesis were discordant and suggested that either the measurement of ureagenesis or leucine oxidation was in error. The results of this study support the fact that leucine oxidation and ureagenesis are closely linked processes and the results of Wolfe's group (1984b) may indicate that the 2 processes are not co-temporal and not that either of the methods are in error. The long time period (>24 h) required to establish an isotopic plateau in [^{15}N]urea during a constant infusion of [^{15}N]glycine provides further evidence that there is a considerable time lag between the turnover of peripheral PRO

and ureagenesis (Golden and Waterlow, 1977).

It has been argued that an increased oxidation of amino acids may confer a physiological advantage to humans by an increased flux through biosynthetic pathways which would provide a buffer to increased demand for amino acids in times of need. This increase in oxidation/flux of amino acids has been termed the "anabolic drive" (Millward and Rivers, 1989). It would seem plausible that the increased oxidation of amino acids at high PRO_{IN} would, as interpreted above, represent an excess as there did not appear to be an anabolic advantage to further increasing the PRO_{IN} after WBPS had plateaued. Increases in WBPS, net synthesis and breakdown for BB in this study consuming the MP and HP diets compared to the LP diet provided evidence of an "anabolic drive" at the MP PRO_{IN} level of 1.43 $g \cdot kg^{-1} \cdot d^{-1}$. If increased oxidation of amino acids *per se* were indicative of an "anabolic drive" conferring some physiological advantage, this would suggest that very high PRO_{IN} would be favourable, which has not been shown to be the case in this and other studies (Tarnopolsky et al, 1988; Meredith et al, 1989). Therefore, it is important to consider all aspects of amino acid turnover in the interpretation of the data.

The calculated PRO_{IN} for zero NBAL was 1.41 $g \cdot kg^{-1} \cdot d^{-1}$ for BB and 0.69 $g \cdot kg^{-1} \cdot d^{-1}$ for S and with a safety margin of

one standard deviation (SD) the estimated requirements were 1.76 and 0.89 g PRO·kg^{-1}·d^{-1} respectively. At zero NBAL the estimate was 104 % and with + 1 SD the estimate was 98 % greater for BB than S. The reasons for inclusion of one standard deviation compared to the usual 2 standard deviations used by national (U.S. Food and Nutrition Board, 1989; Health and Welfare, Canada, 1990) and international (FAO/WHO/UNU, 1985) bodies in setting PRO recommendations is based upon the larger biological variation obtained with small subject numbers and with PRO_{IN} considerably above and below the estimated requirement, as previously described (Tarnopolsky et al, 1988). The fact that the estimate of 0.89 g·kg^{-1}·d^{-1} for the S group in this study is very close to the Canadian R.N.I. of 0.86 g·kg^{-1}·d^{-1} provides further evidence in support of the validity of the estimates put forth. It should also be noted that the NBAL study was conducted with excellent subjective (checklist compliance > 98%) and objective/biochemical (mean CV of creatinine excretion over diet treatments = 3.6 %, Table 3) evidence of compliance. The adaptation period of 10 days should be adequate for both an increasing (Oddoye and Margen, 1979) and decreasing (Rand et al, 1976; Oddoye and Margen, 1979) PRO_{IN}. This was confirmed by the lack of a significant slope in the urinary urea excretion over each of the 3 day

NBAL periods by both groups (Figure 20). In addition, the mean energy intake for both the S group (32.3 $kcal \cdot kg^{-1} \cdot d^{-1}$(Pellett, 1990a; Miller, 1991)) and the BB group (43.2 $kcal \cdot kg^{-1} \cdot d^{-1}$(Tarnopolsky et al, 1991)) would be adequate for their respective activity patterns. The extra daily energy intake for BB vs S would be about 900 Kcal, which is much greater than the clculated energy expenditure during circuit-set weight lifting, of about 240 $Kcal \cdot session^{-1}$ (Tarnopolsky, 1991). The difference ($\sim$600 $Kcal \cdot d^{-1}$) probably relates to the aerobic exercise performed by BB and possibly to their greater LBM (Stunkard et al, 1986). The absence of a significant change in weight for either of the groups (Table 7) provided further support to the adequacy of the dietary energy intake.

Since BB had a significantly greater LBM than did S, it may be informative to express PRO requirements relative to LBM. The zero intercept PRO requirement for BB would be 1.56 g $PRO \cdot kg\ LBM^{-1} \cdot d^{-1}$ and for S would be 0.88 g $PRO \cdot kg\ LBM^{-1} \cdot d^{-1}$. This would reduce the difference between the BB and S groups to + 77 % (BB vs S) from + 104 % (difference using total mass). By correcting for LBM the differences between the groups remains large and supports the argument that the increased PRO requirements for BB are due to their activity patterns and not merely their increased LBM.

From the NBAL data it would be estimated that the BB group would have gained about 1.1 kg of LBM when in fact they lost 1.1 kg, leaving a discrepancy of 2.2 kg. Hence, the LBM data did not support the classical interpretation of the positive NBAL data. This illustrated the value of the leucine turnover values which demonstrated that the HP diet had no ergogenic effect (ie. increased LBM) in the BB group. A failure to consider the leucine turnover data and rely only on the NBAL data may lead one to conclude that an excessive PRO_{IN} is of ergogenic benefit to those involved in resistance exercise. The persistent positive NBAL demonstrated in this study has been demonstrated (Oddoye and Margen, 1979; Tarnopolsky et al, 1988) and commented upon (Hegsted, 1975; Young et al, 1989) before and appears to be an inherent error in the method. In a recent study of endurance athletes, consuming PRO_{IN} above calculated requirements, NBAL was positive and WBPS plateaued (Meredith et al, 1989). The present study and that of Meredith et al. (1989) support the arguments against the NBAL method (Hegsted, 1975; Young et al, 1989) and further demonstrate the need to combine NBAL methodology with tracer techniques to gain a comprehensive understanding of PRO metabolism to determine the PRO requirements for humans (Motil et al, 1981; Young et al, 1981a; Young, 1981b; Young and Bier,

1987a; Meredith et al, 1989; Young et al, 1989).

The PRO requirement for the BB group was 8.6 % and 79 % greater than previously derived PRO_{IN} estimates for novice body-builders and elite body-builders, respectively (Tarnopolsky et al, 1988; Tarnopolsky et al, 1990). The PRO requirements in these two studies mentioned were determined in an identical manner to those in the present study. The 79 % greater PRO requirements determined in this study compared to an earlier study of elite body-builders is probably related to an adaptation phenomenon to the stress of activity. The athletes in this study had experience with weights for between 3 and 9 months compared to about 3 years for elite athletes in our previous study (Tarnopolsky et al, 1988). The adaptation of NBAL to the stress of training was shown longitudinally by Gontzea et al. (1975) and inferred cross-sectionally by Tarnopolsky et al. (1988; 1990). The PRO requirements determined for novice bodybuilders were marginally lower than in the present study in spite of the greater intensity and volume of resistance-type exercise in the study by Tarnopolsky et al. (1990). This is probably due to the fact that in the present study the athletes performed exercise with an endurance component 40 % of the time, which in contrast to circuit weight training (Tarnopolsky et al, 1991), increases leucine oxidation

(Wolfe et al, 1982; Evans et al, 1983; Wolfe et al, 1984b; Knapik et al, 1990) and PRO requirements (Tarnopolsky et al, 1988).

The PRO requirements determined from the NBAL data were in agreement with those estimated from the tracer data. For BB the WBPS and leucine oxidation predicted that the PRO requirement would be at or just above 1.41 g PRO$\cdot kg^{-1}\cdot d^{-1}$ and the NBAL estimate with + 1 SD was 1.76 g$\cdot kg^{-1}\cdot d^{-1}$. The WBPS on the MP diet was non-significantly lower (decrease 8.7 %) than on the HP diet which may indicate that the true PRO requirement is closer to the NBAL derived value of 1.76 g$\cdot kg^{-1}\cdot d^{-1}$ but this can only be determined by studying leucine turnover between these ranges of PRO_{IN}. The WBPS data for S predicted that the PRO requirement would be at or below 0.90 g$\cdot kg^{-1}\cdot d^{-1}$ and the NBAL derived value was 0.89 g$\cdot kg^{-1}\cdot d^{-1}$. It was hypothesized *a priori* that the S group would have significantly greater leucine oxidation on the MP compared to LP diet indicating that MP diet delivered excessive PRO, but this was not found to be significant. However, the oxidation did increase 50 % for S and only 25 % for BB from the LP to MP diet.

The present study clearly demonstrated the need to combine NBAL studies with tracer methodology to comprehensively determine PRO requirements for humans. The

estimated PRO requirement for athletes performing circuit weight training (BB) was about 98 % greater than for age-matched, sedentary controls (1.76 vs 0.89 $g \cdot kg^{-1} \cdot d^{-1}$ respectively) and these estimates were in agreement with the interpretation of the tracer data. Protein consumed in excess of requirements resulted in a plateau in WBPS and a significant increase in leucine oxidation, and did not result in an increased accretion of LBM in body-builders. This conclusion contrasts with the traditional belief of elite athletes engaged in this type of activity (Celejowa et al, 1971; Laritcheva et al, 1978; Faber et al, 1986; Tarnopolsky et al, 1988; van Erp-Baart et al, 1989; Bazzarre et al, 1990).

Chapter 6

OVERALL CONCLUSIONS

6.1 Conclusions

This research has focused on two main areas: 1. The determination of PRO requirements in resistance athletes; and 2. The examination of acute and chronic changes in whole body PRO turnover; using traditional nitrogen balance methods and stable isotope tracer methodology. It was hypothesized, *a priori*, that both PRO requirements and PRO turnover would be greater for resistance athletes as compared to a matched, sedentary, population.

An effort was made to control for a number of factors known to have possible confounding effects on the results and conclusions obtained. Objective evidence for some of these has been documented in the body of the thesis. For the NBAL studies: compliance with and adaptation to the diets was confirmed, sweat losses were measured or estimated (from appropriate data), diet N and energy was biochemically determined, and two or three different PRO_{IN} were studied to determine PRO requirements. For the tracer studies; diet and exercise breath CO_2 enrichment changes were measured and corrected for in leucine oxidation data, the bicarbonate retention factor was measured under the conditions of the experiment and leucine oxidation data were

corrected for this, isotopic plateau was achieved in breath and plasma, exogenous amino acids were provided every 30 min instead of 60 min to ensure a more constant rate of appearance of exogenous leucine, bicarbonate was mixed immediately prior to infusion to avoid losses, and subjects were adapted to the level of dietary PRO_{IN} provided during the infusions. By controlling for these known confounding variables, the validity of the conclusions drawn from the results will be greater.

Several research questions raised prior to the completion of these studies have been answered:

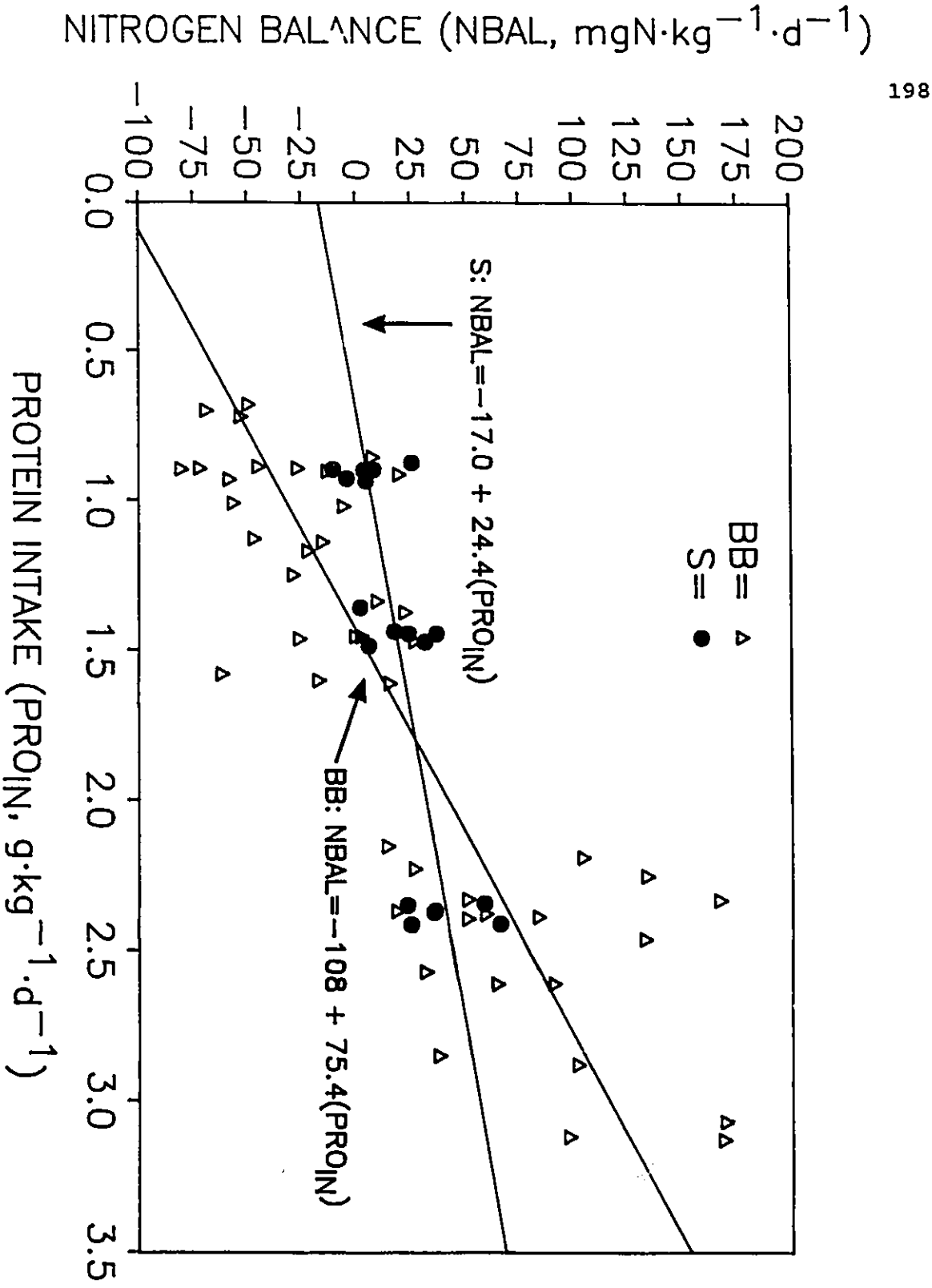
1. Are the PRO requirements for resistance athletes greater than for sedentary subjects? Are the PRO requirements of these athletes greater during the early stages of training (novice)?

For novice resistance athletes performing intensive body building-type, circuit weight training, the NBAL determined safe (+ 1SD) PRO requirement was 1.62 $g \cdot kg^{-1} \cdot d^{-1}$, which was 88 % greater than the Canadian Recommended Nutrient Intake (RNI) for PRO for men greater than 19 years of age (Health and Welfare, Canada, 1990). For young men who habitually engaged in circuit weight training, resistance exercise (>60% of total activity) and other strenuous forms of activity (<40 % of total activity (rugby, football, rowing)), the NBAL determined safe PRO requirement was 1.76 $g \cdot kg^{-1} \cdot d^{-1}$, which was 104 % greater than the Canadian RNI for PRO for men greater than 19 years of age. If we combine the data from the two studies and derive a PRO

requirement based on the pooled data the PRO requirement for zero NBAL was 1.43 $g \cdot kg^{-1} \cdot d^{-1}$, + 1SD = 1.59 $g \cdot kg^{-1} \cdot d^{-1}$, and + 2SD = 1.79 $g \cdot kg^{-1} \cdot d^{-1}$ (Figure 24). As expected, with the greater sample size, the standard deviations were lower and thus the safe requirement estimates were correspondingly lowered (this provides further support to our including 1 SD to our zero NBAL instead of 2 SD, as discussed in the thesis). Therefore, for the resistance athlete performing this type of weight training (even in the early stages of training and combining other strenuous activities) would require a PRO_{IN} of about 1.59 $g \cdot kg^{-1} \cdot d^{-1}$, which is 85 % greater than the Canadian RNI for PRO for men of this age.

The PRO requirement of 1.59 $g \cdot kg^{-1} \cdot d^{-1}$ calculated from the combined data on all of the resistance athletes in this thesis is 62 % greater than the calculated requirement for elite resistance athletes (body builders) of 0.98 g $PRO \cdot kg^{-1} \cdot d^{-1}$ (Tarnopolsky et al, 1988). Since the subjects studied in this thesis had $\leq$ six months of experience with weight training prior to the study (novice), whereas the subjects in the latter study (Tarnopolsky et al, 1988) had at least three years of experience, it is likely that there is a reduction in the need for dietary PRO as the body adapts to the stress of training (Gontzea et al, 1975). A longitudinal study is clearly needed to examine this hypothesis further (see below).

Figure 24. Plot of PRO intake vs NBAL for BB groups from Chapter 3 and 5 and for S from Chapter 5. The predicted PRO_{IN} for zero NBAL (+ 1 and 2 SD) are given in text in Chapter 6 for BB and Chapter 5 for S.


NITROGEN BALANCE (NBAL, mgN·kg^{-1}·d^{-1})
200
175
150
125
100
75
50
25
0
−25
−50
−75
−100
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
PROTEIN INTAKE (PRO$_{IN}$, g·kg^{-1}·d^{-1})
BB= △
S= ●
S: NBAL=−17.0 + 24.4(PRO$_{IN}$)
BB: NBAL=−108 + 75.4(PRO$_{IN}$)

2. Do resistance athletes derive an ergogenic benefit (ie. increased strength or lean body mass) by consuming PRO intakes in excess of calculated requirements?

In agreement with previous work (Tarnopolsky et al, 1988), there was no detectable increase in strength or lean body mass (muscle) in 12 young men starting an intensive, body building-type of circuit weight training when consuming a high (2.62 g PRO$\cdot kg^{-1}\cdot d^{-1}$) compared to a lower (1.35 g PRO$\cdot kg^{-1}\cdot d^{-1}$) PRO_{IN}, each over a one month period. In addition, there was no increase in whole body PRO synthesis, isometric strength, nor lean body mass in 7 young men performing circuit weight training when consuming 2.32 g PRO$\cdot kg^{-1}\cdot d^{-1}$ compared to a PRO_{IN} of 1.42 g$\cdot kg^{-1}\cdot d^{-1}$. Therefore, no ergogenic benefit was apparent in resistance athletes by consuming PRO in excess of calculated requirements.

3. Are the PRO requirements determined from NBAL in agreement with those obtained using amino acid tracers?

Using the measured plateau in WBPS as an indicator of the adequacy of a given PRO_{IN}, it was determined that the PRO requirement for sedentary young men was about 0.90 g$\cdot kg^{-1}\cdot d^{-1}$ and for resistance athletes was about 1.42 g$\cdot kg^{-1}\cdot d^{-1}$, and from the NBAL data the calculated safe PRO requirements were 0.89 g$\cdot kg^{-1}\cdot d^{-1}$ and 1.76 g$\cdot kg^{-1}\cdot d^{-1}$ respectively. The agreement between the two methods was good, however, a quantitative comparison is not yet possible between the methods until more PRO_{IN} are studied at or near the calculated PRO requirements for both groups (see below). The advantage of combining the tracer data was that it provided

additional information on PRO turnover that was useful in the interpretation of NBAL data. For example, without the tracer data, one may conclude that the more positive NBAL measured on the high PRO_{IN} would increase lean body mass (net N retention). Therefore, the advantages of the combined approach to the determination of PRO requirements were greater than when either method was used in isolation.

4. What are the acute effects of resistance exercise on whole body protein turnover?

Acute, circuit-set, weight exercises did not increase WBPS, leucine oxidation, nor PRO breakdown (Ra end leu). In addition, for up to two hours post-exercise, there were no changes in these measured variables. The bicarbonate retention factor (*c*) increased during exercise, and decreased after exercise, and a failure to correct for *c* would result in a false increase in leucine oxidation during exercise and a false decrease in leucine oxidation after exercise. These erroneous changes in leucine oxidation would result in a bias towards and under-estimation of WBPS during, and an over-estimation of WBPS after, acute exercise.

5. What are the chronic effects of resistance exercise on whole body PRO turnover?

There is an increase in both WBPS and PRO breakdown (Ra end leu) in young men performing resistance exercise compared to sedentary young men. The body appears to be in a chronic state

of up-regulation or increased turnover consequent to resistance exercise that results in hypertrophy of skeletal muscle. This state of increased turnover has been demonstrated in animal models of hypertrophy (Laurent et al, 1978b; Laurent and Millward, 1980; Goldspink et al, 1982) and appears to be a conserved process involved in the hypertrophic process (Millward, 1984).

6.2 Future research

This research has added to the growing pool of knowledge on the PRO requirements of resistance athletes, however, there are a number of questions that have been raised in this thesis which warrant further investigation.

1. What is the time course of adaptation to resistance exercise with respect to PRO requirements and PRO turnover and what are the mechanisms involved ?

This would be best addressed using a longitudinal study where previously untrained subjects would start a resistance exercise programme and be followed for a period of one year. The subjects would have NBAL, lean body mass, strength, whole body leucine turnover, 3-MH excretion and MPS determined during the 1st, 3rd, 6th, and 12th month of the study while on a PRO_{IN} of 1.0, 1.3, and 1.6 g $PRO \cdot kg^{-1} \cdot d^{-1}$ given in a randomized, counter-balanced manner. The advantages of this study include: 1. repeated measures design increases the power of the study for inter-individual variance is eliminated; 2. the PRO_{IN} are close

together so it would be possible to get an objective point of inflection in the WBPS and leucine oxidation response curves to determine PRO requirements and the NBAL data would be interpolated from points much closer together (and to the calculated requirement) to derive a more accurate PRO requirement from regression analysis; 3. it would be possible to compare the response in MPS to that of WBPS to determine the contribution of muscle and non-muscle tissues to the response curves; and 4. it would be possible to measure the whole body and muscle PRO turnover changes across time to understand the mechanism of adaptation to training.

2. How does acute resistance exercise influence PRO turnover in specific tissues?

This could be studied by repeating the study presented in Chapter 4 with the following changes: 1. measuring the turnover of PRO synthesised by the liver (ie. albumin, fibronectin, fibrinogen, pre-albumin) using tracer incorporation techniques (Carraro et al, 1990a); 2. measuring MPS and muscle PRO breakdown in an active and an inactive muscle group using the av balance/ stable isotope method (Barrett et al, 1987; Devlin et al, 1990). We now have demonstrated that MPS is increased at four and 24 hours post-resistance exercise in young fed men (Chesley et al, 1991). This demonstrated the need for these studies for in Chapter 4 we did not observe an increase in WBPS by two hours post-exercise which probably indicated that MPS and PRO synthesis

in other tissues changed in opposite directions post-exercise.

3. How do nutrition and resistance exercise affect gene expression?

This study would require muscle biopsy sampling at various times post-exercise and under different dietary conditions and measuring the expression of DNA (Southern blotting), RNA (Northern blotting) and proteins (Western blotting). To first screen for potential proteins that may be up-regulated by resistance exercise muscle biopsy homogenates taken at several time points post-exercise should be subjected to polyacrylamide gel electrophoresis (PAGE) and stained to see which proteins appear to increase at different times post-exercise. It would be important to measure the contractile proteins (and their DNA and mRNA) actin and myosin and their regulatory proteins such as tropomyosin and troponin as well as structural proteins such as titin, actinin, spectrin, dystrophin etc. and finally enzymes such as cytochrome c oxidase, myosin ATPase, PFK, etc. In addition, the role of growth factors and the expression of protooncogenes in skeletal muscle after resistance exercise may help in the elucidation of the mechanisms involved in hypertrophy of skeletal muscle (Booth, 1990). The elucidation of even a few of these will require many experiments and these studies are in their infancy at the present time (Wong and Booth, 1990a,b).

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APPENDIX I.
TRACER METHODOLOGY

1. α-KIC ANALYSIS - (GC/MS METHODOLOGY)

A) Reliability.

i. Intra-test CV* -

sample (n)	mean	SD	CV
4	23.57	0.18	0.77
5	22.53	0.19	0.84

overall (weighted mean) = 0.81 %

ii. Inter-test CV -

sample pairs (n)	mean	SD	CV
10	23.21	0.032	0.14
4	22.5	0.16	0.72

overall (weighted mean) = 0.42 %

* CV = coefficient of variation, CV= standard deviation (SD)·mean $(x)^{-1}$.

B) Accuracy.

Theoretical ^{13}C abundance in *o*-TMS derivitive = 19.63

Baseline plasma α-KIC-*o*-TMS derivitive:

Study	Mean	SD	CV
Chapter 4 (n=6)	19.42	0.34	1.75
Chapter 5 (n=39)	19.34	0.43	2.22
Chesley et al, 1991* (n=12)	19.48	0.18	0.92
Total (n=57)	19.38	0.38	1.98

Baseline α-KIC-*o*-TMS ^{13}C measured enrichment is: 98.72 ± 1.98 % of the theoretical enrichment.

* I ran all of these samples.

C) Plateau in α-KIC Analysis:

By definition plateau has been defined as a CV < 10 % (Thompson et al, 1988) and a slope that is not significantly (P<0.05) greater than 0 (Hoerr et al, 1991).

i) Chapter 4. CV = 5.6 %

Source	SS	DF	MS	F	P
α-KIC	1.88	12	0.157	0.26	0.994
Error	36.78	60	0.613		
Total	38.66	72			

ii) Chapter 5. CV = 6.4 %

Source	SS	DF	MS	F	P
α-KIC	0.20	1	0.20	0.37	0.547
Error	38.465	71	0.54		
Total	38.664	72			

These data provided evidence that the PRO turnover data was obtained under conditions of isotopic plateau.

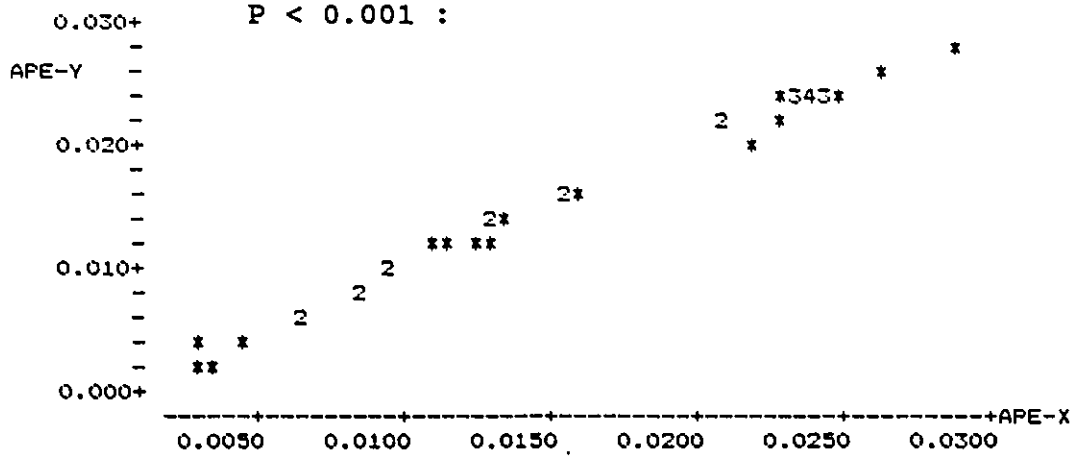
2. BREATH $^{13}CO_2/^{12}CO_2$ ANALYSIS - (IRMS METHODOLOGY)

A) Reliability-

i) Intra-test - correlation between sample pairs (n=38): (CV = 0.889 %)

sample 1= -0.000083 + 1.00 (sample 2)

r^2 (coefficient of determination) = 99.7 %, P < 0.001 :

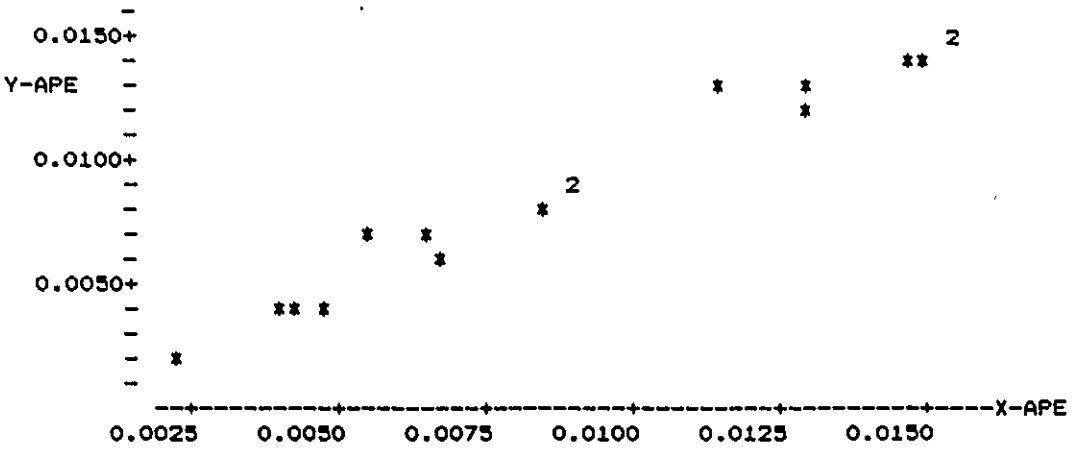


(APE-Y = sample 1; APE-X = sample 2.)

ii) Inter-test - correlation between sample pairs (n=18): (CV = 1.25 %)

sample 1= -0.000031 + 0.985 (sample 2)

r^2 = 98.5 %, P < 0.001:



B) Bicarbonate retention factor (c):

Determined in 4 subjects using a primed-continuous infusion of $NaHCO_3$ (99 % ^{13}C) with an 89:1 prime:continuous infusion ratio and calculated from the equation of Kien et al, 1989 ($c = VCO_2 \cdot (IECO_2 \cdot F^{-1})$)(see Chapter 2 for definitions).
The subjects performed the resistance exercise as described in Chapter 4.

Time Period	Mean c	SD	CV %
-30*	0.84	0.09	10.7
-15*	0.82	0.12	14.6
0*	0.82	0.13	15.8
A	1.72	0.06	3.5
B	1.45	0.39	26.9
C	1.16	0.21	18.1
+ 5min	0.64	0.08	12.5
+15min	0.53	0.04	7.5
+60min	0.53	0.10	18.8
+120min	0.80	0.10	12.5

* - indicates baseline values (pre-exercise)
mean (4 X 3) = .826 (SD = .10) CV = 12.6 %.

C) **Breath background enrichment changes:**

i) Exercise: (see Chapter 4 for details about exercise protocol and graph of data.
(data expressed as change from baseline (pre-exercise)):

Time Period	Mean change (APE)
Baseline	0.0000
A	+0.0003
B	-0.0012
C	-0.0009
+ 5min	-0.0004
+15min	-0.0003
+60min	+0.0023
+120min	+0.0028

(* - Note the greatest background change (+ 120 min) is still only 15.3 % of the breath enrichment APE during the leucine infusion).

ii) Diet changes:

These were studied in 2 subjects under a high PRO and high CHO diet treatment (Chapters 2 and 5) given in q 30 min aliquots:

High CHO diet-

Baseline → 0 (120 min) = +0.0015 APE
+0.0021 APE
mean = +0.0018 APE (SD = 0.00042)

Baseline → +90 min (210 min) = +0.0029 APE
+0.0025 APE
mean = +0.0027 APE (SD = 0.00028)

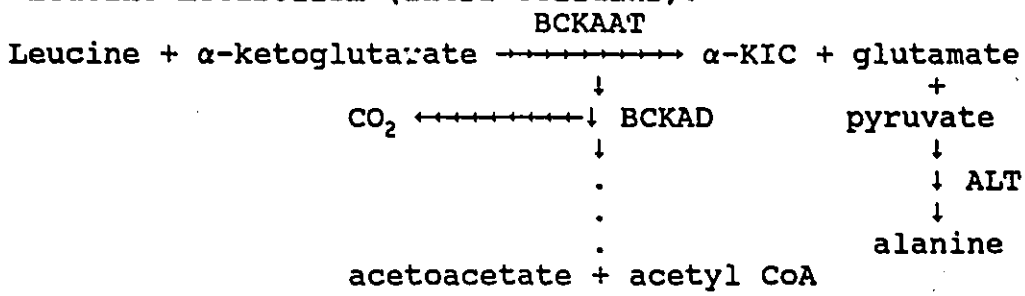
High PRO diet-

Baseline → 0 (120 min) = +0.0015 APE
+0.0018 APE
mean = +0.0016 APE (SD = 0.00021)

Baseline → +90 min (210 min) = +0.0023 APE
+0.0025 APE
mean = +0.0024 APE (SD = 0.00014)

(note - during the high CHO diet these changes accounted for a maximum of 15.8 % and during the high PRO diet for 9.0 % of the APE changes during the leucine infusion).

3. Leucine metabolism (intra-cellular).



BCKAAT - Branched chain keto-acid amino transferase.
BCKAD - Branched chain keto-acid dehydrogenase.
ALT - Alanine amino transferase.

APPENDIX II-
NBAL AND DIET ANALYSIS.

1. NBAL ANALYSIS (Kjeldahl N):

A. Reliability:

i) Intra-assay CV's:

Sample	Mean (mg/ml or mg/g)	SD	CV
Urine (n=11)	3.16	0.18	5.8
Feces (n=9)	64.02	2.43	3.8
Diet (n=22)	2.09	0.09	4.4
Sweat (n=14)	43.71 (mg/100 ml H_2O)	1.75	4.0

ii) Inter-assay CV's:

Sample	Mean (as above)	SD	CV
Urine (n=6 pairs)	3.13	0.034	1.1
Feces (n=5 pairs)	58.61	2.93	5.0
Diet (n=15 pairs)	1.87	0.17	9.2

2. Diet Analyses:

A representative diet for 5 diets in the study in Chapter 3 and 3 diets in Chapter 5 were prepared as duplicate samples. 20 % of the wet diet weight was added and homogenized with distillied water and lyophilized. The dry diet was ground further to homogenize it and the analyses of total N and energy (by bomb calorimetry) were performed on the dry diets.

To compare the N content of the diets as measured by Kjeldahl analysis to that from the corresponding diet record, the total PRO from the calculated (record) was divided by 6.25. To compare the calculated (record) analysis of total diet energy to the measured (bomb calorimetry) value the calculated values were multiplied by the factors 1.00, 1.03, and 1.43 (Merrill and Watt, 1973) to convert metabolizable energy to gross energy so the concordance between the measured and calculated energy could be compared.

i) Total N:

Diet	Calculated N* (gN/diet)	Measured N (gN/diet)	Ratio (M:C)
Chapter 3-1	6.11	5.21	0.85
2	4.93	3.88	0.79
3	3.64	3.89	1.07
4	7.62	7.26	0.95
5	10.54	9.33	0.89
		Total =	0.91
Chapter 5-A(LP)	9.76	10.64	1.09
B(MP)	16.80	17.72	1.055
c(HP)	25.41	25.37	0.998
		Total =	1.048

ii) Energy: C.V's: (n=3 each)

Chapter 3-1	3.1 %
2	5.9 %
3	2.6 %
4	2.8 %
5	0.6 %
mean =	3.0 %

Chapter 5-A	1.9 %
B	0.4 %
C	2.4 %
mean =	1.6 %

Diet	Calculated E* (kcal/diet)	Measured E (kcal/diet)	Ratio (M:C)
Chapter 3-1	1290	1214	0.94
2	1048	878	0.84
3	879	901	1.02
4	1640	1640	1.00
5	2316	2032	0.88
		Total =	0.94
Chapter 5-A(LP)	3169	3126	0.99
B(MP)	3238	3076	0.95
C(HP)	3422	3695	1.08
		Total =	1.00

* - The calculations in Chapter 3 were performed using the ANALYZE programme at McMaster University and for Chapter 5 using Nutritionist III (Silverton, OR).

APPENDIX III-
MISC. ANALYSES:

1. UREA (Urine):

(Sigma kit # 640, St. Louis, MO)
Colorimetric determination at 600 nm using phenol-urease reaction (urea is hydrolyzed to NH_3 by urease and NH_3 + phenol forms indophenol (blue color). Amount of urea is proportional to intensity of blue.

i) Intra-assay CV (n=22): mean = 13.74 mg/ml
SD = 0.55 "
CV = 4.0 %

ii) Inter-assay CV (n=12 pairs):
mean = 12.89 mg/ml
SD = 0.98 "
CV = 7.6 %

2. CREATININE (Urine):

(Sigma kit # 555, St. Louis, MO)
Colorimetric determination at 500 nm using picric acid Jaffe reaction (yellow color creatinine-picric acid is destroyed under acidic conditions and ↓ in color is proportional to [creatinine]).

i) Intra-assay CV (n=12): mean = 78 mg/dl
SD = 2.3 "
CV = 3.0 %

ii) Inter-assay CV (n=12 pairs):
mean = 83 mg/dl
SD = 8.2 "
CV = 9.9 %

3. Creatine phosphokinase (CPK):

(Sigma kit # 47-10, St. Louis, MO)
UV absorbance method at 340 nm using a NAD:NADH linked reaction where CPK activity is proportional to the amount of NAD → NADH in the reactions:
ADP + CP → C + ATP
ATP + glucose → ADP + glucose-6-P
glucose-6-P + NAD → 6-phosphoglucaonate + NADH.

i) Intra-assay CV (n=8): mean = 96 $U \cdot l^{-1}$
SD = 3.3 "
CV = 3.4 %

ii) Inter-assay CV (n=6): mean = 86 $U \cdot l^{-1}$
SD = 4.3 "
CV = 5.0 %

APPENDIX IV-
Chapter 3 DATA.

1. Regression analysis of NBAL vs PRO_{IN}:

$NBAL = -119 + 83.1(PRO_{IN})$:

Predictor	Coeff.	SD	T-ratio	P
Constant	-118.7	19.52	-6.08	0.000
PRO_{IN}	83.1	9.6	8.7	0.000

$s = 41.85$ $r = 0.88$ $r^2 = 0.77$ (77 %)

($NBAL = mgN \cdot kg^{-1} \cdot d^{-1}$; $PRO_{IN} = g \cdot kg^{-1} \cdot d^{-1}$)

Prediction:

zero NBAL = 1.43 $g \cdot kg^{-1} \cdot d^{-1}$.
+ 1 SD = 1.62 " .
+ 2 SD = 1.86 " .

2. Dietary adaptation:

Day to day urea excretion ($g \cdot d^{-1}$):

Diet treatment	Day 1	Day 2	Day 3	CV
CHO	12.55	11.11	11.67	6.2 %
PRO	19.26	20.89	20.14	4.1 %

Significance of slope (if significant there is a trend towards incomplete dietary adaptation):

CHO - P = 0.585 (NS)
PRO - P = 0.637 (NS)

Therefore, dietary adaptation has been acheived on both diet treatments.

C) **Misc. Data:**

Table IV-1. Voluntary strength.

	1 RM[a] Bench Press** (kg)			1 RM Squat** (kg)			Isometric Strength** (N.m)		
	Pre	Post	Change (%)	Pre	Post	Change (%)	Pre	Post	Change (%)
PRO	84.6	89.6	+5.9	123.7	146.4	+18.4	74.4	77.9	+4.7
	±5.7	±5.7		±9.4	±12.5		±3.8	±3.8	
CHO	83.7	91.5	+9.3	133.3	136.5	+2.4	72.4	77.4	+6.9
	±6.1	±6.2		±9.5	±8.6		±3.9	±3.7	

Table IV-2. Body weight, density, % fat, and biceps muscle N.

	Body Weight (kg)			Body Density (g/ml)		
	Pre	Post	Change (%)	Pre	Post	Change (%)
PRO	81.9	82.0	+0.1	1.0764	1.0780	+0.15
	±3.2	±3.2		±0.0047	±0.0043	
CHO	81.9	82.0	+0.1	1.0775	1.0768	-0.06
	±3.3	±3.3		±0.0045	±0.0043	

	Body Fat (%)			Biceps Nitrogen (g/100g dry wt)		
	Pre	Post	Change (%)	Pre	Post	Change (%)
PRO	10.4	9.8	-5.8	14.86	14.85	-0.1
	±1.8	±1.7		±0.12	±0.15	
CHO	10.0	10.3	+3.0	14.64	14.76	+0.8
	±1.8	±1.7		±0.13	±0.18	

values are means±SE
**indicates significant training effect ($P<0.05$)
[a]1 repetition maximum
PRO-Protein treatment
CHO-Carbohydrate treatment

Table IV-3. Neuromuscular Properties (arm flexors). (see Tarnopolsky et al, 1989).

i) % Motor unit activation (Belanger and McComas, 1981):

Variable	Mean	S.E. Mean	Std Dev	Range	Minimum	Maximum	N
MUAA1	87.48	2.66	9.22	28.80	70.9	97.7	12
MUAA2	89.48	1.88	6.44	19.80	77.4	97.2	12
MUAB1	88.13	1.94	6.72	22.70	75.8	98.5	12
MUAB2	92.56	1.88	6.51	21.90	76.5	98.4	12

ii) Maximal Voluntary contraction strength.

Variable	Mean	S.E. Mean	Std Dev	Range	Minimum	Maximum	N
MVCA1	74.41	3.82	13.24	47.20	59.9	107.1	12
MVCA2	77.94	4.03	13.96	49.60	64.1	113.7	12
MVCB1	72.43	3.88	13.45	51.70	53.7	105.4	12
MVCB2	77.43	3.74	12.96	46.50	59.7	106.2	12

iii) Post-tetanic twitch torque.

Variable	Mean	S.E. Mean	Std Dev	Range	Minimum	Maximum	N
PTTA1	12.56	.83	2.89	10.20	6.3	16.5	12
PTTA2	11.23	.66	2.30	7.00	7.6	14.6	12
PTTB1	12.14	.76	2.63	8.30	9.0	17.3	12
PTTB2	12.24	.60	2.07	7.20	8.8	15.8	12

iv) Peak evoked twitch torque.

Variable	Mean	S.E. Mean	Std Dev	Range	Minimum	Maximum	N
TWTA1	8.58	.57	1.97	7.30	5.6	12.9	12
TWTA2	7.50	.54	1.88	6.40	5.7	10.1	12
TWTB1	8.48	.48	1.66	5.70	6.2	11.9	12
TWTB2	8.10	.47	1.62	5.30	5.5	10.8	12

APPENDIX V-
CHAPTER 4 DATA.

1. ANOVA Tables of Protein Turnover Data (all were NS):

i) WBPS:

Source	SS	DF	MS	F	P
WBPS	15464	12	1289	0.62	0.818
Error	125089	60	2085		
Total	140553	72			

ii) Leucine oxidation:

Source	SS	DF	MS	F	P
LEU OXID	3372	12	281	0.31	0.986
Error	55047	60	917		
Total	58419	72			

iii) Protein breakdown (Ra end leu):

Source	SS	DF	MS	F	P
PRO BKD	8763	12	730	0.60	0.830
Error	72557	60	1209		
Total	81320	72			

iv) Total Flux:

Source	SS	DF	MS	F	P
FLUX	8872	12	739	0.52	0.892
Error	84918	60	1415		
Total	92790	72			

2. Correlation between *c* and RER.

$c = -2.75 + 3.87$ (RER)

Predictor	Coef.	SD	t-ratio	P
Constant	-2.75	0.59	-4.61	0.000
RER	3.87	0.62	6.2	0.000

$s = 0.1735$ $r = 0.91$ $r^2 = 0.83$ (83 %).

Therefore, it appears that *c* is strongly correlated to RER and probably relates to the fact that an increased flux of CO_2 (ie. exercise) through the body pools will increase *c* (by definition (Barstow et al, 1989) and RER will increase since it is equal to $(VCO_2 \cdot VO_2^{-1})$.

APPENDIX VI-
CHAPTER 5 DATA.

1. Regression analysis of NBAL vs PRO_{IN}:

i) Sedentary group (S):

$NBAL = -16.8 + 24.3\ (PRO_{IN})$

Predictor	Coeff.	SD	T-ratio	P
Constant	-16.8	9.18	-1.83	0.087
PRO_{IN}	24.3	5.47	4.44	0.000

$s = 14.2$ $r = 0.74$ $r^2 = 0.55$ (55 %)

ii) Resistance athletes (BB):

$NBAL = -71.6 + 50.9\ (PRO_{IN})$

Predictor	Coeff.	SD	T-ratio	P
Constant	-71.6	16.53	-4.33	0.000
PRO_{IN}	50.8	9.99	5.09	0.000

$s = 27.17$ $r = 58$ $r^2 = 0.58$ (58%)

(NBAL = $mgN \cdot kg^{-1} \cdot d^{-1}$; PRO_{IN} = $g \cdot kg^{-1} \cdot d^{-1}$)

Group	PRO_{IN}: zero NBAL	+ 1 SD	+ 2 SD
BB	1.41	1.76	2.33
S	0.69	0.89	1.26
	↑ 104 %	↑ 98 %	↑ 85 %

2. Regression of Oxidation and Urea excretion:

Urea = 1.96 + 0.211 (Oxidation), $r^2 = 0.932$ (93.2 %),
$r = 0.97$
$P < 0.0001$.

3. **ANOVA Tables of Protein Turnover Data:**

i) WBPS:

Source	SS	DF	MS	F	P
Between blocks/subjects					
Group	35390.6	1	35390.6	8.452	0.013
Error	46059.8	11	4187.3		
Within blocks/subjects					
Diet	10500.5	2	5250.2	12.977	<0.001
Groups*Diet	4473.8	2	2236.9	5.529	0.011
Error	8900.5	22	404.6		

Tukey post-hoc analysis: (Bruning and Kintz, 1977)
where, critical difference (c. diff.) =

$$qr \cdot \text{sq. rt.}[(MS_{\text{within group error}} \cdot N(\text{group})],$$

qr = Tukey constant,
MS = Mean Square.

a) Within group (all S were NS).
c. diff = 26.99 (P<0.05)
34.93 (P<0.01)

Condition	Difference	Significance
LP vs MP	45.95	<0.01
LP vs HP	64.75	<0.01
MP vs HP	18.80	NS

b) Between group (BB vs S).
c. diff = 28.06 (P<0.05)
36.33 (P<0.01)

Condition	Difference	Significance
LP	31.23	<0.05
MP	67.76	<0.01
HP	82.3	<0.01

ii) Leucine oxidation:

Source	SS	DF	MS	F	P
Between Blocks/Subjects					
Group	519.8	1	519.8	1.34	0.27
Error	4269.3	11	388.1		
Within blocks/Subjects					
Diet	21218.8	2	10609.4	47.91	<0.001
Group*Diet	308.3	2	154.1	0.70	
Error	4872.2	22	221.5		

Tukey post-hoc analysis:

c. diff (BB) = 19.9 (P<0.05); 25.8 (P<0.01).
(S) = 21.6 (P<0.05); 27.9 (P<0.01).

Condition		Difference	P
LP vs MP	(BB)	10.9	NS
"	(S)	18.3	NS
MP vs HP	(BB)	47.6	< 0.01
"	(S)	52.1	"
LP vs HP	(BB)	58.6	< 0.01
"	(S)	52.1	"

iii) Protein Breakdown:

Source	SS	DF	MS	F	P
Between blocks/subjects					
Group	43937.3	1	43937.3	11.9	0.005
Error	40481.2	11	3680.1		
Within blocks/subjects					
Diet	2259.0	2	1129.5	.81	
Group*Diet	3929.9	2	1964.9	1.4	0.27
Error	30880.3	22	1403.6		

(No interaction or within - thus, marginal mean data must be used - (BB > S)).

iv) Leucine Flux:

Source	SS	DF	MS	F	P
Between blocks/subjects					
Group	17885.2	1	17885.2	13.0	0.004
Error	15172.5	11	1379.3		
Within blocks/subjects					
Diet	40329.9	2	20164.9	39.5	<.001
Groups*Diet	2205.6	2	1102.8	2.2	0.137
Error	11229.5	22	510.4		

Overall BB > S (marginal mean- no interation)

Tukey post-hoc (within group comparisons):

c. diff - BB = 30.31 (P<0.05); 39.24 (P<0.01)
S = 32.74 (P<0.05); 42.38 (P<0.01)

Condition		Difference	P
LP vs MP	(BB)	38	<0.05
	(S)	23.5	NS
MP vs HP	(BB)	58.7	<0.01
	(S)	36.3	<0.05
LP vs HP	(BB)	96.7	<0.01
	(S)	60.1	<0.01

4. Dietary adaptation:

Day to day urea excretion ($g \cdot d^{-1}$):

Group	Diet	Day 1	Day 2	Day 3	CV	P	(sign.?)
BB	LP	9.66	9.42	9.91	2.5 %	0.66	(NS)
	MP	13.32	13.26	13.49	0.9 %	0.50	(NS)
	HP	21.11	21.72	23.21	4.9 %	0.15	(NS)
S	LP	8.85	10.75	9.44	10.0 %	0.80	(NS)
	MP	14.36	15.44	15.71	4.7 %	0.21	(NS)
	HP	22.96	21.10	23.20	5.1 %	0.96	(NS)

Therefore, dietary adaptation has been acheived on all diet treatments for both groups.