PROTEIN TURNOVER IN TRAINED MALE AND FEMALE ENDURANCE ATHLETES

PROTEIN TURNOVER IN TRAINED MALE AND FEMALE ENDURANCE ATHLETES

By

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Abstract

The dietary protein requirements of endurance trained athletes have been previously shown to be higher or no different than those of sedentary persons. However, the current Canadian Recommended Nutrient Intake (RNI) for protein contains no allowance for the effects of habitual physical activity. The discrepancy as to whether the protein requirements of active individuals are elevated, is probably due to: varying study designs, the different training status of the subjects, dissimilar exercise intensities, and the dietary condition of the subjects studied. In addition, previous work (Tarnopolsky, LJ *et al.* J. Appl. Physiol. 68(1):302-308, 1990), has shown that males may catabolize an increased amount of protein, as a result of endurance exercise, compared to females. The present study examined protein turnover in trained male (n=6) and female (n=6) endurance athletes (runners). Athletes were selected for equal training status and conditioning and were placed on a diet, isoenergetic with their habitual intake, containing protein at the Canadian RNI. All female athletes were tested during the mid-follicular phase of their menstrual cycle. After being adapted to the diet for 10 days during which the athletes exercised according to their habitual exercise schedule, each athlete completed a three day measurement of nitrogen balance.

Nitrogen balance showed that the RNI was inadequate for the female athletes (mean ± SE), nitrogen balance = -0.89 ± 0.33 g $N \cdot d^{-1}$ (-15.9 ± 6.0 mg $N \cdot kg^{-1} \cdot d^{-1}$), and for male athletes, nitrogen balance = -1.69 ± 0.64 g $N \cdot d^{-1}$ (-26.3 ± 11.0 mg $N \cdot kg^{-1} \cdot d^{-1}$). To examine the kinetics of leucine metabolism during exercise, each subject received a primed constant intravenous infusion of L-[1-^{13}C]leucine while resting for 2.0 h and then during a 90 minute treadmill run at aproximately 65% of $\dot{V}O_{2max}$. Blood samples were taken at steady state and analyzed for ^{13}C enrichment of α-ketoisocaproic acid and expired gas samples were analyzed for $^{13}CO_2$ enrichment. Corrections were made for changes in background $^{13}CO_2/^{12}CO_2$ and changes in bicarbonate retention factor, during exercise, were also determined. Measurements of whole-body leucine kinetics (flux, oxidation, and non-oxidative leucine disposal) were calculated using the reciprocal pool model. Exercise resulted in a significant increase (P<0.001) in leucine oxidation in both males and females. The increase was 95% above resting in females and 84% above resting in males. Male athletes oxidized a greater amount of leucine during the infusion than female athletes (P=0.004). Leucine flux increased significantly (P<0.001) during exercise in both groups of athletes. The non-oxidative portion of leucine flux did not change significantly throughout the infusion in either male or female subjects.

Oxidation of leucine could account for 88% of the negative nitrogen balance in the female athletes and 90% of the negative nitrogen balance in the male athletes. It is concluded that the Canadian RNI for protein is inadequate for those persons who continually engage in endurance activities. In addition, leucine oxidation during prolonged sub-maximal exercise is greater in males than females and the increase in amino acid oxidation could account for approximately 90% of the negative nitrogen balance observed in these groups of athletes. Future investigations of protein requirements for athletes should consider males and females as distinct groups.

Foreword

This thesis is presented in a form similar to that which would be submitted for publication in a scientific journal. This presentation format was approved in 1986 by the Graduate Council of McMaster University to encourage the development of a writing style suitable for career related writing.

The body of this thesis contains two main sections. Chapter 1 includes: a review of the literature on protein turnover as it relates to athletes; a discussion of the approaches used to determine protein requirements; a review of the methodologies used in this research; an examination of certain gender differences with respect to substrate utilization during endurance exercise. Also included in this chapter are the objectives and hypotheses of this research. Chapters 2 through 4 contain the thesis research presented in manuscript form. Appendices I - XV contain the raw data.

Before reading this thesis a point should be made regarding the use of the Canadian Recommended Nutrient Intake (RNI) for protein. When this study began in the spring of 1990 the RNI was established in Recommended Nutrient Intakes for Canadians (Canada Health and Welfare, 1983). This publication set the RNI for dietary protein for women 19 years and older at 0.74 g protein·kg body weight^{-1}·day^{-1}. Since one of the

objectives of the study was to examine the RNI for protein this value was used as the experimental dietary intake. Five of the twelve subjects were already tested by the time the 1990 version of the RNIs (Health and Welfare, 1990) was made available. The recommended protein intake given for women of 19 years and older established in this newer publication is 0.86 g protein·kg body $weight^{-1}\cdot day^{-1}$. This should not, however, change the conclusions regarding the Canadian RNI for protein since the female subjects in this study actually consumed a protein intake closer to 0.80 g protein·kg body $weight^{-1}\cdot day^{-1}$. All of the male subjects were tested subsequent to the publication of the 1990 version of the RNIs thus, the level of protein consumed by these athletes was 0.94 g protein·kg body $weight^{-1}\cdot day^{-1}$, which is 0.08 g protein·kg body $weight^{-1}\cdot day^{-1}$ greater than that recommended in this publication (Health and Welfare, 1990).

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List of Abreviations

a	artery
ANOVA	analysis of variance
APE	atom percent excess
ATP	adenosine triphosphate
B	protein beakdown
BCAA	branched chain amino acid
BCKAD	branched chain keto-acid dehydrogenase
c	bicarbonate retention factor
^{13}C	stable isotope of carbon (m.w.=13)
°C	degree celcius
CV	coefficient of variation
d	day
DF	degrees of freedom
δ %	delta per mil
EAA	essential amino acid
EE	energy expenditure
EI	electron impact
E_2	17-ß-estradiol
FP	folicular phase (of menstrual cycle)
GC/MS	gas chromatograph/mass spectrometer
I	protein intake
IRMS	isotope ratio mass spectrometer
α-KIC	alpha-keto isocaproic acid
kg	kilogram
LP	luteal phase (of menstrual cycle)
min	minute
mol	gram molecular weight
MPE	mole precent excess
m/z	mass/charge
3-MH	3-methylhistidine
MS	mean square
n	number of subjects
NS	not statistically significant ($p>0.05$)
O	amino acid oxidation
P_4	3-keto-4-diene-pregnenolone (progesterone)
Q	amino acid flux (turnover)
Ra	rate of appearance (of amino acid)
Rd	rate of disappearance (of amino acid)
RDA	recommended dietary allowance (U.S.)
RER	respiratory exchange ratio
RNI	recommended nutrient intake (Canada)
SS	sum of squares
S	protein synthesis
SE	standard error of the mean
v	vein
$\dot{V}CO_2$	volume of carbon dioxide consumed
$\dot{V}O_2$	volume of oxygen consumed

List of Abreviations (cont)

$\dot{V}O_{2max}$	maximal oxygen consumption
WBPS	whole body protein synthesis

Chapter 1

Introduction

1.1 Substrate use during endurance exercise.

Most evidence suggests that all three of the major macronutrients, carbohydrate, fat, and protein, serve as substrates for the production of ATP during endurance exercise. The relative contributions from each substrate during exercise is dependant upon exercise intensity and duration, as well as the state of training of the individual, and diet (Karlsson and Saltin, 1971; Gollnick, 1985; Åstrand and Rodahl, 1986). The major fuels for energy production during endurance exercise are fat and carbohydrate (di Prampero, 1981; Åstrand and Rodahl, 1986).

1.1.1 *Carbohydrate and fat.*

The energy expended by humans can be broken down into three components (Ravussin and Bogardus, 1989; Linder, 1985): resting metabolic rate (RMR); the thermic effect of food (TEF); and the energy cost of physical activity. Encompassed in the RMR are the processes required for rudimentary physiological function, that is the energy required to establish concentration gradients, the synthesis and degradation of cellular proteins, and sympathetic nervous system function, all of which are in a dynamic state and require energy (Ravussin and Bogardus, 1985; Waterlow, 1986). The TEF is the increase in RMR seen upon consumption of food,

which is considered to represent the energy that is mobilized to digest, absorb, distribute, and store the ingested nutrients (Linder, 1985). The energy cost of physical activity would constitute between 10 and 15% of a sedentary persons daily energy expenditure, whereas an elite endurance athlete's energy costs would be somewhere between 20 to 50% of their daily energy expenditure (Heinemann and Zerbes, 1989). Substrate use during the increased physical activity of athletes has been a topic of long term interest.

The intensity and duration of exercise determines the mix of carbohydrate and fat that are used as substrates. High intensity (>65% $\dot{V}O_{2max}$) exercise results in carbohydrate usage, either in the form of muscle glycogen or blood glucose, as the predominant fuel (Bergström et al, 1967; Hultman, 1989). Endurance exercise of lower intensities (and thus increasing length) result in fat, as either free fatty acids (FFA) or intra-muscular triglyceride, being the main energy source (Ahlborg et al, 1974; Hultman, 1989). The maximum work capacity that can be sustained deriving approximately 50% of the calories from fat is 50 to 55% of $\dot{V}O_{2max}$ in untrained subjects, but this increases to 65% after training due to an increased capacity to oxidize FFA as fuel (Holloszy and Coyle, 1984). The increased capacity for FFA oxidation is due (in the long term) to an increased oxidative enzyme capacity that

occurs with training (Åstrand and Rodahl, 1986; Holloszy and Coyle, 1984). As a result of the increased fatty acid oxidation an athlete can achieve an increased endurance capacity due to the "sparing" of carbohydrate (Hultman, 1989; Coggan et al, 1990; Green, et al, 1991). Preservation of carbohydrate as glycogen stores is important, since muscle glycogen concentration is a limiting factor in endurance capacity at moderate intensities of $\dot{V}O_{2max}$ (65-85%; Hultman, 1989). Diet can also markedly affect endurance performance. A diet high in carbohydrate will increase muscle glycogen and use of blood borne glucose during endurance exercise (Bergström et al, 1967), which can cause a significant improvement in endurance performance (Sherman et al, 1981).

While it acknowledged that carbohydrate and fat are the predominant fuels during endurance exercise, increasing evidence suggests that protein too plays a role in energy producing pathways during exercise (Lemon, 1987). The use of protein during endurance exercise is not thought to account for a significant fraction of exercise energy expenditure (Hood and Terjung, 1987; Lemon, 1987; Paul, 1989). Even though protein may only contribute as little as 1-2% (Hood and Terjung, 1987) to the energy cost of exercise, it is believed by some investigators that the protein requirements of endurance trained athletes are greater than those of sedentary

individuals (Tarnopolsky et al, 1988; Friedman and Lemon, 1989; Meredith et al, 1989).

1.1.2 *Protein use during endurance exercise.*

Views on the use of protein during endurance exercise and consequently the protein requirements of athletes have changed throughout history. In 460 BC, Dromeus of Stymphalus, a trainer and champion of the long footrace at Olympia endorsed a diet rich in meat for his athletes (Evans et al, 1983). The opinion that protein was important for endurance activities persisted through the mid 19th century when von Liebig (1842) proposed that protein was the primary fuel for working muscle. Subsequently, it was shown by other investigators (Fick and Wislicenus, 1865; Cathcart, 1925), that post exercise urinary urea (considered the major metabolite of protein catabolism) excretion was only slightly elevated. Cathcart (1925) states, "...muscle activity does increase, if only in a small degree, the metabolism of protein." Thus, the conclusion was reached that protein does not contribute to the energy cost of exercise to any significant extent. Based on the results of several experimental procedures other investigators have unaminously concluded that protein contributes only a small extent to the energy cost of endurance activity (Dohm et al, 1977; Rennie et al, 1981; Lemon and Mullin, 1980; Lemon, 1987; Henderson

et al, 1985; Hood and Terjung, 1985). It is important to realize, however, that even a small percentage of a high exercise energy expenditure could produce a significant increase in protein use (Lemon, 1987).

1.2 **Approaches to studying protein metabolism.**

Investigations of protein metabolism have used a variety of techniques to establish the utilization of protein during exercise. A review of the common approaches used to study protein metabolism is given below.

1.2.1 *Urea excretion.*

All urea formed in humans is synthesized in the liver, since only this organ has the necessary enzymes to convert toxic ammonia to urea (Guyton, 1986). Studies employing urea excretion to study protein metabolism do so because the urine is the primary site of protein excretion (approximately 70-80% of all protein under normal conditions; Rand et al, 1976) and urea forms the vast majority of nitrogen (N) in the urine (Lemon, 1987). Urinary urea is also a sensitive indicator of protein intake, responding to changing protein intakes over a few days (Tarnopolsky et al, 1988; Rand et al, 1976). Urea formation takes place in the liver mitochondria, where the N removed from amino acids combines with CO_2 to form carbamoyl phosphate, which is the source of one the two N groups utilized in the cycle, while the second N group comes from

Aspartate (Stryer, 1988). The formation of urea results in the consumption of four ATP making the process essentially irreversible.

Energy balance can also alter the excretion of urinary urea. Surfeit energy above requirement will result in a decreased urinary urea excretion, but the converse is also true: a deficit in energy below requirement results in an increased urinary urea N excretion (Todd et al, 1984; Göranzon and Forsum, 1985; Hoffer and Forse, 1990). Whether the energy excess or deficit is created by feeding or activity (or fasting or inactivity) seems not to be important to the effects described above. The proportion of food stuffs ingested can also impact on urinary urea excretion. Richardson and colleagues (1979) showed that an isoenergetic exchange of fat for carbohydrate resulted in a significant decrease in urinary urea excretion and a significantly lower fasting serum urea. Lemon and Mullin (1980) showed that employing a carbohydrate loading protocol to increase muscle glycogen concentration (Åstrand and Rodahl, 1986) resulted in a significantly lower sweat and urinary urea excretion than during the non-carbohydrate loaded state.

An important aspect of using urea excretion to study protein metabolism, during exercise, is the large quantity of N (primarily as urea) excreted in sweat during exercise

(Butterfield and Calloway, 1984; Lemon and Mulin, 1980; Lemon et al, 1986; Lemon, 1987; Tarnopolsky et al, 1988). Failure to account for this loss can underestimate the contribution of protein to exercise. Sweat urea is likely elevated during exercise due to the reduction in blood flow to the kidney that accompanies exercise (Poortmans, 1984). Shifts in blood flow and dehydration associated with exercise reduce post-exercise urine volume and urinary urea production would also be decreased. Additionally, the slow turnover of the urea pool would result in post-exercise urinary urea not being reflective of exercise urea production (Waterlow et al, 1978; Lemon and Mullin, 1980; Lemon, 1987). It is also possible that urea production is suppressed during exercise (Wolfe, 1982). Dolan and Lemon (1987) have shown that the state of hydration can be important in determining protein utilization as result of urea excretion.

Urinary urea gives a good estimate of protein metabolism and the procedure is fairly convenient for both subjects and experimenters. Since urea is only a fraction (70%) of the total N excreted, its quantification will underestimate total N losses.

1.2.2 *Nitrogen balance.*

Nitrogen balance (NBAL) is based on the premise that the unique dietary source of N is protein. Nitrogen balance involves the quantification of all sources of protein (on average protein is 16% N; FAO/WHO/UNU, 1985) intake (diet) and excretion (urine, feces, and sweat). The overall balance is determined as shown in equation 1, below:

NBAL = N in - [N urine + N feces + N sweat] (equation 1)

A net positive balance indicates a state of net protein accretion or an increase in lean body mass (growth or an expansion of protein pools), while a net negative balance indicates protein depletion usually seen as a decreased lean body mass (60-70% of the body's protein) in the long term (Waterlow, 1986; Young, 1987).

Balance studies are rigorous but difficult to conduct due to the completeness of the collections needed to obtain accurate estimates of protein utilization (Young, 1987; Lemon, 1987; Bier, 1989). In addition nitrogen balance studies are subject to the same need for control of energy intake and nutrient proportions as are urea excretion studies (see section 1.2.1; Richardson et al, 1979; Todd et al, 1984; Göranzon and Forsum, 1985; Chiang and Huang, 1988). The quantification of sweat N especially with exercise treatment is also of importance for accurate nitrogen balance (Lemon, 1987). Failure to include estimates of sweat loss has resulted

in earlier studies reporting a falsely positive nitrogen balance (Cathcart, 1925, cited in Lemon, 1987). Collection of sweat N using different procedures can result in a wide variation in the estimates of sweat N losses during exercise (Costa et al, 1969; Consolazio et al, 1975; Lemon et al, 1986), although the whole body washdown procedure of Lemon (1986) appears to be the most accurate and reproducible.

Although nitrogen balance has provided the great bulk of information on whole body protein metabolism the procedure is not without problems, most of which have been recognized (Hegsted, 1975; Young, 1986; Young, 1987). An analysis of nitrogen balance studies that have been performed, lead Hegsted (1975) to the conclusion that nitrogen balance has a systematic error which results in the continuous overestimation of balance. This is most likely a methodological error resulting from the routine underestimation of N losses and overestimation of N intake, thus, falsely increasing balance estimates (Bier, 1989). Although these errors may be small since balance represents the small difference between a large intake and output, the relatively small errors in measurement of intake and output (in opposing directions) can produce large errors in balance (Kopple, 1987).

The response to altered intakes of protein also represents a problem in balance studies, since choice of the point at which balance will be studied can affect the outcome (Bier, 1989). This is due to the time course of the changes in protein metabolism following increased or decreased intakes of N. The "lag" in response of protein metabolism to altered protein intakes has to be accounted for in the interpretation of nitrogen balance studies (Rand et al, 1989; Bier, 1989).

One of the most perplexing problems with nitrogen balance is the large positive balances seen in non-growing adults with stable body weight and composition (Tarnopolsky et al, 1988; Tarnopolsky, 1991a). Positive nitrogen balances most likely reflect the systematic intake and output errors mentioned above. Young (1986) and Waterlow (1985) have argued that "balance" per se may not be a desirable state, in other words the mechanisms to achieve balance may not be benefiting the individual and even though they are in balance they have had to undergo a physiological compromise to achieve that state. This situation raises the concepts of *Adaptation* and *Accommodation*. Adaptation as it applies to protein metabolism is observed when the protein intake of an individual is decreased and that individual responds such that they retain all biochemical and physiological indices in an "acceptable" or normal range. Accommodation may occur if the protein intake

of the above individual were lowered beyond adaptable levels. At this protein intake all physiological functions could not be maintained without compromising some function such as protein synthesis, which would be decreased to prevent a state of net protein loss (Waterlow, 1985; Waterlow, 1986). Balance studies cannot distinguish states of adaptation and accomodation, such that a person consuming a sub optimal protein intake may be in positive nitrogen balance but at the same time be experiencing a decreased rate of whole body protein synthesis to achieve a "balanced" state.

Beaton (1986) proposed a framework to describe the concepts of adaptation and accomadation, which has been subsequently modified by Young (1987). The framework describes protein homeostasis as part of a continuum, where a state of adequate protein intake results in a well nourished state with ample "reserve." Excess intake, on the other hand, results in a nutrient overload (described as nutriture level +1). A reduction in protein intake results in progressive decreases in protein reserve (nutriture levels -1, -2,..., -n), until a clinical nutrient deficiency results, where obvious loss of protein has occurred (i.e. kwashiorkor). The various states of protein nutriture described by Young (1987) cannot be distinguished using nitrogen balance. Since adaptation will result in all persons being in balance (Young, 1987) despite

having to compromise some physiological process to achieve equilibrium (Waterlow, 1986). The accurate quantitation of the processes (e.g. decreased catabolism or synthesis) to achieve balance cannot be made using nitrogen balance (Bier, 1989; Young, 1987). Balance studies represent a "black box" in which something is fed in one side and appears transformed at the other (Bier, 1989).

Given the limitations and problems associated with nitrogen balance, the procedure has yielded information which has been used as a starting point for subsequent studies (Young, 1987). The nitrogen balance method is used in this thesis primarily for two reasons: despite the shortcomings it remains one of the most accepted methods for evaluation of protein turnover (FAO/WHO/UNU, 1985; Pellet, 1990); and the method is also used by both international (FAO/WHO/UNU, 1985) and national (U.S., Food and Nutrition Board, 1989; Canadian, Health and Welfare, 1990) committees to set dietary guidelines.

1.2.3 *N^{τ}-Methylhistidine excretion.*

The breakdown of skeletal myofibrillar proteins results in the formation of $N\tau$-methylhistidine (3-methylhistidine or 3-MH). The source of 3-MH remains controversial and estimates range from 75 and 90% from the skeletal muscle (Young and Munro, 1978; Rennie and Millward,

1983; Lemon, 1987) with the remainder being derived from rapidly turning over pools in skin and intestine (Rennie and Millward, 1983). Once formed, however, the amino acid cannot be reutilized and is excreted virtually completely (99-100%) in the urine (Young and Munro, 1978). When a meat free diet (essentially 3-MH free) is utilized the amount of 3-MH excreted in the urine will reflect skeletal muscle protein breakdown (Young and Munro, 1978). Urinary 3-MH has been used by many investigators as an index of muscle protein breakdown during endurance activities (Rennie et al, 1981; Dohm et al, 1982; Calles-Escandon et al, 1984; Plante and Houston, 1984; Dohm et al, 1985). The results of such studies have been contradictory, however, with some showing an increase (Dohm et al, 1982; Dohm et al, 1985), or no change (Rennie et al, 1981; Calles-Escandon et al, 1984; Plante and Houston, 1984) in 3-MH excretion. The variability of these results could be due to the variation in the collection time of post-exercise urine which can affect the pattern of 3-MH excretion (Dohm et al, 1985). Also Rennie and Millward (1983) have argued that skin and the musculature of the gastrointestinal tract can contribute a significant amount of 3-MH to the excreted pool and thus, 3-MH is an unreliable marker of muscle protein turnover. The use of 3-MH does, however, provide a useful, non-invasive measure of protein bound 3-MH regardless of its

origin (Plante and Houston, 1984; Sjolin et al, 1989).

1.2.4 *Tracer methodologies.*

Tracer methodologies to study metabolism involve the administration of labelled, distinguishable tracer to determine the kinetics or metabolism of an endogenous tracee. Probes to study amino acid and protein metabolism have used either radioactive (^{14}C or ^{35}S) or stable (^{13}C or ^{15}N) isotope labelled amino acids (Wolfe, 1984a; Picou and Taylor-Roberts, 1969). Measurement of specific activity or enrichment of the tracer, in accessible body compartments (blood, urine, air or muscle), allows the calculation of fluxes of the endogenous tracee (Wolfe, 1984a; Rennie, 1984). A more detailed discussion of tracer methodologies will be presented in section 1.3 (see below).

1.3 **Tracer methodology.**

The availability of stable isotopes and the instruments with which to measure them has meant that pathways once studied using radioactive tracers can now be studied using stable isotope probes. The advantage of using stable isotope probes is that they present no health risks, that accompany exposure to radioactive sources (Wolfe, 1984a; Rennie, 1984), and can be used in a situation where a radioactive label would be unacceptable (such as with a pregnant mother or a young infant; Wolfe, 1984a; Rennie,

1984).

1.3.1 *Isotope terminology.*

Stable isotopes are isotopes of the same element with a different number of neutrons, which do not spontaneously decay and so, on a global scale, form an invariant fraction of the elements abundance. For example, the stable isotope of hydrogen, deuterium, makes up 0.015% of all hydrogen (Wolfe, 1984; Thompson et al, 1985). Carbon also has a stable isotope, as ^{13}C, which forms 1.11% of all carbon (Wolfe, 1984a; Thompson et al, 1985; Schoeller et al, 1980). This naturally occurring background of ^{13}C complicates studies involving the use of ^{13}C, since the background enrichment must be accounted for (Thompson et al, 1985; Pérronnet et al, 1990). Compared to the background counts of radioactivity which are subtracted from each measurement of radioactivity (of which there is considered to be almost zero natural abundance in the case of ^{14}C; Wolfe, 1984a), one can appreciate what an abundance in the order of 1.11% can mean in terms of a background level (Wolfe, 1984a).

Radioactive tracers are measured using (in the case of ß-emitters) a scintillation counter, their activity is usually expressed as counts or disintegrations per minute (cpm or dpm). Stable isotopes are measured using mass spectroscopy (for details see section 1.3.3) and their activity is

expressed as atom or mole percent excess (APE or MPE), or delta per mil ($\delta^{13}C$ ‰) depending on the sample type (Wolfe, 1984a; see 1.3.4 for more details). Stable isotopes are expressed relative to another isotope as a ratio, in the case of carbon the ratio is $^{13}C/^{12}C$. By subtracting the enrichment of a baseline sample from the sample enriched (above baseline) with ^{13}C, the excess (of ^{13}C), APE, or MPE in the sample is obtained. The use of APE or MPE makes the terminology practically interchangeable with radio-tracer terminology (Wolfe, 1984a). For example, a sample which contains 10,000 cpm is twice as radioactive as a sample with 5,000 cpm. Similarly, a sample which is enriched to 10 APE is twice as enriched with isotope as a sample with 5 APE.

1.3.2 *Model of protein turnover.*

Whole body protein turnover have been measured for 150 years using the nitrogen balance technique (Bier, 1989). The problems with this technique (see section 1.2.2 for discussion) have stimulated researchers to use new techniques to examine whole body protein turnover (Young, 1989). Whole body protein turnover is the overall rate at which protein is synthesized or catabolized in the body. The rate is the sum of all individual proteins over the range from the fastest to the slowest (Waterlow et al, 1978). One of the most widely used procedures to measure whole body protein turnover is the

primed-continuous infusion of an amino acid (commonly either L-[1-^{13}C] leucine or L-[α-^{15}N or 1-^{13}C] lysine). The technique, described by Matthews and colleagues (1980), involves the intravenous administration of the labelled amino acid until an isotopic plateau or "steady state" is achieved. An isotopic steady state occurs when the rate of appearance (Ra) into the sampled pool is equal to the rate of disappearance (Rd) from the sampled pool (Golden and Waterlow, 1977; Matthews et al, 1980; Wolfe, 1984a). This means that an isotopic steady state can be established in the absence of a physiological steady state, since the only criterion for an isotopic steady state is that the enrichment of the sampled pool is constant over time (Wolfe, 1984a). The primed constant infusion has been used to measure whole body protein kinetics in a variety of conditions, in burned victims and patients with sepsis (Wolfe et al, 1989), and also during exercise (Rennie et al, 1981; Hagg et al, 1982; Millward et al, 1982; Wolfe et al, 1982; Wolfe, et al, 1984b; Evans et al, 1983; Knapick et al, 1991; Tarnopolsky et al, 1991b).

The model used to measure whole body protein turnover, in this thesis, is the single pool model described by Waterlow *et al* (1978). This model is based on a stochastic analysis, dealing only with the overall in and out of the system and does not include separate compartments or pools (Waterlow et

al, 1978; Wolfe, 1984a). The advantage of using this type of analysis is that it requires sampling of only a limited number of pools in the body, which is an advantage when dealing with human subjects since access to many compartments is obviously restrictive (Waterlow et al, 1978).

If delivery of a tracer substrate results in the excretion of a labelled metabolite then the disposal rate of the label can be determined (Waterlow et al, 1978). For instance, ingestion of ^{15}N glycine results in the excretion of the ^{15}N label in urine as ^{15}N labelled urea or ammonia (Picou and Taylor-Roberts, 1969; Waterlow et al, 1978). When steady state or plateau exists the dilution of the isotope tracer can be measured and the rate of entry or appearance of the endogenous tracee into the sampled pool can be calculated this is referred to as the Ra or total flux (Matthews et al, 1980; Wolfe, 1984a). Using the basic principles outlined above the model of whole body protein turnover generates the relationship given in equation 2, below and shown schematically in Figure 1a (Waterlow et al, 1978; Matthews et al, 1980):

$$Q = \underbrace{S + C}_{A} = \underbrace{B + I}_{B} \quad \text{(equation 2)}$$

A - rate of disappearance from the sampled pool (Rd)

B - rate of appearance into the sampled pool (Ra)

where, Q is equal to the flux (turnover of the tracer); S is equal to the rate of incorporation of tracer into proteins (synthesis or non-oxidative leucine disposal, NOLD; see below); C is the rate of catabolism (or oxidation, O) of the tracer; B is the rate of breakdown of endogenous proteins (also referred to as the Ra of endogenous leucine); and I is the rate of exogenous protein intake. If L-[1-^{13}C] leucine is the tracer then the rate of catabolism, C, is expressed as oxidation, O, of the tracer and is measured by the excretion of $^{13}CO_2$ in expired breath. Since the flux through the pool can be calculated by tracer dilution (see above), at isotopic plateau (Ra=Rd) the rate of synthesis can be calculated by simply subtracting C from Q (Waterlow et al, 1978; Wolfe, 1984; Matthews et al, 1980). The rate of synthesis is only an approximation, however, and is more commonly being referred to as the non-oxidative disposal (NOLD) of leucine flux (Tarnopolsky, 1991a). In the absence of any intake (either dietary or via infusion) of protein or amino acids then Q simply equals B, the breakdown of endogenous protein.

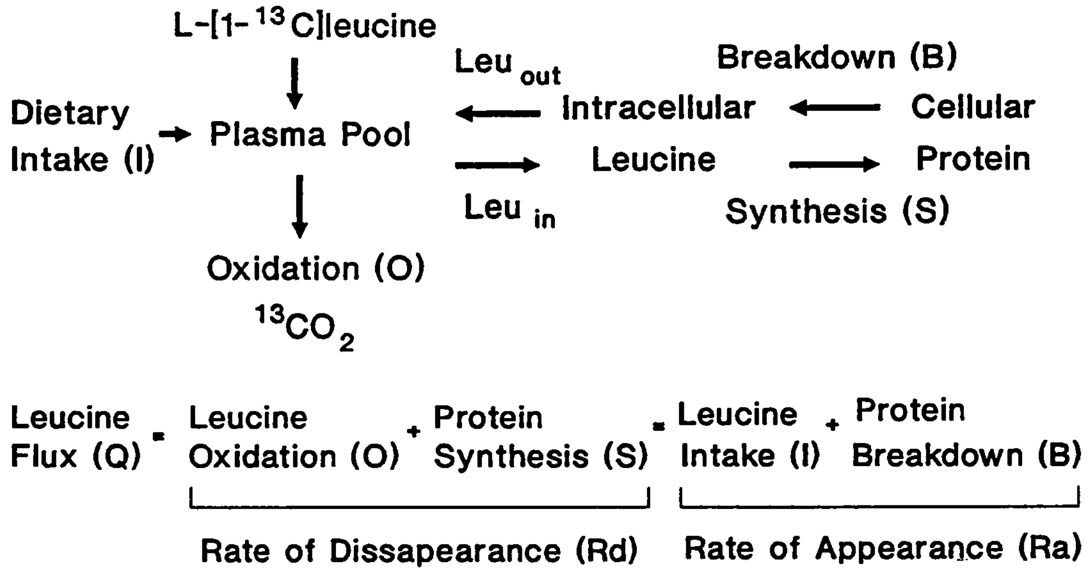
The calculation of leucine flux (Q) is measured from the dilution of the tracer at isotopic plateau according to the formula given below in equation 3 (Matthews et al, 1980):

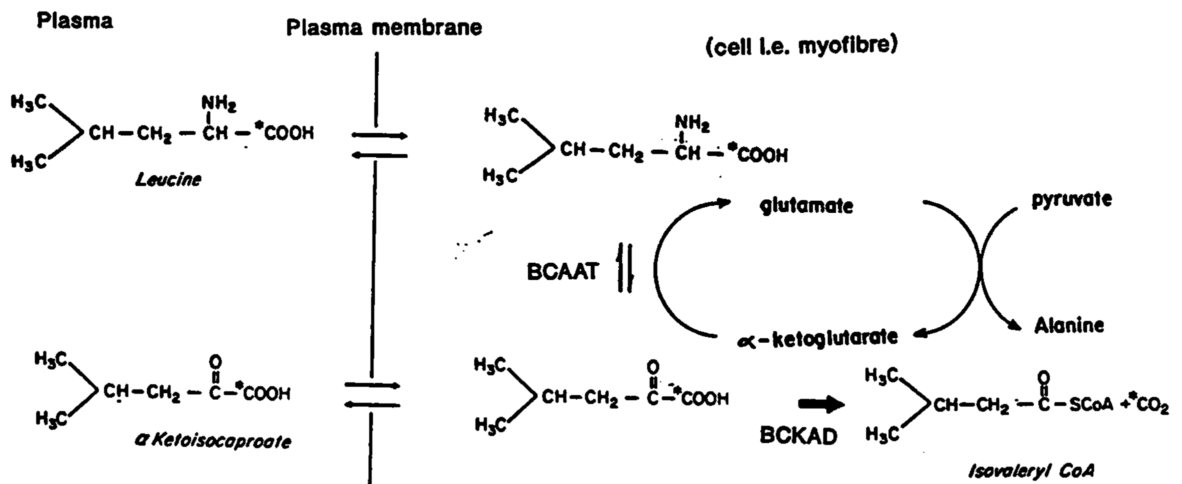
$$Q = i \cdot [E_i/E_p - 1] \text{ (equation 3)}$$

where, i is the infusion rate of the tracer (1-[1-^{13}C] leucine)

Figure 1a. Model of whole body leucine kinetics (Waterlow, 1978).

Figure 1b. Intracellular leucine metabolism. BCAAT - branched chain amino acid transferase, BCKAD - branched chain keto-acid dehydrogenase, * - ^{13}C label. Adapted from Wolfe (1984a) and Goldberg and Chang (1978).


A
L-[1-13C]leucine
Dietary Intake (I)
Plasma Pool
Oxidation (O)
13CO2
Leu out
Leu in
Intracellular Leucine
Breakdown (B)
Cellular Protein
Synthesis (S)
Leucine Flux (Q) = Leucine Oxidation (O) + Protein Synthesis (S) = Leucine Intake (I) + Protein Breakdown (B)
Rate of Dissapearance (Rd)
Rate of Appearance (Ra)


B
Plasma
Plasma membrane
(cell i.e. myofibre)
Leucine
BCAAT
glutamate
pyruvate
α-ketoglutarate
Alanine
α Ketoisocaproate
BCKAD
Isovaleryl CoA

in $\mu mol \cdot kg^{-1} \cdot h^{-1}$; E_i is the enrichment of the infused leucine in APE; E_p is the enrichment of the sampled plasma pool (either $[^{13}C]$ leucine or $[^{13}C]$ alpha-keto isocaproic acid or α-KIC; see 1.3.3); and the term - 1 corrects for the contribution of tracer infusion to flux. All parameters of equation 2 will be expressed as micromoles of leucine per kilogram per hour ($\mu mol \cdot kg^{-1} \cdot h^{-1}$), throughout this thesis unless otherwise specified.

It is recognized that the single pool model represents an over-simplification of the complicated process of protein turnover (Waterlow et al, 1978; Bier, 1989). The model has been validated to some extent using experimental techniques, however, more complicated (multi-compartment) analysis at this time is only possible theoretically (Bier, 1989). The basic assumptions of the model are discussed below.

1.3.3 *Assumptions of the model.*

To apply any of the equations to calculate protein turnover using the single pool model an isotopic plateau in the sampled pools must be achieved (Wolfe, 1984a). This has been defined as a regression line joining the enrichment values over time as having a slope not significantly ($p>0.05$) different from zero (Hoerr et al, 1991) or a pooled coeficient of variation of less than 10% (Thompson et al, 1988). It was determined previously that a plateau in both breath and plasma

pools occurs only 90 minutes after isotope infusion begins (Tarnopolsky, 1991b).

While it is not an assumption, the choice to use L-[1-^{13}C] leucine as an amino acid tracer is an important one for several reasons. Leucine is one of the nine essential amino acids (EAA), that cannot be synthesized by humans (FAO/WHO/UNU, 1985; Linder, 1985). Thus, dilution of the tracer can only arise from endogenous protein and not by *de novo* synthesis. Leucine is also interesting from the standpoint of its metabolism since it is oxidized, to some degree, in muscle whereas most other amino acids are oxidized in the liver (Hutson et al, 1978; Schneible et al, 1981; Harper et al, 1984). Leucine is also one of the principal sources of the α-amino N of gluconeogenic amino acids released from muscle (Felig and Wahren, 1971; Odessey et al, 1974; Goldberg and Chang, 1978; Paul, 1989; Hood and Terjung, 1990).

Other assumptions of the model are outlined below. For the model of protein turnover (Waterlow et al, 1978) to be valid all of these assumptions should be met.

1) The tracer enrichment of the sampled pool is representative of the tracer's intracellular enrichment. Figure 1b shows how leucine is metabolized, in muscle, at the cellular level. Since leucine is the labelled tracer many investigators have used the plasma leucine enrichment to

estimate intracellular leucine enrichment (Matthews et al, 1980; Wolfe et al, 1982; Evans et al, 1983; Wolfe et al, 1984b). However, Wolfe *et al* (1982) have argued that the plasma enrichment of leucine leads to an underestimation of flux (especially during exercise) and that plasma leucine enrichment does not reflect the intracellular enrichment of the labelled amino acid. As Figure 1b shows the intracellular transamination product of leucine is α-KIC (α-keto isocaproic acid; IUPAC, 4-methyl-2-oxo-pentanoate). The plasma enrichment of α-KIC during a primed constant infusion of ^{13}C leucine has been shown to be more representative of the intracellular leucine enrichment than plasma leucine (Wolfe et al, 1982; Matthews et al, 1982; Rennie, 1984; Schwenk et al, 1985a; Vazquez et al, 1986; Thompson et al, 1988). The use of plasma α-KIC enrichments to predict intracellular leucine enrichments in solving the equations for protein turnover is known as the reciprocal pool approach (Schwenk et al, 1985a). The conversion of leucine to α-KIC is not rate limiting and is readily reversible in non-hepatic tissues (Harper et al, 1984; Thompson et al, 1988), resulting in the rapid conversion of leucine to α-KIC during infusion of leucine. Thompson *et al.* (1988) gave a bolus dose of L-[1-^{13}C, ^{15}N] leucine to post-absorptive subjects and by sampling venous plasma they showed the rapid disappearance of the original tracer and rapid (peak

at 6 minutes) appearance of [^{13}C] α-KIC and subsequent appearance (peak at 10 minutes) of [^{13}C] leucine. The results of this experiment demonstrate how rapid and reversible the transamination reaction is. The relationship between plasma α-KIC and intracellular α-KIC has been shown to be unity with respect to both muscle in rats, whereas ratios of plasma leucine to intracellular leucine concentrations were consistently less than one (Vazquez et al, 1986). Consequently, it appears that intracellular α-KIC is in rapid equilibrium across the cellular plasma membrane. The use of plasma α-KIC to estimate intracellular leucine concentration is also thought to better reflect the enrichment of the immediate precursor for the irreversible decarboxylation and subsequent release of the label (Wolfe et al, 1982; figure 1). The use of plasma α-KIC enrichment to predict the intracellular leucine enrichment appears to be the most appropriate method for assessing whole body protein turnover. Therefore, α-KIC enrichments have been used in this thesis. The plasma α-KIC enrichment is used in the calculation of plasma leucine enrichment ($E_{p;}$ see equation 3 in section 1.3.2).

2) The tracer is rapidly and completely mixed within the sampled pool. The validity of this assumption has already been shown. The rapid appearance of both [^{13}C]α-KIC and [^{13}C]

leucine following a bolus dose of L-[1-^{13}C,^{15}N]leucine showed that the peak time of appearance for [^{13}C]α-KIC was only six minutes (Thompson et al, 1988).

The other consideration is the sample site which gives a representative enrichment (or concentration) of the infused tracer (Layman and Wolfe, 1987; Pell et al, 1983). The procedure used for the infusion of isotope in this research is outlined in chapter 2 (see section 2.2.2 for details of the infusion procedure). The tracer was infused via a forearm vein and sampled from the contralateral "arterialized" hand vein (Abrumand et al, 1981). This approach of infusion and sampling is known as the va (vein, artery) method. Layman and Wolfe (1987) reported that the va method of plasma sampling, during a L-[1-^{13}C]leucine infusion, resulted in arterialized venous plasma α-KIC enrichment being more reflective of tissue leucine enrichment than that of leucine. In addition, α-KIC enrichment was insensitive to sampling site (Layman and Wolfe, 1987).

3) Over the time course of the experiment there is no recycling of the isotope tracer. One of the fates of an infused tracer such as L-[1-^{13}C]leucine is that it can be incorporated into protein (used in synthesis, see equation 2, 1.3.2). If during the same infusion the same tracer molecule were to re-enter the free amino acid pool this would

cause flux (dilution) to be underestimated (Wolfe et al, 1984a; Schwenk et al, 1985b). The degree of recycling is directly dependent upon the length of the protocol (Schwenk et al, 1985b), although even during a short protocol there will be some degree of amino acid recycling due to the incorporation of isotope into rapidly turning over visceral proteins (Carraro et al, 1990a). The degree of recycling at any given time has not been determined, but it appears that protocols of short duration such as the one described in this thesis (3.75 hours) should not be affected to a significant degree by recycling.

4) There is no difference in the treatment of the isotope label as opposed to the most abundant isotope. The use of a labelled tracer requires that it is not discriminated from the unlabelled tracee and that it will trace the movement of the endogenous molecule (Wolfe, 1984a). Some isotope effects (due to the increased binding affinity of the heavier atom) have been shown to occur in *in vitro* systems where the isotope was fractionated from its more abundantly occurring counterpart (Wolfe, 1984a), however, the magnitude of these effects *in vivo* should be negligible. Furthermore, in the case of ^{13}C labelled compounds the isotopic effects would be less compared to the isotopic effects when using radioactive, ^{14}C labelled compounds, due the greater difference in mass between

the labelled atom and the common ^{12}C molecule.

5) There is no change in the amount of expired $^{13}CO_2$ label during the course of the study. When L-[1-^{13}C] leucine is infused the irreversible (Harper et al, 1984) decarboxylation (oxidation) of the molecule precedes the release of $^{13}CO_2$ into the breath (see Figure 1b). The amount of infused $^{13}CO_2$ that is retained within the body is termed the bicarbonate retention factor (c). This factor is obtained by infusion of a known amount of [^{13}C]labelled sodium bicarbonate ($NaH^{13}CO_3$) at a known rate and by analyzing the breath $^{13}CO_2/^{12}CO_2$ ratio to determine enrichment, one can then calculate the amount of $^{13}CO_2$ excreted versus that infused, the resulting value gives the amount of excreted or retained label (Wolfe, 1984a). The formula to calculate the bicarbonate retention factor (c) is given below:

$$c = \dot{V}CO_2 \cdot [IE^{13}CO_2 \cdot F] \cdot 100 \quad \text{(equation 4)}$$

where, $\dot{V}CO_2$ is the volume of CO_2 produced in $\mu mol \cdot min^{-1}$; IE $^{13}CO_2$ is the isotopic enrichment of the expired breath, at plateau, in APE; and F is the rate of infusion of the [^{13}C]$NaHCO_3$ in $\mu mol \cdot min^{-1}$; multiplying by 100 converts the retention factor to a percentage.

The retention of bicarbonate is used in correcting oxidation values of the labelled L-[1-^{13}C] leucine. Upon oxidation of α-KIC the ^{13}C label is lost as $^{13}CO_2$, but not all

of the label is excreted and some becomes trapped in slowly turning over metabolic pools of bone or becomes incorporated into Krebs cycle intermediates (Irving et al, 1983). Hence, a resting retention of 0.81 (81% of the infused label being excreted) is the most commonly used factor in correcting resting oxidation measurements. Leucine oxidation is calculated from the amount of excreted $^{13}CO_2$ label during the infusion of leucine according to the following formula:

$$\text{Leucine O} = [(IE_{CO2} \cdot \dot{V}CO_2) \div (IE_{\alpha\text{-KIC}} \cdot c)] \quad \text{(equation 5)}$$

where, IE_{CO2} is the isotopic enrichment, at plateau, of the breath $^{13}CO_2$ in APE; $IE_{\alpha\text{-KIC}}$ is the isotopic enrichment, at plateau, of the plasma α-KIC in APE; c is the bicarbonate retention factor; and $\dot{V}CO_2$ is the rate of carbon dioxide produced in $\mu mol \cdot kg^{-1} \cdot min^{-1}$. Using 0.81 as the correction factor to correct for the resting retention of $^{13}CO_2$ seems to be valid only in the resting state (Wolfe, 1984a; Hoerr et al, 1989). Physiological perturbations such as exercise cause a change in the amount of $^{13}CO_2$ excreted (Wolfe et al, 1984b). Wolfe *et al* (1984b) showed that exercise as mild as 35% of VO_{2max} was enough to cause an almost 100% increase in the amount of $^{13}CO_2$ recovered in breath initially which fell exponentially to a new plateau at about 90 minutes of exercise. The bicarbonate retention factor was corrected in this study by infusing subjects (n=3 per group) with

$[^{13}C]NaHCO_3$ during the same exercise protocol undergone during the infusion of L-$[1-^{13}C]$leucine. Hence, although assumption 5 may not always be valid it can be corrected for by performing a separate study to determine the bicarbonate retention factor to appropriately correct the substrate oxidation values (Wolfe, 1984b). Other studies which have examined leucine oxidation during exercise (Rennie et al, 1981; Wolfe et al, 1982; Millward et al, 1982; Hagg et al, 1982; Evans et al, 1983; Wolfe et al, 1984b; Knapik et al, 1991) have not accounted for the retention of $^{13}CO_2$ during exercise and thus, have reported falsely elevated oxidation values.

6) The background ratio of $^{13}CO_2/^{12}CO_2$ does not change appreciably during the course of the experiment. The background ratio of the sample is used in converting the sample from, in the case of a breath sample, delta per mil or the $^{13}C/^{12}C$ ratio to APE or MPE. Since ^{13}C forms 1.11% of all carbon, background enrichment is important and changes in enrichment can mean a significant difference in APE (Wolfe, 1984a; Wolfe et al, 1984c; Pérronet et al, 1990). Additionally, the natural enrichment of carbon from various sources, such as different plants, atmospheric CO_2, fat, and carbohydrate varies widely (Schoeller et al, 1980; Wolfe, 1984a; Wolfe et al, 1984c). The result of these differences

in ^{13}C enrichments is that different metabolic fuels have different isotopic enrichments (Wolfe, 1984a; Schoeller et al, 1984). Therefore, any protocol using stable isotopes that perturbs the pattern of metabolic fuel use from that when the background enrichment was determined, will have to account for the change in background enrichment (Wolfe et al, 1984c; Péronnet et al, 1990).

Depending on the intensity and duration of exercise the pattern of fuel will shift to a greater use of carbohydrate or fat as a metabolic fuel (Hultman, 1989). The shift in the pattern of fuel use means that the background enrichment of ^{13}C will change according to the amounts of fat and carbohydrate being used. Carbohydrates are more enriched with ^{13}C than are fats, due to the slight isotopic fractionation that occurs during certain biochemical reactions (Wolfe, 1984a; Péronnet et al, 1990). If an intervention such as an exercise treatment is used during infusion of a ^{13}C-labelled substrate, then the change in isotopic enrichment should be accounted for to correct the values of expired $^{13}CO_2$ during the infusion protocol (Wolfe et al, 1984c). Wolfe *et al* (1984c) showed that exercise (which resulted in a increased respiratory exchange ratio, RER, from 0.74 to 0.97) significantly increased the $^{13}C/^{12}C$ ratio in the expired breath of humans. Diet can aslo have a effect on breath ^{13}C

enrichment. Tarnopolsky (1991a) showed that when subjects consumed a high carbohydrate diet for 10 days their breath $^{13}CO_2$ enrichment was significantly higher than their breath $^{13}CO_2$ enrichment after 10 days of a high fat and protein diet. The change in expired CO_2 enrichment means that any protocols which collect CO_2 as a measure of tracer oxidation, such as [^{13}C] leucine infusion, have to account for changes in breath enrichments. A separate experiment was conducted in this thesis to account for the changes caused by the exercise intervention and the resultant changes in background enrichment were subtracted from oxidation breath enrichments.

Most of the model's (equation 2) assumptions can be either tested or accounted for by using separate experiments as shown above. Leucine kinetics were measured using a primed, continuous infusion of L-[1-^{13}C] leucine to examine the changes that occur in whole body leucine metabolism during 90 minutes of exercise (~65% of $\dot{V}O_{2max}$).

1.3.4 *Instrumentation.*

The use of stable isotope probes has only been possible due to the advances in the equipment necessary to analyze the samples (Wolfe, 1984a). Since stable isotopes do not spontaneously emit any particles which can be easily counted they can only be distinguished from their more abundant counterpart using mass spectroscopy. Mass

spectroscopy uses a variety of machines, but the most commonly used machines for stable isotope labelled, amino acid studies are isotope ratio mass spectrometer (IRMS) and gas chromatography mass spectrometer (GC/MS). The basic principles of these two machines and their associated techniques will be discussed below.

1.3.5 *Isotope ratio mass spectrometer (IRMS)*.

An IRMS is used to measure the isotopic abundance of gaseous samples of elements such as: H_2, CO_2, N_2 and O_2 (Wolfe, 1984a). I have used the IRMS at the McMaster Geology department (directed by Dr. H.P. Schwarz) to determine the $^{13}CO_2$ enrichment of the breath samples collected from the subjects in this study during the protocols necessary to determine breath enrichment (see sections 2.2.2; 2.2.3; and 2.2.4 for details of the protocols in which breath samples were collected).

The $^{13}CO_2/^{12}CO_2$ ratio in the breath sample is expressed as delta per mil or $\delta^{13}C$ % (part per thousand). The sample to be determined is compared to an international standard (reference gas), of known $^{13}CO_2$ composition, and the sample's enrichment is expressed relative to the standard (Wolfe, 1984a; Thompson et al, 1985). By subtracting the $\delta^{13}C$ % of the background or baseline sample the sample's enrichment can be converted to APE or MPE.

The IRMS machine uses pure gaseous samples which either have to be converted, in the case of some samples, or isolated to yield the gas to be analyzed. The sample is introduced into the IRMS using a vacuum-line and is held prior to it's ionization in a bellows chamber to equilibrate the pressures between the sample gas and the reference. The sample and reference gas are then admitted into the ionization source in alternating sequence for several comparisons (M. Knyf, 1991, personal communication). The ionization source is a stream of electrons from a tungsten filament that bombard the sample causing it to be ionized. The positively charged ions produced are accelerated through a curved magnetic mass analyzer, and then are deflected by the magnetic field according to the square root of their mass (Wolfe, 1984a). Since $^{13}CO_2$ (m/z, mass/charge, =45)and $^{12}CO_2$ (m/z=44) differ by one atomic mass unit, they are deflected to different degrees, resulting in sufficient separation for the ions to be collected on spatially distinct Faraday collection cups. Thus, the signal emitted by the cups is proportional to the abundance of the ions and the ratio of the two signals can easily be calculated (Wolfe, 1984a). The sensitivity and precision of the IRMS is high, having the ability to detect changes of 0.001 APE and a CV of only

± 0.00001% (Wolfe, 1984a; Thompson et al, 1985; Scrimgeour and Rennie, 1988).

Since samples for IRMS analysis must be pure gas, this means that they must first be prepared for analysis. In the case of $^{13}CO_2$ there exist two methods for sample preparation: either by bubbling the sample through NaOH to trap the CO_2 as $NaHCO_3$ and then subsequently liberating the CO_2 with concentrated phosphoric acid; or by cryogenically trapping the CO_2 (Wolfe, 1984a). The latter procedure was used in this research (see Appendix XXX for the details of the procedure).

1.3.6 Gas chromatography/mass spectrometer (GC/MS).

The GC/MS is a machine used to measure the enrichment of species that cannot be converted to a gaseous form. It utilizes the principles of two machines, the gas chromatograph and the mass spectrometer. The GC portion of the machine is used to separate the various species in the sample and the MS portion of the machine to determine the isotopic abundance of the various species (Wolfe, 1984a).

The compound of interest is separated from the biological sample (urine, blood, or tissue e.g. muscle) and derivatized to form a volatile compound for separation by the GC. The GC column is usually either a narrow glass column or a very thin capillary silica column, both of which vary in dimension according to the sample and type of separation

needed. Once injected into the GC column the sample moves over the stationary phase of the column via a mobile carrier gas. The compounds in the sample are then separated according to their partition coefficients (Halliday and Read, 1981; Wolfe, 1984a), which are inversely proportional to their vapour pressures (a sample with a lower partition coefficient will elute from a GC column first). Various increases in temperature (ramps) will facilitate the movement of later eluting compounds from the column (B. Hill, 1991, personal communication). Once the sample has eluted from the GC column it directly enters the ionization source of the MS portion of the machine. The sample is ionized using one of two methods: chemical impact (CI) ionization, which uses high pressure gases such as methane to create fragment ions from the eluting sample; or electron impact (EI) ionization, which uses a stream of electrons from a filament (e.g. tungsten) to create fragment ions. The fragment ions are actually pieces or fragments of the original injected compound that are created upon collision with the ionization source. The fragments are then bounced through an oscillating field of four large magnets, quadropoles, which cause fragments to oscillate with a characteristic frequency, the machine can be set so as to allow only specific ions (specified oscillating frequency) to be monitored (selected ion monitoring or "SIM"; Wolfe, 1984a).

The ions that are "selected" are detected and that signal is then amplified and translated to give an ion chromatograph. The area under the chromatograph or the height of the mass spectrum at the apex of the chromatograph can be used to determine the ratio of certain fragment ions which can yield the APE once the background enrichment is subtracted from the sample (Wolfe, 1984a).

The sensitivity and precision of the GC/MS are not as high as that of the IRMS. The GC/MS can detect changes in sample enrichment in the order of 0.2 APE, and CV of ± 0.1% (Wolfe, 1984a; Thompson et al, 1985). The GC/MS requires only a small sample, if used with a capillary column it can determine enrichments of picomolar quantities of compounds (Thompson et al, 1985). One limit placed on the kinds of samples that can be analyzed using GC/MS is that the compound of interest should be of a sufficiently high atomic weight (>175 amu) to avoid interference from background fragment ions in the spectrometer (Thompson et al, 1985).

In this investigation, plasma α-KIC enrichments were detemined using the o-quinoxalinol-trimethylsilyl derivative of α-KIC (Ford et al, 1985). This derivative yields a very simple mass spectra and the reaction to produce the derivative is highly efficient (Ford et al, 1985). The procedure for the derivatization of the sample is shown in Appendix XXVII.

1.4 Protein metabolism in endurance athletes.

Endurance exercise results in an increased amino acid oxidation in humans (Rennie et al, 1981; Wolfe et al, 1982; Hagg et al, 1982; Evans et al, 1983; Wolfe et al, 1984b; Knapik et el, 1991) and rats (Dohm et al, 1977; White and Brooks, 1981; Lemon et al, 1982; Lemon et al, 1985; Henderson et al, 1985) and also an increased oxidation of lysine in humans (Wolfe et al, 1984b). An increased excretion of urinary urea, suggestive of an increased protein utilization, has also been observed following endurance exercise (Décombaz et al, 1979; Lemon and Mullin, 1980; Dohm et al, 1982; Calles-Escandon et al, 1984; Lemon et al, 1986; Dolny and Lemon, 1988). Whether an increased amino oxidation or an increased urea excretion would result in an increased protein requirement for those persons performing exercise, has been questioned by some (Butterfield and Calloway, 1984; Todd et al, 1984; Butterfield et al, 1987). The current national, Canadian RNI for protein (Health and Welfare, 1990) the U.S. Recommended Dietary Allowance (RDA) for protein (Food and Nutrition Board, 1990) and the international (FAO/WHO/UNU, 1985) recommendations for protein make no allowance for an effect of exercise on protein requirements. The methods and rationale of how the protein requirements for Canadians are set are given below.

1.4.1 *The Canadian RNI for protein: methods and rationale.*

Before beginning this section the reader is referred to the note in the foreword of this thesis for the explanation of the RNI values used. The current Canadian RNI for protein is 0.86 g protein$\cdot kg^{-1}\cdot d^{-1}$ for all individuals greater than 19 years of age, regardless of gender (Health and Welfare, 1990). The Canadian RNI for protein as it stood up until 1990 was 0.82 g protein$\cdot kg^{-1}\cdot d^{-1}$ for males over 25 and was 0.74 g protein$\cdot kg^{-1}\cdot d^{-1}$ for females over 25.

The 1983 Canadian RNI for protein was based on the amended reports of the FAO and WHO (1975; 1979) and followed the rationale in these reports. The 1990 Canadian RNI uses the figures outlined in the 1985 FAO/WHO/UNU report (FAO/WHO/UNU, 1985). The FAO/WHO/UNU (1985) used figures from nitrogen balance studies that were unavailable to the earlier committee (FAO/WHO, 1975; FAO/WHO, 1979) and as such there is a slight increase in protein requirements.

The FAO/WHO/UNU defines protein requirements as the need for total amino N and EAA (FAO/WHO/UNU, 1985). The approach used in this report to establish protein requirements was a combination of the factorial method and nitrogen balance. The factorial method of determining protein requirements uses the principles of the nitrogen balance method to establish the protein losses that occur when protein

intake is essentially zero, that is: nitrogen balance, when N in = 0 (see equation 1 in section 1.2.2). These losses are then multiplied by factors to account for growth (in the case of humans less than 19 years of age), protein accretion in the case of pregnant women, protein required for milk formation in the case of lactating women, individual variability, and quality (FAO/WHO/UNU, 1985; Health and Welfare, 1990). The losses that occur when there is no N intake are termed the obligatory N losses. It is important to realize that while the obligatory losses are being determined the diet must supply adequate levels of all other nutrients especially energy (FAO/WHO/UNU, 1985). The problem with the use of obligatory losses as a reference point, is that at intakes below requirement the efficiency of protein use increases and less protein is required to maintain most physiological processes. This phenomenon of increased efficiency of protein use, relates to the concepts of adaptation and accommodation discussed earlier (Young, 1989; see discussion in section 1.2.2). Hence, when intake is increased from zero to near requirement level the efficiency of protein utilization decreases and more protein is required for physiological processes. The 1985 FAO/WHO/UNU report recognized the fact that nitrogen balance could not, therefore, be achieved simply by replacing the obligatory losses (FAO/WHO/UNU, 1985). Thus,

an approach was adopted that combined the nitrogen balance and factorial techniques.

The starting point for the FAO/WHO/UNU board members, in setting protein requirements, was the direct measurement of the N needed for zero balance in short-term or long-term nitrogen balance studies. This level was established as 0.63 g protein$\cdot$kg$^{-1}\cdot$d^{-1} for short-term balance studies and 0.58 g protein$\cdot$kg$^{-1}\cdot$d^{-1} for long-term balance studies, the mean value (adopted by the FAO/WHO committe) being 0.605 g protein$\cdot$kg$^{-1}\cdot$d^{-1}, which was rounded to 0.60 g protein$\cdot$kg$^{-1}\cdot$d^{-1} (FAO/WHO/UNU, 1985). This requirement represents the "average requirement" for high quality protein, such as those from meat, milk, fish, and egg. The issue of gender and its effects on protein requirements was considered, but it was concluded, "...there is no evidence to suggest that the efficiency of utilization of dietary protein for meeting their [females] physiological requirements is substantially different from that of young adult men..." (FAO/WHO/UNU, 1985). The basal requirement of 0.60 g protein$\cdot$kg$^{-1}\cdot$d^{-1} forms the basis of the current Canadian RNI (Health and Welfare, 1990). The estimate of 0.60 g protein$\cdot$kg$^{-1}\cdot$d^{-1} or 100 mg N$\cdot$kg$^{-1}\cdot$d^{-1} was defined as maintenance requirement and it was assumed that this requirement remained constant throughout the lifespan (Health and Welfare, 1990). Factors that accounted for the above mentioned situations:

growth, tissue accretion during pregnancy, milk production, individual variability, and quality, were then used to correct the estimate of 100 mg $N \cdot kg^{-1}$. The factors are given below for the protein requirements of males from 16 to 18 years of age:

Maintenance requirement = 100 mg $N \cdot kg^{-1}$ +

Growth requirement = 7 mg $N \cdot kg^{-1}$ = 107 mg $N \cdot kg^{-1}$ +

2 standard deviations[1] = 132 mg $N \cdot kg^{-1}$ = 0.83 g protein$\cdot kg^{-1}$ x

Quality adjustment[2] = 1.12 = 0.93 g protein$\cdot kg^{-1}$

An identical approach was taken to determine the protein requirements of those individuals greater then 19 years of age. All of the estimates of the protein requirements are based on sedentary individuals and this may have led to a bias in requirements. The estimates of protein requirement contain no specific allowances for a possible effect of physical exercise, despite evidence that the protein requirements of athletes may be increased.

1.4.2 *The effects of endurance exercise on protein*

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[1] Assuming the CV for maintenance requirements was 11.9%, or 12.5% for adults over 19, two standard deviations is equal to 23.8%. If the protein requirements are distributed as a Gaussian curve then the addition of two standard deviations to the mean requirement should cover the protein requirements of 97.5% of the population (FAO/WHO/UNU, 1985; Health and Welfare, 1990).

[2] The adjustment for protein quality takes into account the EAA composition of the average diet and on the protein digestibility, since the FAO/WHO/UNU requirement represents high quality protein. The factor will vary according to the age of the group specified and the type of diet consumed by each group (Health and Welfare, 1990).

requirements.

In estimating the protein requirements for those individuals engaged in habitual endurance exercise the results of various studies have lead to a wide variety of recommendations from 109% (Meredith et al, 1989) up to a maximum of 209% of the current RNI (Brouns et al, 1989). The influence of several important variables should also be taken into account when examining studies of protein requirements of endurance exercising humans. These variables include: the intensity and duration of exercise; the state of training of the subjects; and the amount of protein and relative proportion of foodstuffs being ingested by the subjects at the time of the study.

The evidence for an increased protein requirement due to the effects of endurance exercise can only be based on the results of nitrogen balance experiments, since an increased excretion of urea is only reflective of an increased protein catabolism and not an increased need for protein (Butterfield, 1987). Thus, only the results of well controlled nitrogen balance studies can show an increased protein requirement. Brouns *et al*. (1989a) studied 13 highly trained cyclists completing intense (exhaustive) cycle ergometer exercise. The diet of the subjects was monitored and protein intake was 1.7 g protein$\cdot$kg^{-1}. The nitrogen balance of the subjects showed

this protein intake was equal to the N (protein) excreted by the subjects. The authors concluded that endurance cyclists would have an increased protein requirement (Brouns et al, 1989a). Subjects engaging in their habitual exercise regime were studied by Friedman and Lemon (1989). Subjects consumed two levels of protein, one thought to be sufficient and the other, 0.86 g protein$\cdot kg^{-1}\cdot d^{-1}$, close to the current U.S. RDA. The nitrogen balance of the athletes was positive on the high protein intake and was negative when consuming the low protein diet. The investigators concluded that the current RDA may be inadequate for athletes. Meredith *et al*. (1989) also studied habitually training endurance athletes. The nitrogen balance of each athlete on three different protein intakes was measured. The mean protein intake to maintain zero nitrogen balance was 0.94 g protein$\cdot kg^{-1}$ (109% of protein RNI; Meredith et al, 1989). In a further study of trained male endurance athletes, Tarnopolsky *et al*. (1988) showed, using nitrogen balance, that endurance athletes required 1.67 times more daily protein than did a sedentary control group. The authors suggested that endurance exercise resulted in a net protein catabolism that caused the incresed protein requirement for endurance athletes (Tarnopolsky et al, 1988).

In contrast to the findings of many researchers that athletes have an increased requirement for protein

(Tarnopolsky et al, 1988; Meredith et al, 1989; Friedman and Lemon, 1989; Brouns et al, 1989a), others have found that exercising persons could maintain nitrogen balance on protein intakes less than the RNI (Butterfield and Calloway, 1984). Butterfield and Calloway (1984) fed young men a low protein intake, while they engaged in mild intensity endurance exercise. When the subjects were in energy equlibrium the mean nitrogen balance was not different from zero. The investigators concluded that the improvement in N retention (nitrogen balance), accompanying exercise, suggested that the protein requirement to maintain lean tissue in a chronically active individual may be less than one who is inactive (Butterfiel and Calloway, 1984).

It has been demonstrated that the training state of the subjects studied can have an impact on their protein requirements. Gontzea *et al.* (1974) studied the nitrogen balance of young men who consumed 1.0 or 1.5 g protein·kg^{-1} at the initiation of an exercise program. Prior to engaging in the exercise program both groups were in positive nitrogen balance. However, during the exercise period nitrogen balance became negative in the group consuming 1.0 g protein·kg^{-1}. The same response to the exercise challenge was seen in the group consuming the higher intake, however, this group maintained a positive nitrogen balance. The cessation of exercise

resulted in an a return to positive nitrogen balance in both groups. It is important to realize that if the negative nitrogen balance, at the begining of an exercise program, were to persist then undesirable losses of lean body mass would occur. Hence, at some point following the initiation of an exercise program an individual must adapt to be able to retain N, since the proposed loss of lean mass does not occur (Lemon, 1987). Gontzea *et al.* (1975) showed that during a more prolonged exercise study the initial negative nitrogen balance was reduced over time. It is apparent, however, that the increase in protein retention does not persist indefinitely and that with chronic exercise an increase in protein requirement does occur (Tarnopolsky et al, 1988; Brouns et al, 1989a; Meredith et al, 1989; Friedman and Lemon, 1989).

It appears that with the initiation of an exercise program there is a transient negative nitrogen balance that dissapears with time (Gontzea et al, 1974; Gontzea et al, 1975). This increased N retention does not appear to continue indefinitely, however, since many researchers have shown that the protein requirements of endurance athletes are elevated with continual training (Tarnopolsky et al, 1988; Meredith et al, 1989; Friedman and Lemon, 1989; Brouns et al, 1989a). Paradoxically, others (Butterfield and Calloway, 1984) have shown that exercise results in an increased retention of

dietary N, which has been interpreted to mean that the protein requirements of those that are active are no different from those that are sedentary.

1.4.3 *Amino acid metabolism during endurance exercise.*

The oxidation of amino acids, such as leucine, has been shown to increase during endurance exercise, in humans (Rennie et al, 1981; Hagg et al, 1982; Millward et al, 1982; Wolfe et al, 1982; Evans et al, 1983; Knapik et al, 1990) and rats (Dohm et al, 1977; White and Brooks, 1981; Lemon et al, 1985; Henderson et al, 1985; Vazquez et al, 1986). In addition, the oxidation of lysine has also been shown to increase slighly with exercise (Wolfe et al, 1984b). The tissue sites where this increased amino acid oxidation occurrs are liver and muscle (Goldberg and Chang, 1978). Muscle has the capacity to oxidize several amino acids including: leucine, isoleucine, valine, aspartate, asparagine, and glutamamte (Odessey et al, 1974; Goldberg and Chang, 1978). Although of the amino acids oxidized by muscle only the oxidation rates of leucine, isoleucine, and valine (branched-chain amino acids, BCAA), are significantly increased during catabolic states (Goldberg and Chang, 1978), with leucine catabolism being increased to the greatest extent.

The reason for an increase in leucine oxidation during exercise is probably indirectly as a source of gluconeogenic

precursors to sustain blood glucose production (Goldberg and Chang, 1978). The transamination of leucine inside the cell by BCAA aminotransferase (BCAAT) is rapid and reversible in muscle (Harper et al, 1984; Thompson et al, 1988). Muscle is also the primary tissue in which metabolic rate is elevated during exercise and is the primary site of leucine oxidation during muscular work (Hood and Terjung, 1987).

Figure 1b shows how leucine (the mechanism can be applied to all BCAAs) is transaminated with α-ketoglutarate by BCAAT to give glutamate, glutamate is then either transaminated itself with pyruvate to give alanine or it may incorporate free ammonia to form glutamine (Odessey et al, 1974; Goldberg and Chang, 1978). The release of both alanine and glutamine from exercising muscle exceeds that which would be expected from muscle cell proteolysis, thus, both amino acids are synthesized de novo (Felig and Wahren, 1971; Odessey et al, 1974; Goldberg and Chang, 1978). Leucine oxidation increases with increasing work rate (Millward et al, 1982) and increasing frequency of muscular contraction (Hood and Terjung, 1987). At the same time alanine output from skeletal muscle also increases, proportional to exercise intensity (Felig and Wahren, 1971). The subsequent uptake, by the liver, of the alanine produced is proportional to exercise intensity up to an intensity representing a 7-8 fold increase in resting

O_2 consumption at which point hepatic alanine uptake plateaus (Felig and Wahren, 1971). The alanine reaching the liver can serve as a source of pyruvate, once deaminated, for the production of glucose (Odessey et al, 1974; Goldberg and Chang, 1978; Lemon, 1987). This "glucose-alanine cycle" can theoretically provide 42 mol ATP·mol leucine^{-1} (if leucine is completely oxidized to isovaleryl CoA) compared to the 2 mol ATP·mol glucose^{-1} (obtained from lactate production; Goldberg and Chang, 1978).

The increase in leucine oxidation that occurs with exercise is due to the activation of the rate limiting enzyme involved in BCAA metabolism, branched-chain α-ketoacid dehydrogenase (BCKAD or BCOAD - branched-chain 2-oxo acid dehydrogenase; Wagenmakers et al, 1984; Kasperek et al, 1985; Kasperek and Snider, 1987 Wagenmakers et al, 1989). The activation of this enzyme is well correlated with the increase seen in leucine oxidation (Kasperek and Snider, 1987) and the activation is proportional to exercise intensity (Kasperek and Snider, 1987). The BCKAD complex is located on the inner mitochondrial membrane and is a five subunit enzyme which is very similar in nature to the pyruvate dehydrogenase complex (Harper et al, 1984). The BCKAD complex is highly regulated through a reversible phosphorylation (inactivation) - dephosphorylation (activation) mechanism (Harper et al ,1984;

Randle et al, 1984; Kasperek, 1989). The activation of this enzyme during exercise, in rats, has been shown to range from 27% (Wagenmakers et al, 1984) to 142.5% (Kasperek and Snider, 1987) above resting. The only report of the level of activity of the BCKAD complex in humans showed that subjects completing 120 minutes of cycle ergometry at 70% of $\dot{V}O_{2max}$, had 13.5% more BCKAD complex active at the end of exercise compared with resting activity (Wagenmakers et al, 1989).

1.5 Gender differences in substrate use during endurance exercise.

One aspect that has not, to my knowledge, been adressed in the investigation of the protein requirements of active individuals is the possibility of gender differences in the requirement for dietary protein. The current Canadian RNI makes no distinction for males and females as to their protein requirements (Health and Welfare, 1990) nor does it make any allowance for the effects of habitual physical activity. Some investigations have shown that there are definite gender differences in the pattern of fat and carbohydrate use during endurance exercise (Froberg and Pederson, 1984; Tarnopolsky et al, 1990), but none have systematically examined the response of protein metabolism to endurance exercise.

Substrate use during endurance exercise is influenced by many factors (Åstrand and Rodahl, 1986). To make a valid comparison between genders the influence of factors such as: training status, menstrual phase, and diet, must be controlled (Tarnopolsky et al, 1990). The possible influence of these factors and how they might affect gender comparisons is considered.

1.5.1 *Training status.*

Almost all research in the area of gender differences has focussed on the difference in the use of fat and carbohydrate during endurance exericse. There are well recognized training effects on the pattern of usage of these two substrates (reviewed in section 1.1.1; Holloszy and Coyle, 1984; Åstrand and Rodahl, 1986; Coggan et al, 1990). Thus, when conducting comparisons of metabolic responses of male and female athletes, groups should be matched for training status.

The most reflective measure of training status is a persons $\dot{V}O_{2max}$ ($ml \cdot kg^{-1} \cdot min^{-1}$) which represents the function of a number of integrated systems (Åstrand and Rodahl, 1986) and is the primary determinant of work capacity in weight bearing exercise (Sparling, 1980). Matching males and females on the basis of this parameter, however, will not ensure that the two sexes are evenly matched, since males will most times have a greater $\dot{V}O_{2max}$ than females given the larger body size and

greater lean mass of the males (Sparling, 1980; Cureton and Sparling, 1980; Cureton, 1981). Expression of $\dot{V}O_{2max}$ relative to lean body weight (LBW) has been proposed by some researchers as the most valid measure of training state upon which members of the opposite sex should be matched (Pate et al, 1985). However, Sparling (1980) has argued that training history is the best predictor of training equality between genders, since matching for training status resulted in a 9% difference between men and women in VO_{2max} when expressed relative to LBW. This is in contrast with an average difference of 15% in $\dot{V}O_{2max} \cdot kg\ LBW^{-1}$ oberved when subjects were selected and matched using $\dot{V}O_{2max}$ as the initial selection criterion.

1.5.2 *Menstrual phase.*

The female menstrual cycle is characterized by the fluctuation in stimulatory and ovarian hormones that bring about menstruation and ovulation (Guyton, 1986). The first phase of the cycle is known as the follicular phase (FP, days 1-14) which begins at the onset of menses and ends at ovulation and is characterized by low concentrations of progesterone (P_4) and estradiol (E_2) ($<3\ ng \cdot ml^{-1}$ of P_4 and $<200\ pg \cdot ml^{-1}$ of E_2; Guyton, 1986). The concentration of estradiol rises during the FP peaking at approximately day 12-14 along with a peak in concentration of follicle stimulating hormone

and lutenizing hormone. The phase following ovulation is known as the luteal phase (LP, days 14-28). At ovulation E_2 concentration declines and P_4 concentration begins to increase, E_2 also increases slightly, with both peaking around day 22 of the cycle and then decreasing rapidly prior to menstruation.

Some evidence suggests that there may be differences in fat, carbohydrate, and protein usage across the menstrual cycle. The LP phase of the menstrual cycle has been associated with an increased endurance due primarily to a decreased reliance on carbohydrate as a fuel. This has been shown to result in decreased plasma lactate concentrations in women exercising during this phase of their menstrual status (Nicklas et al, 1989; Jurkowski, 1981). In contrast, exercise during the LP phase resulted in a decreased plasma glucose concentration compared to the same intensity exercise during the FP (lavoie, 1986). The decrease in glucose was particularly apparent after 70 minutes of exercise (Lavoie, 1986). Hatta *et al.* (1988) studied glucose and FFA oxidation in ovariectomized rats. Rats who exercised after receiving 17-ß-estradiol had a higher oxidation of FFA and lower oxidation of glucose compared to control rats. However, comparing ammenorrheic and eummenorrheic athletes, de Souza *et al.* (1990) showed that there was no effect for either menstrual

phase (LP versus FP) or status (ammenorrheic versus eummenorrheic) on time to fatigue or plasma lactate.

Menstrual phase can also alter protein metabolism in exercising and sedentary women. Calloway and Curzer (1982) examined protein metabolism during all phases of the menstrual cycle in six sedentary women. They showed that urinary N excretion followed a significant biphasic pattern in each subject. Peak urinary N excretion occured during the mid LP and mid FP of the cycle. Lamont *et al.* (1987) examined the effects of exercise on protein catabolism during both phases of the menstrual cycle. Urea excretion (sweat plus urinary) was greatest on the exercise day during the mid LP versus urea excretion during menses. Excretion on control days (pre- and post-exercise) was not, however, significantly different between menstrual phases.

The evidence for an effect of menstrual cycle on substrate utilization remains unclear. What is recognized is that the two phases are characterized by different hormonal concentrations with respect to both the gonadotropic and gonadotropic releasing hormones (Guyton, 1986; Bonen et al, 1991). These different hormone levels may not only affect substrate metabolism themselves, but may exert their effects through a permissive action on other hormones such as growth hormone, insulin, or glucagon (Bunt, 1990). Since the

possibility of a different substrate response during different phases of the menstrual cycle exists, studies should control for this effect by testing all females during the same phase of their menstrual cycle.

1.5.3 *Dietary effects on endurance exercise*.

An increased consumption of carbohydrate as a regimented protocol or simply as a snack prior to engaging in endurance exercise can increase endurance performance (see section 1.1.1 for a detailed discussion; Bergström et al, 1967; Karlson and Saltin, 1971; Sherman et al, 1981; Hultman, 1989). Accordingly, failure to adequately control for the effects of increased carbohydrate consumption could alter endurance performance.

The proportion of energy derived from dietary macronutrients has not been shown to be significantly different between male and female athletes (Brotherhood, 1984; van Erp-Baart et al, 1989; Worme et al, 1990). Day-to-day intakes can vary widely, however, and it has been shown that when carbohydrate intake becomes a significant portion of an athlete's diet (>65% of total adequate calories), endurance exercise performance can be enhanced (Costill, 1988; van Erp-Baart, 1989). To control for the influence of diet, adherence of a subject to an experimetally controlled diet would decrease the day-to-day variation associated with intake

(Brotherhood, 1984) and would also eliminate the possibility of high carbohydrate intake affecting performance.

Most studies examining the effects of exercise have studied overnight fasted subjects. This practice has presumably been used to standardize the subject biochemically and physiologically (Kuipers et al, 1989; Brouns et al, 1989b). Very rarely, when competing, do endurance athletes actually compete in the fasted state (Brouns et al, 1989b). Many athletes now recognize that the consumption of a high carbohydrate meal prior to competition can restore depleted liver glycogen (Hultman, 1989) and ensure adequate blood glucose (Bonen et al, 1980; Brouns et al, 1989b). Consequently, studying athletes in the fasted state may represent an artificial situation in which the results are not applicable to the fed state.

1.5.4 *Differences in carbohydrate and fat usage during endurance exercise in males and females.*

Female athletes have, in some cases, shown a lower RER than males athletes (Tarnopolsky et al, 1990). A lower RER is indicative of a greater fat utilization under steady state conditions (McCardle et al, 1986). Thus, the interpretation has been that females derive a greater proportion of exercise energy from fat (Froberg and Pederson, 1984; Blatchford et al, 1985; Bunt et al, 1986). Other

investigators have reported no differences in RER between genders, implying no difference in fat and carbohydrate use (Costill et al, 1979; Graham et al, 1986). It is possible that the discrepancies in these findings could be accounted for by the varying exercise intensities used and the training states of the subjects. While the RER can give an estimate of carbohydrate and fat oxidation, tissue substrate concentrations will reveal the tissue-specific pattern of substrate use. Muscle glycogen concentration in males was shown to be significantly lower, following 90 minutes of treadmill running, than muscle glycogen concentration in female athletes (Tarnopolsky et al, 1990). A greater muscle glycogen depletion in males versus females has not been shown by other invesigators (Jansson, 1986). Tarnopolsky *et al.* (1990) also showed that during the exercise bout the level of FFA and glycerol were not significantly different between males and females. This finding conflicts with some *in vitro* evidence, which showed that the lipolytic response of fat cells was greater in cells from trained females versus cells from trained males (Crampes et al, 1989). No study yet has looked at the usage of intra-muscular triglyceride in males and females. The use of this substrate could explain the lower RER seen in females during exercise, despite there being no differences in plasma levels of FFA and glycerol response

(Tarnopolsky et al, 1990).

A comparison of male and female endurance athletes is difficult due to the number of factors which must be controlled (Sparling and Cureton, 1981). Differences in carbohydrate and fat usage during endurance exercise have shown that females can utilize more fat than males (Froberg and Pederson, 1984; Blatchford et al, 1985; Bunt et al, 1986; Tarnopolsky et al, 1990), or do not differ from males (Costill et al, 1979; Graham et al , 1986). The oxidation of muscle glycogen use during exercise has been shown to be greater in males than females (Tarnopolsky et al, 1990), or to show no difference (Jansson, 1982).

Differences in protein utilization and the effect of endurance exercise on protein metabolism between genders has not, to my knowledge, been systematically studied. Some indirect evidence indicates that a difference might exist.

1.5.5 *Protein metabolism in males and females: possible gender differences.*

Under conditions of starvation (and nutrient deprivation) the metabolic response of many mammals is similar to that induced by exercise (Ahlborg et al, 1974; Lemon and Nagle, 1981). The metabolic events during starvation appear to follow two phases (Saudek and Felig, 1976). In the early phase (1-5 days) hepatic gluconeogenesis is increased to

maintain plasma glucose, at the expense of substrates such lactate (Cori cycle), alanine (glucose-alanine cycle), and glycerol (Saudek and Felig, 1976; Stryer, 1988). The secondary response is a conservation of protein and a dramatic increase in ketogenesis. During this phase the use of fat for synthesis of ketones is increased and the adaptation of the central nervous system to use this fuel is also increased (Saudek and Felig, 1976). At the same time protein degradation falls to about 20 $g \cdot day^{-1}$ and urinary N losses are only about 5 $g \cdot day^{-1}$ (Owen et al, 1969). This pattern of protein use has a close parallel to the events described by Gontzea *et al.* (1975), who showed an initial increase in protein catabolism during the early stages of training and then a decrease in protein catabolism.

Evidence for gender differences during starvation could support the theory of gender differences during exercise. During a period of total starvation female rats relied on lipid metabolism to a greater extent and protein catabolism to a lesser extent than starved male rats (Triscari et al, 1980). Moreover, following exercise training, exhaustive exercise resulted in an increased urea excretion in male but not female rats (Dohm and Louis, 1978). The same lab (Tapscott et al, 1982) has shown that exercise training resulted in an increased muscle protein degradation in trained

male rats by 130% over untrained controls, whereas female rats showed no significant difference. Male athletes had an increased excretion (32%) of urea as a result a 90 minute exercise bout, whereas, female athletes completing an equivalent amount of exercise did not increase their excretion of urinary urea N (Tarnopolsky et al, 1990). It could not be assertained from the data, however, whether the protein catabolism had occured during the exercise or at another time during the day (Tarnopolsky et al, 1990).

Since in male and female non-human mammals there appear to be differences during starvation (Triscari, 1980) and differences in protein metabolism using exercise (Dohm and Louis, 1978; Tapscott et al, 1982), it is not surprising that humans respond in a similar fashion (Tarnopolsky, 1990). The underlying processes that result in an increased protein catabolism in males but not in females have not been studied.

1.6 **Rationale for research.**

Exercise has been shown to increase protein catabolism. This increase in protein catabolism has been demonstrated by both an increase in amino acid oxidation during exercise (Wolfe et al, 1982; Hagg et al, 1982; Evans et al, 1983; Wolfe et al, 1984b; Knapik et al, 1990) and as an increased excretion of post-exercise urinary urea (Décombaz et al, 1979; Lemon and Mullin, 1980; Dohm et al, 1982; Calles-

Escandon et al, 1984; Lemon et al, 1986; Dolny and Lemon, 1988; Brouns et al, 1989a). Some investigators have shown that the increased protein catabolism occuring in athletes results in an increased dietary requirement for protein in this group (Tarnopolsky et al, 1988; Meredith et al, 1989; Brouns et al, 1989a; Friedman and Lemon, 1989). Other investigators maintain that the protein requirements of athletes are no greater than those of sedentary individuals (Butterfield and Calloway, 1984; Butterfield, 1987).

Despite the evidence of an increased protein requirement for athletes, current national, RNI and RDA, and international, FAO/WHO/UNU, recommendations for protein consumption include no allowance for the effect of habitual physical activity. The increased protein requirements of other groups such as growing children, and pregnant or lactating women are recognized (Health and Welfare, 1990). The reason behind the decision to disregard the protein requirements of athletes is the supposition that exercise does not result in an increased protein requirement. Some investigators feel that since most habitually exercising persons consume greater than their requirement for protein there is no need for studies of the protein requirements of this group (Butterfield, 1987).

In addition, the possible effects of gender on protein requirements has not been systematically studied. It was found

that protein utilization in male athletes may be significantly greater compared to female athletes as a reult of exercise (Tarnopolsky et al, 1990). Such a difference in protein requirements could result in a lower protein requirement for females athletes compared to male athletes.

The conflicting evidence concerning the protein requirements of physically active individuals is probably a result of: the variety of study designs used; the training status of the individuals studied; and the macronutrient composition of the diets consumed by the subjects. The present study has examined differences in protein turnover between trained male and female endurance athletes. The level of training of the subjects was controlled for by selecting subjects in a steady state of training, that were matched between genders. Subjects were provided with a pre-packaged diet, that provided their habitual energy intake and protein close to the RNI, to minimize fluctuations in dietary intake. The subjects were given time to adapt to the diet (10 d) and the adequacy of the protein level of the diet was determined using nitrogen balance. Differences in leucine kinetics between men and women were also studied using a constant primed intravenous infusion of L-[1-^{13}C]leucine during a 90 minute run at approximately 65% of the subjects $\dot{V}O_{2max}$.

1.7 Hypotheses and Objectives.

1.7.1 *Hypotheses.*

Given the evidence from previous investigations of protein metabolism the following hypotheses were proposed:

1) When endurance athletes undergoing training consumed a diet providing their habitual energy intake and protein at the current Canadian RNI, female endurance athletes would be in more positive nitrogen balance than their male counterparts. Furthermore, both groups would be in negative nitrogen balance when consuming the diets provided.

2) Using L-[1-^{13}C] leucine as a tracer, trained female athletes would have a lower total oxidation of leucine during a 90 minute run than matched male athletes exercising at the same relative intensity.

3) The oxidation of l-[1-^{13}C] leucine during the 90 minute run would account for the negative nitrogen balance in both male and female athletes.

1.7.2 *Objectives.*

The specific objectives were:

1) To examine, using l-[1-^{13}C] leucine, whole body leucine metabolism in trained males and females during the 90 minute run to determine any gender differences that occur in whole body leucine metabolism during exercise.

2) To determine using nitrogen balance, the adequacy of the Canadian RNI for protein (0.86 g protein$\cdot$kg$^{-1}\cdot$day^{-1}) for habitually exercising endurance male and female athletes.

Chapter 2

Methodology

2.1 Subjects.

2.1.1 *Subject recruitment and selection.*

Subjects were recruited from the campus at McMaster University via posters and presentations. Broad eligibility criteria were used to get the maximum number of applicants, as well as finding "suitable" recruits for the study. The eligibility criteria were as follows: 1) Subjects were at a constant weight, that is not trying to gain or lose weight, and not weight cycling; 2) Subjects were habitual runners - running at least 3 times per week for a minimum of 6 to 8 months; 3) Female athletes were not taking oral contraceptives and were eummenorheic; an additional criteria was added when the male subjects were recruited, that was 4) That male subjects weighed 72 kg or less. Subjects that fit these criteria were then asked to fill out a preliminary questionnaire (see Appendix XVIII for a sample copy of the questionnaire) detailing their training characteristics. Subjects were then evaluated with respect to their training status according to their responses on the questionnaire. Minimal requirements for entry into the study were as follows: 1) Both male and female athletes had to be running at least 30 $km \cdot week^{-1}$; 2) Female athletes that specified a 10 km time

should have a personal best 10 km time of less than 45 minutes; 3) Male athletes that specified a 10 km time should have a personal best 10 km time of less than 40 minutes. Subjects were excluded if they did not meet these criteria or if their weekly running mileage exceeded 90 $km \cdot week^{-1}$. As an additional criterion to try and match male and female subjects for weight, larger (>60 kg) female subjects and smaller (<72 kg) male subjects were deemed more suitable.

Eligible subjects were then advised of the risks associated with the study and signed written consent form approved by the McMaster University Human Ethics Committee (see Appendices XVI and XVII for ethics forms).

Subjects were then given a maximal aerobic power (VO_{2max}) test to determine their overall fitness. The $\dot{V}O_{2max}$ test was performed according to the procedure of Thoden *et al.* (1982) and took place no fewer than two weeks prior to the subject's entry into the testing phase of the study.

Respiratory measurements were evaluated on an open circuit system. A Zenith IBM-compatible computer was interfaced with: a Validyne pneumotach that measured the volume of expired air (Validyne, Northridge, California); a Rapox oxygen analyzer (Rapox, Bilthoven, Holland); and a Hewlet Packard carbon dioxide analyzer (Hewlet Packard, Avondale, Pennsylvania).

To be eligible female subjects were required to have $\dot{V}O_2$ max values of 50 $ml \cdot kg^{-1} \cdot min^{-1}$ or greater and male subjects to have VO_2 max values of 55 $ml \cdot kg^{-1} \cdot min^{-1}$ or greater. In order to select "matched" subjects from a total of 45 wholly eligible subjects (26 female and 19 male) $\dot{V}O_2$ max tests were given to 15 females and 12 males respectively. Training status (determined from the questionnaire and later verified with an actual written record by the subject) was considered equal if there were no important differences in: running distance per week; level of competition in running or other endurance sport; and running duration per workout. The training characteristics of the subjects are shown in Table 1.

All female subjects reported having a normal menstrual cycle ranging from 27 to 33 days in length and their mean age at menarche was 13.6±1 years.

2.2 **Design.**

2.2.1 *Experimental protocol.*

The protocol included two phases. The first phase was an adaptation phase and the second phase was a nitrogen balance experiment (Figure 2). Prior to entering the study protocol subjects completed 4 day weighed dietary records (Basiotis et al., 1987; Karkeck, 1987). These records were then analyzed using a computer based nutrient analysis program

Table 1. *Subjects' training characteristics.*

	Males (n=6)	Females (n=6)	p
Years running	5.17±1.19	5.58±1.12	0.810
Mileage ($km \cdot wk^{-1}$)	50.33±9.13	43.33±4.66	0.520
Training duration ($min \cdot d^{-1}$)	41.25±4.27	37.50±2.14	0.460
Training frequency ($d \cdot wk^{-1}$)	4.41±0.58	5.00±0.51	0.470
Pace ($min \cdot km^{-1}$)	3.70±0.17	4.35±0.32	0.110
Most frequent event	10 km	10 km	
Best 10 km time (min)	36.97±1.31	41.03±0.96	0.040
Competitive events ($event \cdot yr^{-1}$)	5.0 ±1.5	2.5 ±0.5	0.150

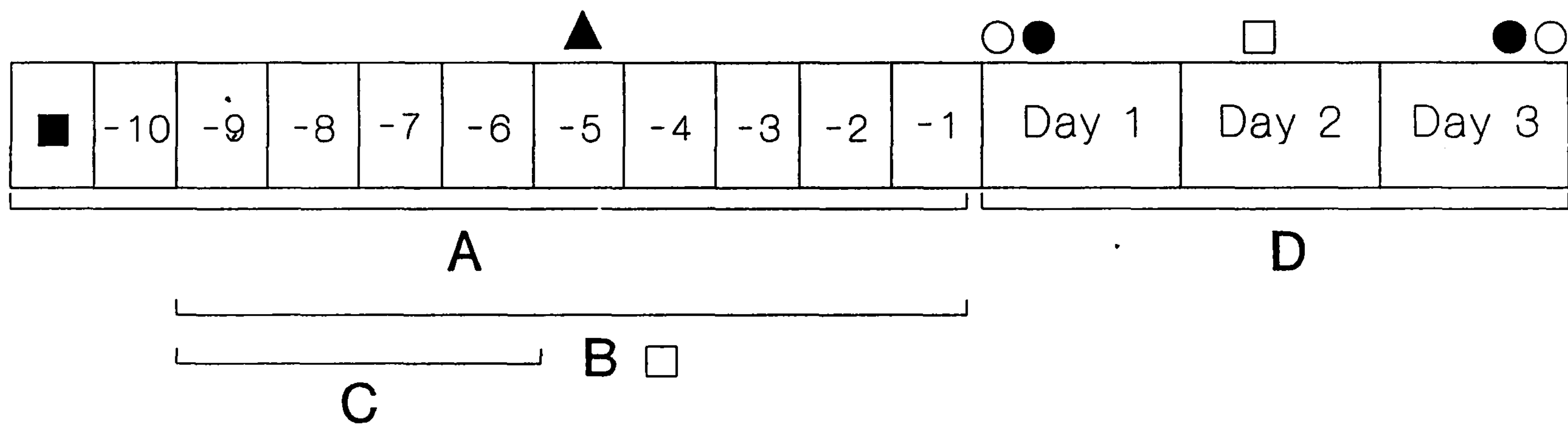
Figure 2. Study design. All subjects completed 13 days of controlled dietary treatment.

A - days -10 to -1 - adaptation period: diet was pre-packaged, pre-weighed, exercise was in accordance with habitual schedule.

B - days -9 to -1 - background breath enrichment test: n=3 subjects per group were tested once for changes in breath enrichment caused by the protocol. Exercising sweat losses were determined after this test.

C - days -9 to -6 - bicarbonate retention test: n=3 subjects per group were tested once for changes in CO_2 retention caused by the protocol.

D - days 1 to 3 - nitrogen balance determination: n=6 subjects per group collected urine and stool for determination of nitrogen balance.



■ - Pre-tests: VO_{2max}, training status assessed

□ - Whole body sweat washdown (rest or exercising)

○ - 3 x 24 h continuous urine collection

● - 72 h continuous fecal collection (between carmine markers)

▲ - Day 1 (approximate) of female subjects menstrual cycle

(Nutritionist III, N-squared computing Co., Silverton, Oregon) to determine the subject's habitual dietary protein and energy intake. Table 2 shows the subjects' habitual dietary characteristics. Subjects were then provided with a pre-weighed (diets were weighed to the nearest 0.1 g; O-Haus, Florham Park, New Jersey), pre-packaged diet that supplied 89±4.5% of the subject's habitual energy (the remainder was made up by the subject from a list of food exchanges), and 98.8±0.2% of the subject's protein energy at the level of the Canadian RNI. Table 3 shows the subjects' dietary data whilst consuming the defined study diet. Efforts were made not to alter the relative percentages of energy as carbohydrate and fat since this can affect nitrogen balance (Richardson et al, 1979). The diets were a two day rotating menu for the entire 10 day adaptation, but were a fixed composition during the nitrogen balance period. Diets consisted of standard foods that were lacto-ovo-vegetarian in nature and contained 28.6±1.93% of the female subjects and 16.0±1.17% of the male subjects habitual energy intake as a liquid supplement (Ensure, Ross Laboratories, Montreal). Subjects were required to keep a record of their compliance with the diet, which entailed checking off items once they were consumed from a dietary list. Compliance according to the dietary lists ranged from 97 to 100%. Compliance was also assessed by measurement

Table 2. *Subjects' habitual dietary data.*

	Males (n=6)	Females (n=6)	p
Energy intake ($kJ \cdot d^{-1}$)	13319±889	7455±530	0.0005
Energy intake ($kJ \cdot kg^{-1} \cdot d^{-1}$)	207.4±14.5	128.3±7.9	0.0005
Protein intake ($g \cdot d^{-1}$)	121.5±12.5	58.0±3.1	0.0021
Protein intake ($g \cdot kg^{-1} \cdot d^{-1}$)	1.89±0.19	1.00±0.05	0.0068
Carbohydrate intake ($g \cdot d^{-1}$)	424.8±26.2	255.0±29.7	0.0140
Fat intake ($g \cdot d^{-1}$)	113.0±5.2	64.1±4.7	0.0021
Protein:Energy (g PRO·240 kJ^{-1})	37.6±1.8	33.2±2.5	0.1900
Carbohydrate:Fat (% energy CHO:% energy fat)	1.67±0.05	1.77±0.24	0.7200

Table 3. *Subjects' dietary data during the study.*

	Males (n=6)	Females (n=6)	p
Energy intake ($kJ \cdot d^{-1}$)	13394±937	7653±514	0.0005
Energy intake ($kJ \cdot kg^{-1} \cdot d^{-1}$)	208.9±14.6	131.6±8.8	0.0005
Protein intake ($g \cdot d^{-1}$)	60.4±1.5	46.4±1.5	0.0001
Protein intake ($g \cdot kg^{-1} \cdot d^{-1}$)	0.94±0.01	0.80±0.01	0.0001
Carbohydrate intake ($g \cdot d^{-1}$)	470.4±34.0	265.8±28.0	0.0012
Fat intake ($g \cdot d^{-1}$)	125.1±11.0	67.4±4.8	0.0028
Protein:Energy ($g\ PRO \cdot 240\ kJ^{-1}$)	19.4±1.8	25.9±1.7	0.0310
Carbohydrate:Fat (% energy CHO:% energy fat)	1.65±0.1	1.75±0.2	0.6700

of 24 hour urinary creatinine, which should be constant on a meat free diet (Heymsfield et al, 1983). Subjects consumed this diet for 10 days to adapt to the level of protein in the diet (Rand et al, 1976; FAO/WHO/UNU, 1985). Duplicate portions of the diets were kept for chemical analysis. Ten percent of each duplicate diet was homogenized, lyophilized, and stored at -70°C until analysis for energy and nitrogen content.

During the adaptation to the diet, subjects exercised in accordance with their habitual pattern and kept an accurate record of the exercise they completed during this time period. This record was compared with the subjects response to the questionnaire completed prior to entering the study. Male subjects completed 108±3% of their reported weekly mileage and female subjects completed 101±3.5% of their reported weekly mileage during the adaptation period. Compliance with the diet during this period was reinforced by frequent contact with the investigators and often having more than one subject participating at the same time which provided peer pressure to comply. Subjects also received $200.00 at the completion of the study.

Following the adaptation phase, subjects completed a 3 day nitrogen balance experiment during which compliance with the diet was strongly emphasized. The pattern of exercise was restricted during this phase of the study such that the

subjects followed their habitual exercise schedule on day 1, refrained from running (and any other strenous activity) on day 2, and on day 3 subjects reported to the testing centre for the infusion of isotope. To determine total nitrogen excretion during the nitrogen balance period subjects collected urine for 3 sequential 24 hour periods and collected all stool for the entire 72 hour period. Representative resting and exercising sweat samples were also obtained. Urine was collected into 4 litre acid washed containers, treated with 5 ml of glacial acetic acid as a preservative. Urine was stored at less than 5°C by the subjects and was delivered to the testing centre within 24 hours of being collected. The volume of the urine collections were determined and aliquots of the collections were taken and stored at -70°C until subsequent analysis. Fecal samples were collected between carmine markers and were kept frozen by the subjects until delivery to the testing centre (within 24 hours). Each fecal collection was weighed and diluted with an equal volume of distilled water, homogenized, and lyophilized. Aliquots of the homogenate were taken and stored at -70°C until subsequent analysis.

Sweat collections were made according to the whole-body washdown technique described and validated by Lemon *et al.* (1983 and 1986). Aliquots of the washdown water were

collected and stored at -70°C for subsequent analysis. Sweat loss of urea N, exercising or resting, was made in 3 subjects in a randomized counterbalanced manner. The subjects that were to receive an exercise washdown had their exercising sweat production determined after completing the background breath test (see section 3.2.2 for details). Subjects receiving a resting sweat washdown had their resting sweat production determined on day 2 of the nitrogen balance period. The resting sweat collection was made over a minimum of three hours, which was considered necessary to obtain a representative resting sweat samples (M. Tarnopolsky, 1991, personal communication). Subjects exercised in either their own clothes that had been acid washed (by the investigators) or in a set of clothes provided for them, which were also acid washed. During the run subjects were continually exposed to a large fan to minimize run off sweat losses. The temperature during these tests ranged from 21°C to 24.5°C, humidity was not controlled. Fluid intake of the subjects was controlled on days 2 and 3 of the nitrogen balance period. On day 3 subjects consumed fluid equal to their day 2 intake plus an additional 30 $ml \cdot kg^{-1}$ after the exercise bout on day 3. This added fluid intake post exercise was to minimize dehydration effects on the kidney which occur during exercise and can result in delayed excretion metabolites formed as a result of

exercise (Poortmans, 1984; Lemon, 1987; Dolan, 1987).

2.2.2 *L-[1-^{13}C] Leucine infusion protocol.*

On day 3 of the nitrogen balance period subjects were infused with L-[1-^{13}C] leucine to determine whole body leucine turnover according to the method described by Waterlow (1978). The infusion protocol is shown in Figure 3. At 0700 h subjects consumed a high carbohydrate breakfast, consisting of 1443 kJ (345 kcal; 4% protein, 82% carbohydrate, and 14% fat). Subjects then reported to the testing centre at 0800 h. Subjects rested for 30 minutes and then breathed into a one way valve on-line with the gas analysis system described (see section 3.2.1 for a detailed description of the on-line gas system). A minimum of six stable values were attained prior to the subject being switched into the collection apparatus. Expired air was collected into a 150 l meterological balloon. Duplicate gas samples were injected into 20 ml evacuated tubes (Vacutainer; Becton Dickinson, Rutherford, New Jersey). Breath samples were subsequently analyzed for $^{13}CO_2/^{12}CO_2$ within 72 hours of being obtained. After the baseline breath sample was obtained the subject had a 22-gauge plastic catheter inserted into a hand vein and a baseline "arterialized" blood sample (hot box at 65±5°C; Abumrad et al, 1981) was obtained. This catheter was kept patent throughout the infusion protocol by

Figure 3. Infusion day protocol. All subjects were infused with L-[1-^{13}C]leucine to determine whole body leucine kinetics. - blood sample for α-KIC enrichment, - breath sample for breath $^{13}CO_2$ enrichment.

A - At 0700 h the subject consumed a high carbohydrate snack (1493 kJ).

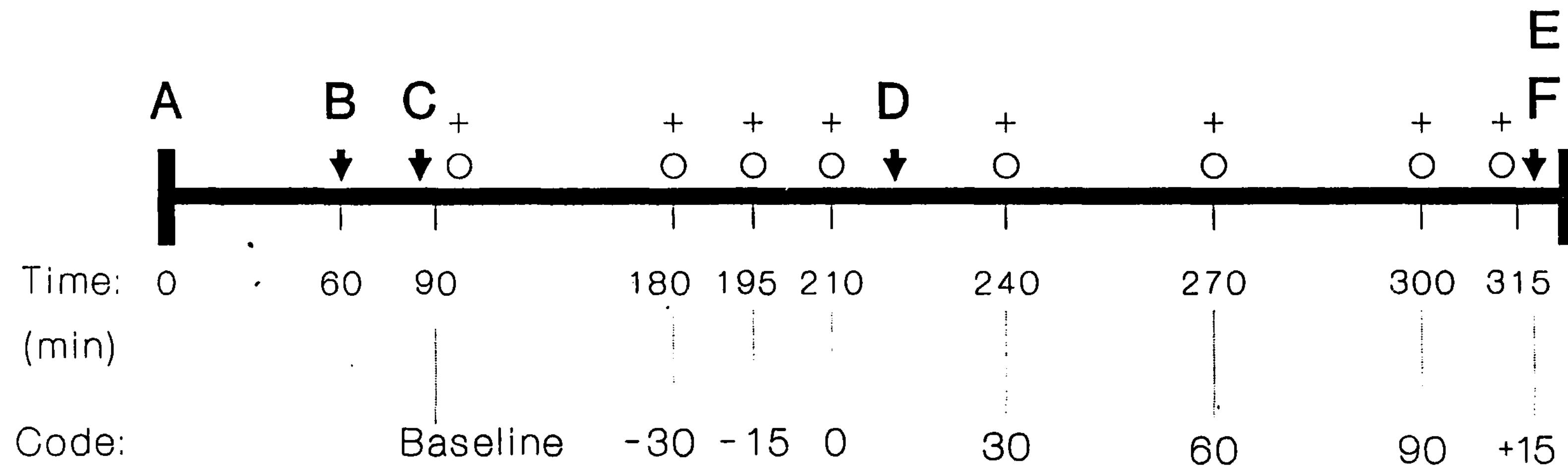
B - At 0830 h the subjects arrived at the testing centre and stretched in preparation for the 90 minute run.

C - At 0900 h a dorsal hand vein catheter was inserted and a "baseline" arterialized blood sample was obtained. Isotope was infused via a second catheter into a contralatertal forearm vein.

D - At 1100 h the subjects began to run at 65% of their pre-determined VO_{2max}.

E - At 1230 h the subject completed the run and one post-exercise blood sample was taken.

F - The subjects was weighed and had their body density determined by hydrostatic weighing.



○ - Breath sample to determine $^{13}CO_2$ enrichment.

+ - Blood sample to determine alpha-KIC enrichment

a continuous drip of 0.9% saline. Blood samples were placed into chilled, heparinized tubes and centrifuged immediately, the plasma was stored at -70°C until subsequent analysis of α-KIC enrichment. 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 $mg \cdot kg^{-1}$) and [^{13}C] sodium bicarbonate (0.295 $mg \cdot kg^{-1}$) (both 99% atom percent; MSD Isotopes, Pointe Claire, Quebec) was administered over 1 min, followed by a constant infusion of L-[1-^{13}C] leucine (1 $mg \cdot kg^{-1} \cdot min^{-1}$) for 4.5 hours, delivered by a calibrated syringe pump (Harvard Apparatus, Boston, Massachusetts). The L-[1-^{13}C] leucine was diluted into sterile, non-pyrogenic 0.9% saline the day prior to infusion and the [^{13}C] sodium bicarbonate was diluted immediately prior to being infused. Both infusates were prepared under aseptic conditions and were sterilized by microfiltration immediately prior to infusion (0.1 μm; Gelman Sciences, Ann Arbor, Michigan). At 90 (t=-30 minutes before exercise), 105 (t=-15), and 120 (t=0) minutes after the bolus injection gas and blood samples were taken to ensure isotopic plateau (Tarnopolsky et al, 1991b). Immediately following the t=0 collection, subjects began to exercise on a treadmill at a velocity requireing approximately 65% of their predetermined $\dot{V}O_2$ max for 90 minutes. Blood and gas samples were taken again

at 150 (t=30 minutes of exercise), 180 (t=60), and 210 (t=90) minutes after the bolus injection to ensure isotopic plateau and to calculate flux, oxidation, and NOLD (1.3, chapter 1). One post-exercise blood and breath sample was taken 225 (t=+15 minutes after exercise) minutes after the bolus. After the exercise subjects consumed 30 $ml \cdot kg^{-1}$ of water to compensate for exercise water losses. At this time subjects were weighed and had their body density estimated by hydrostatic weighing. Each subject also had their residual volumes measured by the helium dilution method (W.E. Collins, Braintree, Massachusetts) and their percent body fat was estimated according to the equation of Siri (1966) after correcting for resdiual volume.

2.2.3 *Background test.*

Between days 2 and 10 of the adaptation period (Figure 1) 3 male and 3 female subjects were randomized to undergo either a background test run or a bicarbonate infusion (subjects undergoing the background protocol also received an exercise sweat washdown, 2.2.1). This test was conducted in exactly the same manner as the infusion of l-[1-^{13}C] leucine (2.2.2). Subjects reported to the testing centre at 0900 after having ingested a high carbohydrate breakfast (identical to that listed in section 3.2.2) at 0700. Subjects then ran for 90 minutes at approximately 65% of their predetermined VO_2

max. During this test the speed of the treadmill for subsequent testing was adjusted to elicit approximately 65% of the subject's $\dot{V}O_2$ max. Breath samples were collected as described above (2.2.2). The subject received no isotope infusion, however, since only background changes were being assessed. This test was used to establish the changes in $^{13}CO_2$ enrichment caused by the protocol itself (see section 1.3 for more details).

2.2.4 *Bicarbonate infusion protocol.*

Subjects randomized to receive the bicarbonate infusion were infused on or between days 2 to 5 of the adaptation period (Figure 2). This test was performed far enough in advance of the labelled leucine infusion so as to allow the subject to "washout" any excess [^{13}C] sodium bicarbonate, which might have affected $^{13}CO_2$ enrichment during the labelled leucine infusion. The protocol during this test is identical to that outlined in section 2.2.2, except that no blood samples were drawn and only breath samples are collected and analyzed. Subjects undergoing this protocol received the following: a priming dose of 99 atom percent [^{13}C] sodium bicarbonate (0.295 $mg \cdot kg^{-1}$), administered over 1 minute; followed by a constant infusion of [^{13}C] sodium bicarbonate (0.443 $mg \cdot kg^{-1} \cdot min^{-1}$) for 4.5 hours. Both the prime and the infusate were prepared immediately prior to the infusion. This

test was used to determine the specific bicarbonate retention factor (c) unique to this type of exercise (1.3, chapter 1). Since this test was only done in three subjects the specific retention factors obtained for these subjects were used in correcting their oxidation values, whereas the means of the three determinations per group was used in correcting the other subjects.

2.3 Analyses.

2.3.1 *Nitrogen analysis.*

The total nitrogen content of the diets, feces, and urine were analyzed using the micro Kjeldahl method (Association of Official Analytical Chemists, 1965; Appendix XXIX). The diets were classified into two main diets based on their common contents, these were female 1, female 2, male 1, and male 2. The analyzed nitrogen content of these diets was 6.5%, 7.2%, 3.5%, and 2.2% greater than that estimated by the computer based dietary analysis program, Nutritionist III (assuming a nitrogen content of 16% for all protein).

2.3.2 Urea analysis.

The urea nitrogen content of all urine and sweat samples was analyzed using a colorimetric determination (Sigma Diagnostics kit no. 640; see Appendix XXVI for details of the method).

2.3.3 *Creatinine analysis.*

The creatinine content of all urine samples was analyzed using a colorimetric determination (Sigma Diagnostics kit no. 555; see Appendix XXVIII for details of the method).

2.3.4 *Bomb calorimetry.*

All duplicate portions of the diets ingested by the subjects were combusted in a adiabatic bomb calorimeter (Parr Instruments, Moline, Illinois) to determine gross energy content. The ratio of metabolizable to gross energy was taken overall as being 1.045 (values for carbohydrate were multiliplied by a factor 1.00, values for fat were multiplied by a factor of 1.03, and values for protein by a factor of 1.06; Merrill and Watt, 1973). The calculated gross energy from calorimetry was 6.2% and 4.3% greater than estimated for the diets female 1 and female 2 respectively (see section 2.3.1 for an explanation of the diet name system) and was 1.2% lower for male 1 and 3.2% greater for male 2 than calculated.

2.3.5 *Breath analysis.*

Carbon dioxide from the evacuated tubes was cryogenically extracted (Appendix XXX) and the $^{13}CO_2$ isotope enrichment of the sample was determined using a gas-isotope ratio mass spectrometer (VG Isogas, SIRA 10, Chesire, England) set at m/z=44/45 (Scrimgeour and Rennie, 1988). Values are expressed as APE relative to baseline samples.

2.3.6 *Plasma Analysis.*

The o-quinoxalinol-trimethylsilyl derivative of α-ketoisocaproic acid was prepared using the procedure of Wolfe (1984a), with the modifications of Tarnopolsky *et al.* (1991b) (see Appendix XXVII for detials of the derivatization procedure). The derivatized samples (0.3 μl) were injected into a 15 m, fused silica capillary column (0.25 mm internal diameter with 0.25 μm film thickness; DB 5, J.W. Scientific, Rancho Verda, California). The oven program for the gas chromatograph was set an initial temperature of 120°C, ramped to 160°C at a rate of $8°C \cdot min^{-1}$, and then ramped to 310°C at a rate of $20°C \cdot min^{-1}$ and held at 310°C for 2 minutes. Helium was used as the carrier gas ($32\ cm \cdot s^{-1}$) and the head pressure was less than 2 psi. The column exited directly into the mass spectrometer ion source, and electron impact was used to ionize the sample (70 eV, trap current 170μA, source current 1.6 mA). The m/z ratio of 232.2/233.2 was monitored by scanning the instrument over a narrow mass range (228 to 238 amu: cycle time 1.35 s; 1.25 s scan, 0.05 s interscan). The abundance of the ^{13}C enriched species (233.2 amu) of α-KIC was taken from the listing of the mass spectrum acquired at the apex of the area under the chromatographic peak. Using these methods the intra assay coefficient of variation was less than 1%.

2.4 Calculations.

2.4.1 *Whole body leucine kinetics.*

The equations for calculating leucine kinetics are presented in section 1.3, chapter 1 (see Appendix XXII-XXIV).

2.4.2 *Energy expenditure.*

Energy expenditure during exercise was calculated from RER measurements (Zuntz, 1901; McArdle et al., 1986; Appendix XIX). The contribution of protein to the energy expenditure was calculated for 3 subjects from each group that underwent an exercise sweat washdown (2.2.1). The assumptions made in calculating the contribution of protein to the energy cost of the exercise are outlined in Appendix XXV.

2.5 Statistical Analysis.

Differences between group means were assesed using Students t-test for unpaired samples. A two-way analysis of variance implementing a between/within split-plot design was used to determine whether significant differences existed between the factors (gender and time). When a significant F ratio was observed, a Tukey post hoc test (see Appendix XXI for calculation) was used to isolate the means that were significantly different. Linear regression to determine plateau enrichment of plasma α-KIC and breath $^{13}CO_2$, was analyzed using the least squares method of regression. Statistics were analyzed using either Minitab (MINITAB v 7.0;

Minitab Inc., Pittsburgh, Pennsylvania) or Anova II (ANOVA II; Apple Computing). Ventilatory threshold was calculated by using a custom designed regression analysis program that was able to perform two compartment regression analysis. Briefly, ventilation ($l \cdot min^{-1}$) was plotted against VO_2 ($l \cdot min^{-1}$) two separate regression equations were developed for the portions of the above plot, these two equations had significantly different slopes ($p<0.05$). The intersection point of these two equations was defined as the subjects ventilatory threshold. A confidence level of $p<0.05$ was taken to indicate significance, however, p values are given where appropriate.

Chapter 3

Results

3.1 Subjects' descriptive characteristics.

Table 4 gives the descriptive characteristics of the subjects (all p values are from between group comparisons by unpaired students t-test). Subjects did not differ in age, height, or weight. Male subjects did have significantly less body fat and consequently a greater lean body mass than females. Males also had a significantly higher absolute $\dot{V}O_{2max}$ ($l \cdot min^{-1}$), but when expresssed per kilogram total body weight or lean body weight there was no significant difference between males and females. Both groups of subjects had similar ventilatory thresholds, when expressed as a percentage of their $\dot{V}O_{2max}$, of approximately 80% of their respective $\dot{V}O_{2max}$. Subjects did not significantly differ with respect to their $\dot{V}O_2$, % of $\dot{V}O_{2max}$, speed, or distance completed during the 90 minute run.

3.2 Energy expenditure.

Table 5 gives the subjects' energy expenditure (EE) during the 90 minute run. Males had a greater total energy expenditure, as a result of the exercise, than females. When EE was expressed as a function of body weight or lean body weight there was no significant difference between the EE of both groups. The contribution of fat and carbohydrate to EE

Table 4. *Subjects' descriptive characteristics.*

	Males (n=6)	Females (n=6)	p
Age (yr)	23.33±1.63	23.00±2.00	0.900
Height (cm)	172.83±2.06	167.67±1.91	0.099
Weight (kg)	64.13±2.26	58.15±2.24	0.093
Body fat (%)	10.51±0.53	18.80±0.76	0.001
Lean body mass (LBM, kg)	57.40±2.25	47.25±2.07	0.009
$\dot{V}O_2$ ($l \cdot min^{-1}$)	3.79±0.14	3.15±0.21	0.036
$\dot{V}O_2$ ($ml \cdot kg^{-1} \cdot min^{-1}$)	59.05±2.58	54.66±1.82	0.200
$\dot{V}O_2$ ($ml \cdot kg\ LBM^{-1} \cdot min^{-1}$)	66.11±3.11	67.52±2.18	0.720
$\dot{V}O_{2\ VT} \cdot \dot{V}O_{2\ max}^{-1}$ (%)[a]	79.42±0.61	80.17±1.11	0.570
Test intensity (% $\dot{V}O_{2\ max}$)	64.30±1.40	62.75±0.5	0.340
Test $\dot{V}O_2$ ($ml \cdot kg^{-1} \cdot min^{-1}$)	38.01±1.66	34.37±1.19	0.110
Treadmill speed ($km \cdot h^{-1}$)	11.02±0.36	10.07±0.34	0.089
Distance completed (km)	16.52±0.54	15.10±0.51	0.089

[a] VT - Ventilatory threshold, shown as a percentage of $\dot{V}O_{2\ max}$

Table 5. *Energy expenditure and substrate utilization during the exercise bout.*

	Males	Females	p
Energy expenditure (kJ)	4551±143	3678±193	0.005
Energy expenditure ($kJ \cdot kg^{-1}$)	63.3±2.5	71.3±2.9	0.067
Energy expenditure ($kJ \cdot kg\ LBW^{-1}$)	18.6±0.7	19.1±0.8	0.700
Lipid utilization (g)	59.7±2.9	59.6±5.8	0.980
Carbohydrate utilization (g)	127.4±12	71.5±7.8	0.004
Protein utilization (g)	8.33±0.5	4.40±1.0	0.008
Lipid:Carbohydrate	0.499±0.1	0.894±0.1	0.048
Protein contribution (%)[a]	3.05±0.3	2.03±0.5	0.034

[a] - The contribution of protein to total enegy expenditure was calculated from leucine oxidation during the exercsie bout. (see Appendix XXV for calculation).

was calculated from the subjects' RER data. Males and females did not oxidize a significantly different amount of fat during exercise; males did, however, oxidize a greater amount of carbohydrate during exercise. The ratio of carbohydrate to fat oxidized shows, however, that females utilized a greater proportion of fat during the exercise period. The finding that females derived a greater proportion of their exercise energy from fat compared to carbohydrate is supported by the RER measurements during exercise. Figure 4 shows the RER data for both groups pre-exercise (0), at 30, 60, and 90 minutes during exercise, and 15 (+15) minutes post-exercise. Females had a consistently lower RER ($p<0.001$) than males at all time points throughout exercise.

3.3 Creatinine excretion.

Figure 5 shows the daily creatinine excretion during the balance period. Creatinine excretion was variable between days for certain subjects, the day-to-day variation ranged from 1.5 to 18% in female subjects and from 1.9 to 9.8% in male subjects. There were no significant differences observed between males and females. In addition, there were no significant differences in day-to-day creatinine excretion.

3.4 Urea N excretion.

Figures 6a and 6b show the urea N excretion during the balance period for both males and females. Males excreted a

Figure 4. RER ($VCO_2 \cdot VO_2^{-1}$) measurements taken at rest (t=0) and at 30 minute intervals during (t=30, 60, and 90), and 15 minutes after (t=+15) the 90 minute run. ○ - males; ● - females. G - represents a significant ($p<0.001$) gender difference Values are means±SE.

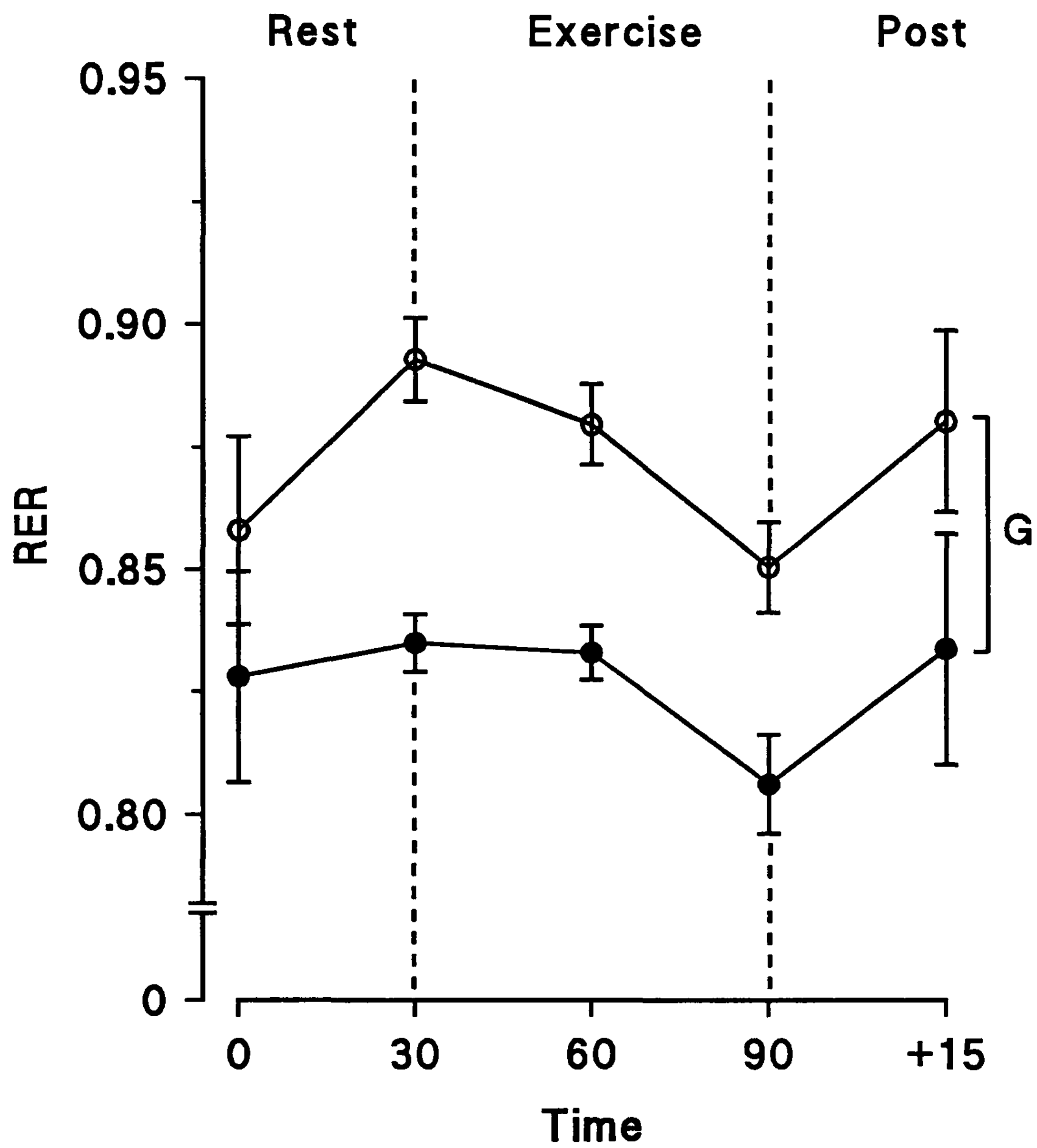
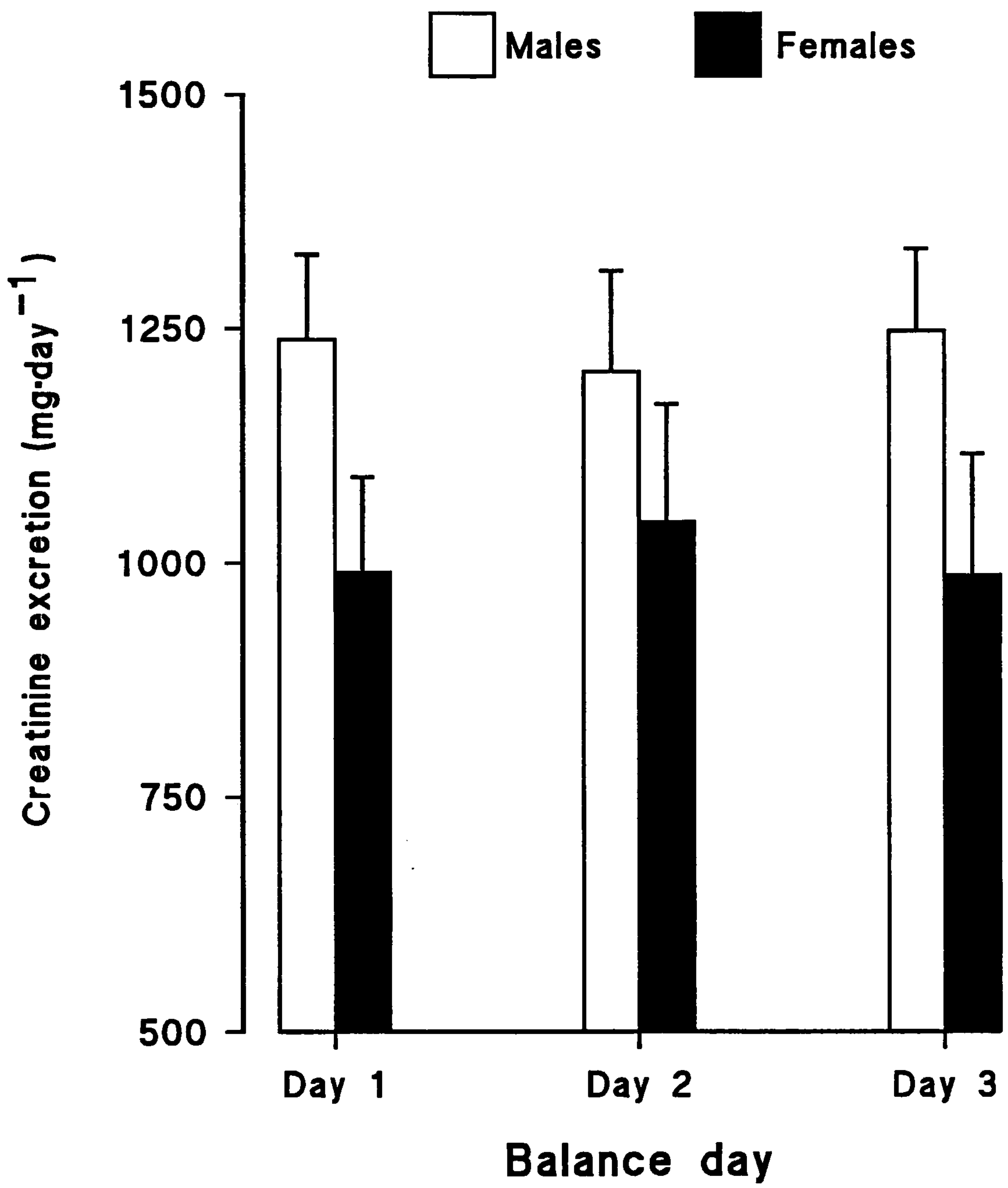

Rest
Exercise
Post
0.95
0.90
0.85
0.80
0
RER
G
0
30
60
90
+15
Time

Figure 5. Daily urinary creatinine excretion ($mg \cdot d^{-1}$) during the balance period. Values are means±SE.


Males
Females
1500
1250
1000
750
500
Creatinine excretion (mg·day⁻¹)
Day 1
Day 2
Day 3
Balance day

greater amount of urea on all balance days compared to females but the difference was not significant ($p>0.05$).

3.5 Nitrogen balance.

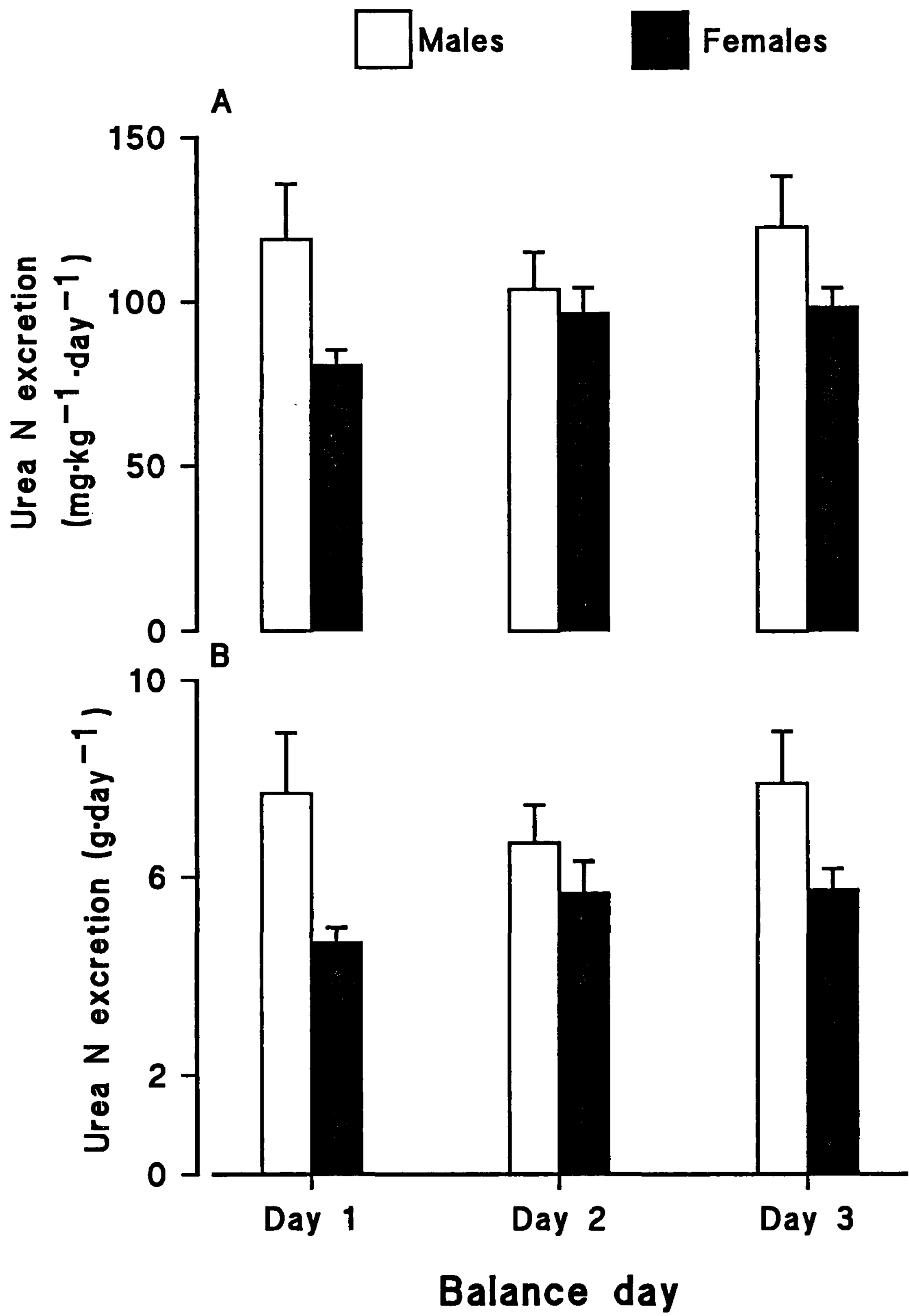
Three day nitrogen balance revealed no significant differences between genders for NBAL expressed in absolute terms or as a function of body weight (Figures 7a, and 7b respectively). Males had a greater: urinary N loss ($p=0.011$); daily absolute intake of N (0.0001); and resting sweat N excretion ($p=0.0009$; Figure 7a). Expressing NBAL as a function of body weight did not change any of the results: males had greater N intakes ($p<0.001$), urinary N losses ($p=0.04$), and resting sweat losses ($p=0.015$). The total amount of N lost by males was significantly greater than females, expressed absolutely ($g\ N \cdot day^{-1}$, $p=0.0057$), or as a function of body weight ($mg\ N \cdot kg^{-1} \cdot day^{-1}$, $p=0.031$).

3.6 Background $^{13}CO_2$ breath enrichment and bicarbonate retention factor.

Exercise caused a significant increase in endogenous breath $^{13}CO_2$ enrichment in both males and females (Figure 8a). Breath enrichment decreased significantly ($p=0.002$) with exercise in both groups although the female subjects showed a 23% increase in breath enrichment from t=60 to t=90 minutes that was not observed in the males. Breath enrichments were significantly lower than the t=30 enrichment at t=60, 90, and

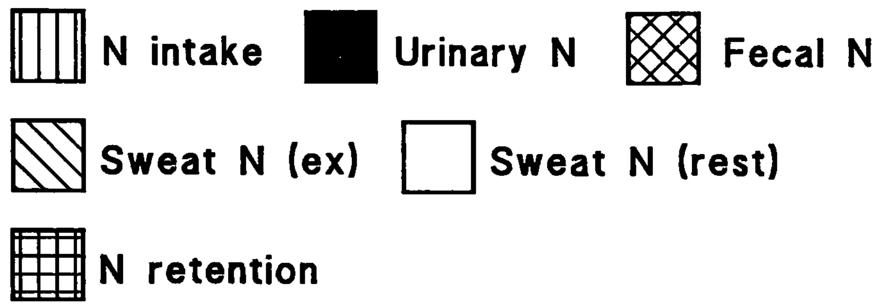
Figure 6a. Daily urea N excretion (urinary plus sweat, $mg \cdot kg^{-1} \cdot d^{-1}$) during the balance period. Values are means±SE.

Figure 6b. Daily urea N excretion (urinary plus sweat, $g \cdot d^{-1}$) during the balance period. Values are means±SE.



Males
Females
A
150
100
50
0
Urea N excretion
(mg·kg⁻¹·day⁻¹)
B
10
6
2
0
Urea N excretion (g·day⁻¹)
Day 1
Day 2
Day 3
Balance day

Figure 7a. Three day nitrogen balance (g N·d^{-1}). Values are means±SE. a: significant ($p=0.0001$) gender difference, b: significant ($p=0.011$) gender difference, and c: significant ($p=0.0009$) gender difference.


N intake
Urinary N
Fecal N
Sweat N (ex)
Sweat N (rest)
N retention

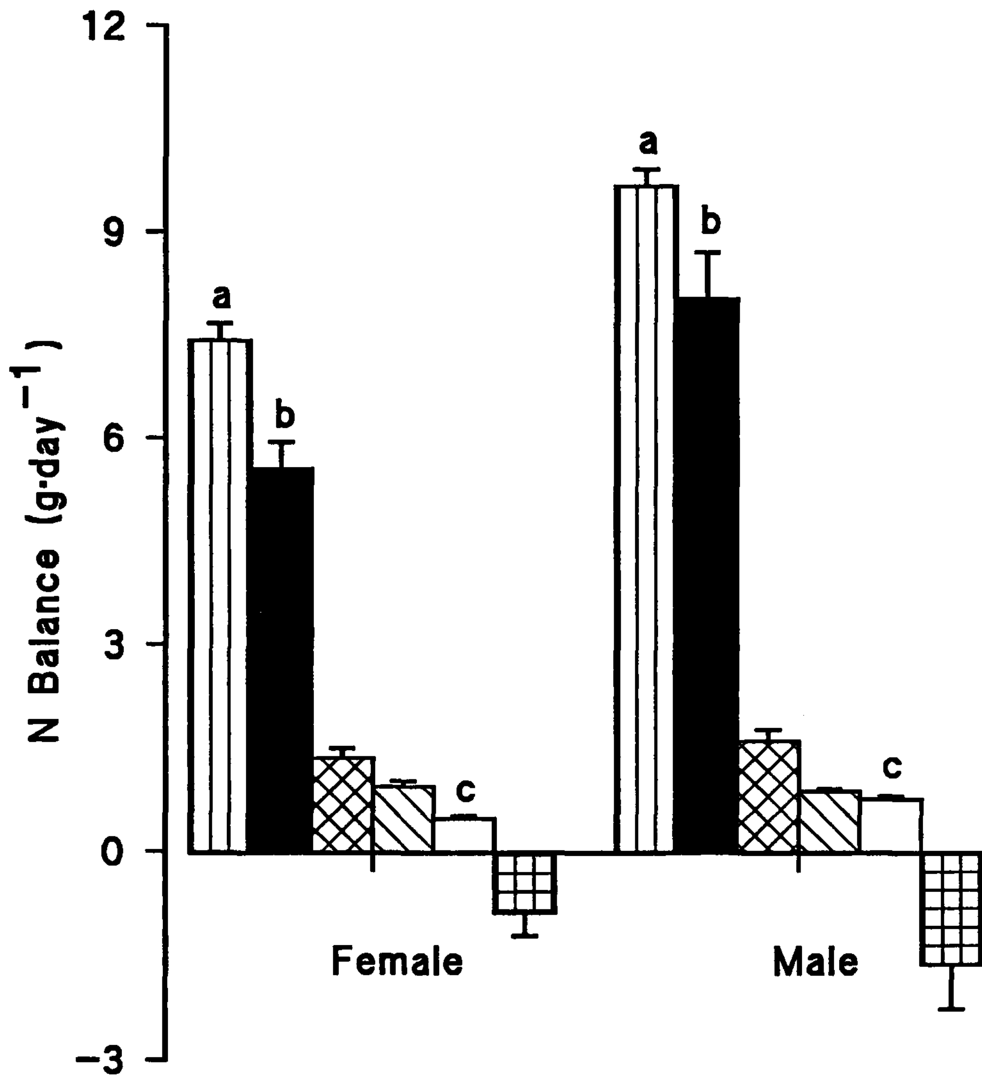

N Balance (g·day^{-1})
12
9
6
3
0
-3
a
b
c
a
b
c
Female
Male

Figure 7b. Three day nitrogen balance (mg $N \cdot kg^{-1} \cdot d^{-1}$). Values are means±SE. a: significant ($p<0.0001$) gender difference, b: significant ($p=0.04$) gender difference, and c: significant ($p=0.015$) gender difference.

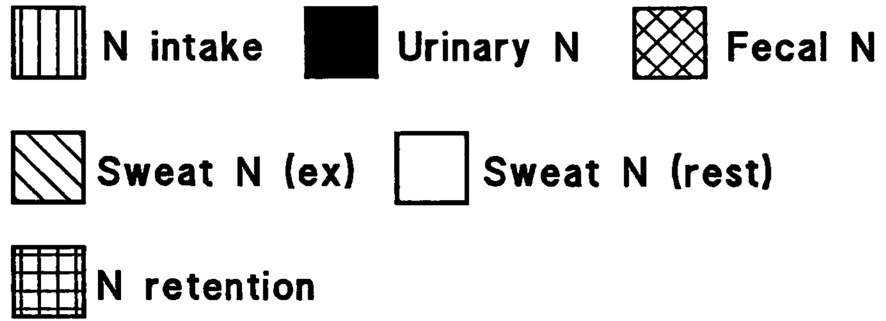
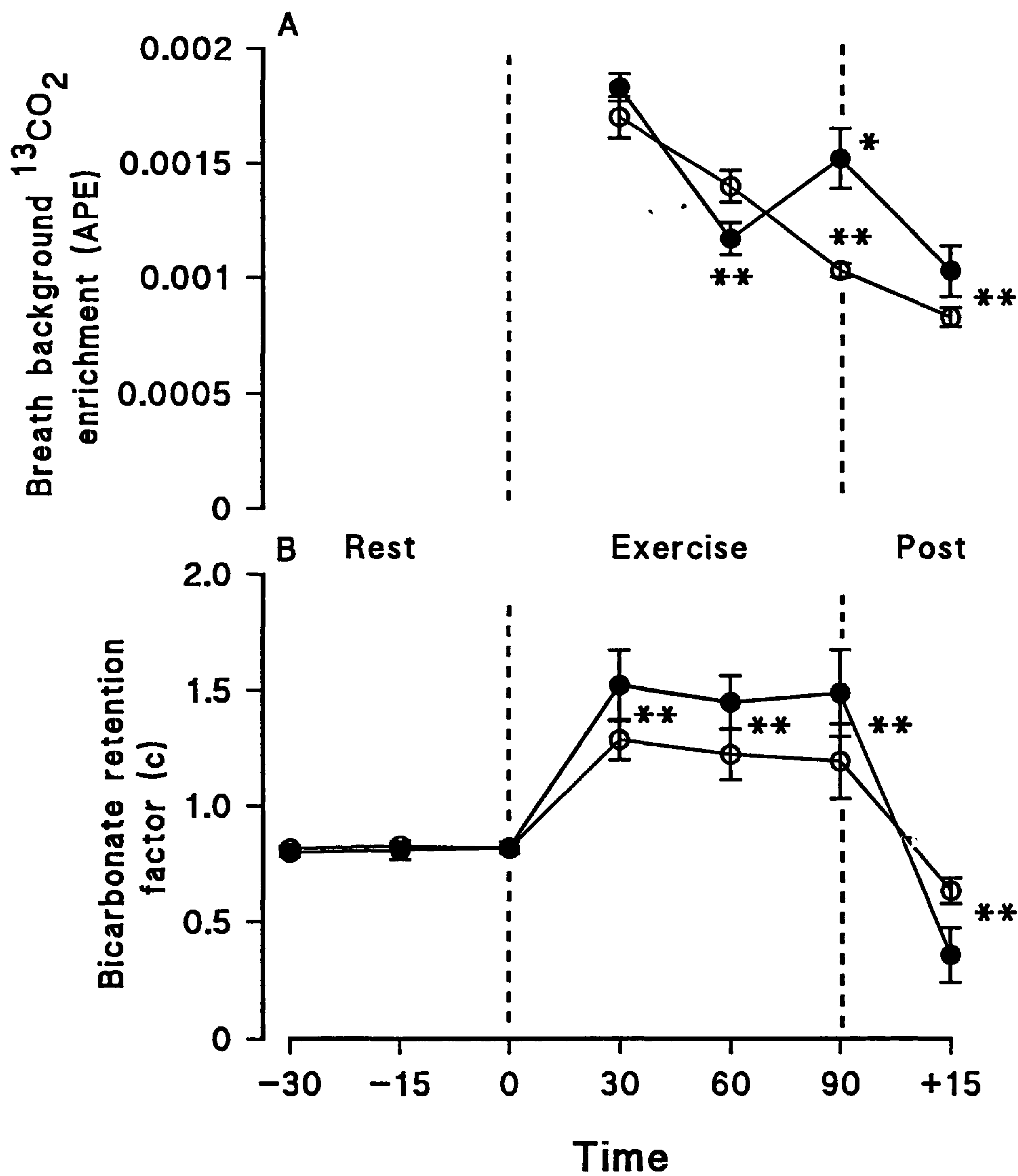

N intake
Urinary N
Fecal N
Sweat N (ex)
Sweat N (rest)
N retention
N Balance (mg·kg⁻¹·day⁻¹)
160
120
80
40
0
−40
a
b
c
a
b
c
Female
Male

Figure 8a. Background $^{13}CO_2/^{12}CO_2$ breath enrichment (APE) at 30 minute intervals (t=30, 60, and 90) during and 15 minutes post-exercise (t=+15). ○ - males, ● - females. Values are means±SE. *: significantly ($p<0.05$) different from t=30 enrichment, **: significantly ($p<0.01$) different from t=30 enrichment.

Figure 8b. Bicarbonate retention factor (c) at rest (t=-30, -15, and 0), during (t=30, 60, and 90), and 15 minutes post-exercise (t=+15). ○ - males, ● - females. Values are means±SE. *: significantly ($p<0.01$) different from resting (t=-30, -15, and 0) bicarbonate enrichment.


A
0.002
0.0015
0.001
0.0005
0
Breath background $^{13}CO_2$ enrichment (APE)
*
**
**
**
B
Rest
Exercise
Post
2.0
1.5
1.0
0.5
0
Bicarbonate retention factor (c)
**
**
**
**
−30
−15
0
30
60
90
+15
Time

+15 time points ($p<0.05$) in the female subjects. Males showed a 104% decrease in breath enrichment from t=30 to t=+15, the difference was significant at t=90 and t=+15 ($p<0.01$). There was no significant difference between genders in background breath enrichments.

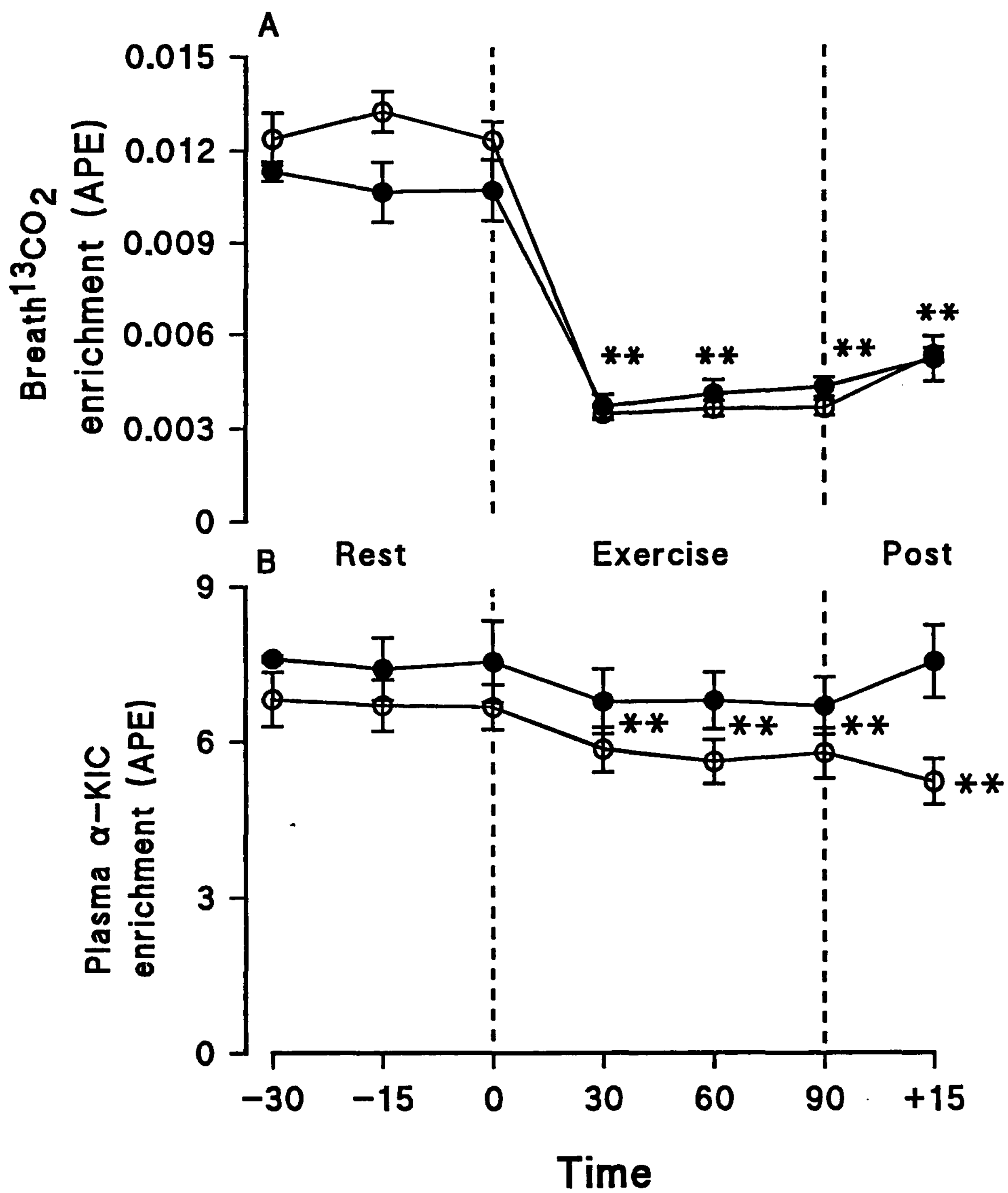
Bicarbonate retention factor (c, corrected for background enrichment) was measured in three subjects in each group (Figure 8b). The resting retention of bicaronate, calculated as the mean retention at -30, -15, and 0 time, in males was 0.82±0.0039 and in the females was 0.81±0.0069 (NS). Exercise caused a significant ($p<0.01$) increase in the bicarbonate retention factor with both groups having higher exercise retention values at all time points versus resting retention. There was a significant gender time interaction during exercise as a result of the difference in post exercise values. Post exercise (+15) bicarbonate retention was significantly ($p<0.01$) depressed versus resting values in females. Males also demonstrated a significantly ($p<0.05$) decreased retention value versus resting values at +15.

3.7 $^{13}CO_2$ breath enrichment and α-KIC enrichment.

Figure 9a shows the isotopic enrichment of breath $^{13}CO_2$ during the infusion protocol, which has been corrected for background breath enrichment. Isotopic plateau occured in both males and females pre-exercise (CV=4.11% and 3.44% for males

Figure 9a. Breath $^{13}CO_2$ enrichment at rest, during the exercise bout, and 15 minutes post-exercise (see figures 8a and b for explanation). ○ - males, ● - females. Values are means±SE. **: significantly ($p<0.01$) different from resting breath enrichment.

Figure 9b. Plasma α-KIC enrichment at rest, during the exercise bout, and 15 minute post-exercise (see figures 8a and b for explanation). ○ - males, ● - females. Values are means±SE. **: significantly ($p<0.01$) different from resting plasma enrichment.


A
0.015
0.012
0.009
0.006
0.003
0
Breath$^{13}CO_2$ enrichment (APE)
**
**
**
**
B
Rest
Exercise
Post
9
6
3
0
Plasma α–KIC enrichment (APE)
**
**
**
**
–30
–15
0
30
60
90
+15
Time

and females). The regression lines joining the mean values at -30, -15, and 0 had slopes of -0.0113 and -0.2077 for males and females respectively, both of which were not significantly different from zero (p=0.979 and 0.376). There was a significant (p<0.05) gender time interaction that occured pre-exercise that was not apparent at any other time points. Exercise resulted in a significant (p<0.01) drop in breath enrichment from pre-exercise values for both males and females. Isotopic plateau was reestablished during exercise in breath $^{13}CO_2$ enrichment with the CVs being 2.97% and 7.88% and the regression slopes of these lines being 0.0333 and 0.1055 (p=0.235 and p=0.096) for males and females respectively. Post exercise breath enrichment was also significantly lower (p<0.01) than pre-exercsie breath enrichment for both males and females.

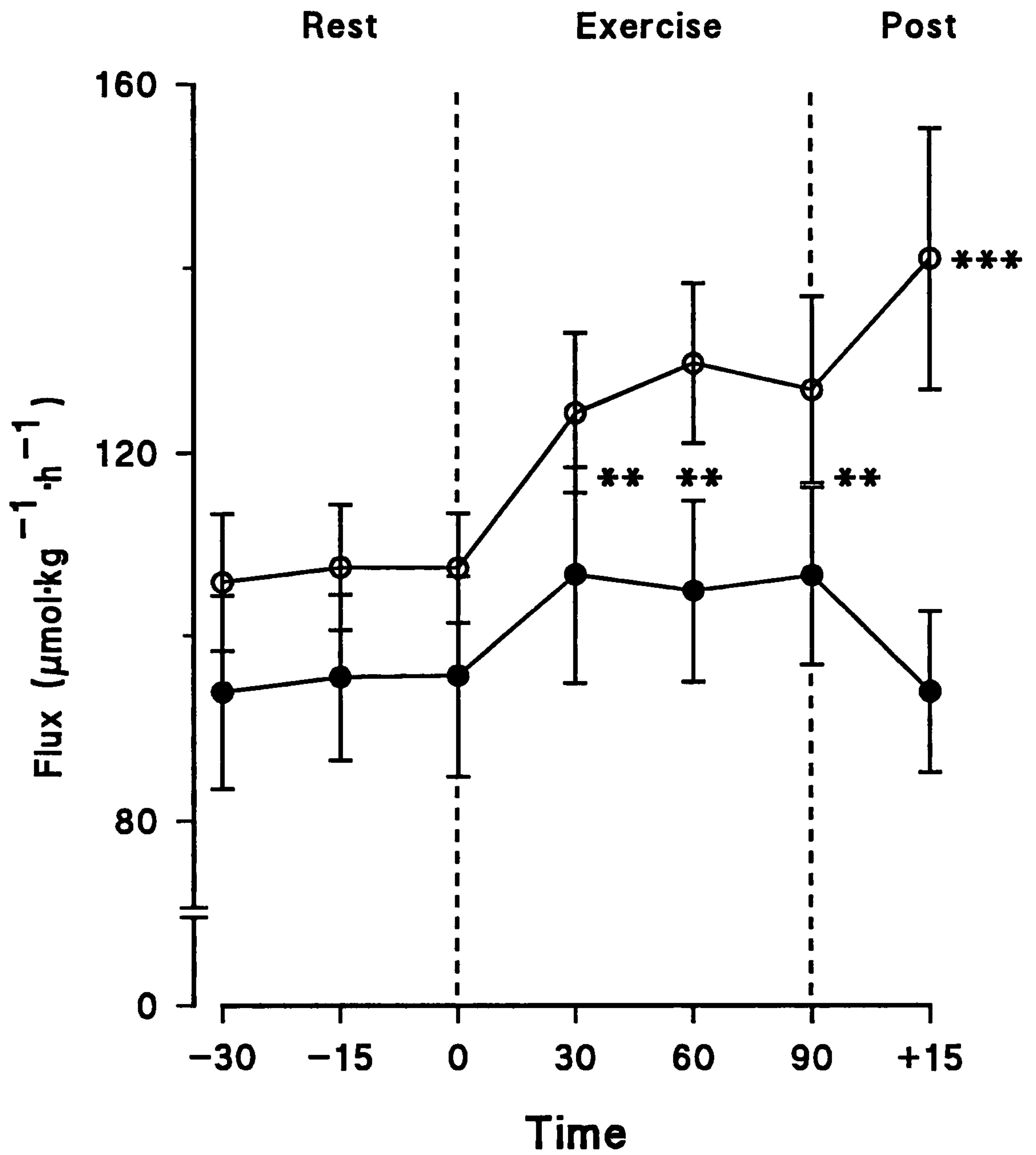
The enrichment of α-KIC was at isotopic plateau prior to exercise in both groups of subjects. The CV of the values obtained prior to exercise were 1.18% and 1.29% and the slopes of the lines joining the -30, -15, and 0 values were -0.005 and -0.002 (neither of which were significantly different from zero, p=0.212 and p=0.80) respectively for males and females respectively. All of these criteria by definition indicate a plateau (Thompson et al., 1988; Hoerr et al., 1991). Exercise resulted in a significant (p<0.01)

drop in α-KIC enrichment in both males and females; all exercise enrichments were significantly lower than pre-exercise enrichments (Figure 8b). During exercise isotopic plateau enrichment in both males and females was maintained; the CV was less than 3% in both cases and the slopes were -0.0023 and -0.003 ($p=0.808$ and $p=0.391$ respectively). Post exercise males showed a further drop in enrichment of α-KIC ($p<0.01$), whereas females showed an increase in α-KIC enrichment to pre-exercise values (NS). There was also a significant gender time interaction as a result of the post exercise values ($p<0.01$).

3.8 Leucine flux.

Since isotopic plateau was achieved in all sampled pools equations (Golden and Waterlow, 1977; Wolfe, 1984a; Wolfe et al., 1982) were applied to calculate leucine flux, oxidation, and non-oxidative disposal (see section 1.3, chapter 1 for details). Figure 10 shows leucine flux during the infusion protocol. Flux in this condition also represents protein breakdown, since there is no exogenous intake of leucine (see section 1.3, chapter 1 for an explanation). Flux exhibited a significant ($p<0.01$) gender time interaction as a result of the difference in post-exercise flux in males and females. Exercise caused a significant ($p<0.01$) increase in flux compared to the mean resting (mean of flux at t=-30, -15, and

Figure 10. Leucine flux (Q, $\mu mol \cdot kg^{-1} \cdot h^{-1}$) at rest, during the exercise bout, and 15 minutes post-exercise (see figures 8a and b for explanation). O - males, ● - females. Values are means±SE. **: significantly ($p<0.01$) different from resting flux, ***: significantly ($P<0.01$) different from exercising flux.


Rest
Exercise
Post
160
120
80
0
Flux (μmol·kg⁻¹·h⁻¹)
***
**
**
**
−30
−15
0
30
60
90
+15
Time

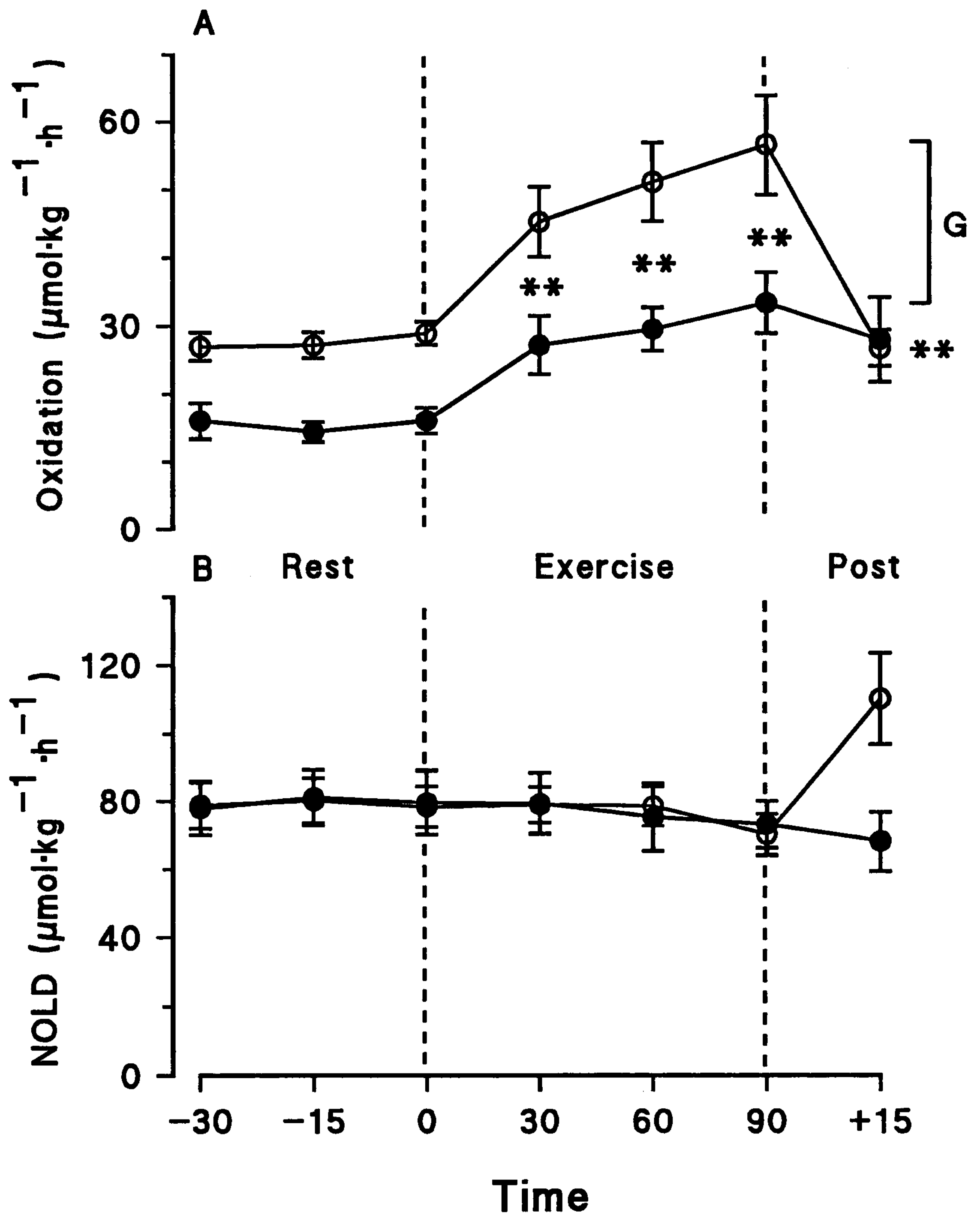
0) flux in both males and females. Post exercise flux returned to pre-exercise values in females (NS). Males, however, showed a large (11.6%), significant ($p<0.01$) increase in flux compared to the mean exercising flux (mean of flux at t=30, 60, and 90).

3.9 Leucine oxidation and non-oxidative leucine disposal.

Figure 11a shows leucine oxidation by males and females during the infusion protocol. Leucine oxidation was greater at all time points, except post exercise, for males versus females and there was a significant main effect for gender ($p=0.04$). Leucine oxidation also showed a significant gender time interaction, which was evident throughout the infusion. Leucine oxidation was 15.46±0.59 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ at rest (calculated as the mean of the values at t=-30, -15, and 0), for the females and 27.75±0.63 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ at rest for the males. Exercise significantly ($p<0.01$) elevated leucine oxidation by 95% in the females and 84% in males, oxidation was greater at all times during exercise compared to the mean resting oxidation values. The mean exercising leucine oxidation (calculated as the mean of values at t=30, 60, and 90) was almost 70% greater in the males than the females (30.15±3.14 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ versus 51.13±5.68 $\mu mol \cdot kg^{-1} \cdot h^{-1}$). Post exercise oxidation in males returned to pre-exercise values (NS). In females, however, oxidation was still elevated at +15

Figure 11a. Leucine oxidation ($\mu mol \cdot kg^{-1} \cdot h^{-1}$) at rest, during the exercise bout, and 15 minutes post-exercise (see figures 8a and b for explanation). ○ - males, ● - females. Values are means±SE. **: significantly different from resting oxidation, G: significant (p=0.004) gender difference.

Figure 11b. Non-oxidative leucine disposal (NOLD, $\mu mol \cdot kg^{-1} \cdot h^{-1}$) at rest, during the exercise bout, and 15 minutes post-exercise (see figures 8a and b for explanation). ○- males, ●- females. Values are means±SE.


A
Oxidation (μmol·kg⁻¹·h⁻¹)
60
30
0
**
**
**
G
**
B
Rest
Exercise
Post
NOLD (μmol·kg⁻¹·h⁻¹)
120
80
40
0
−30
−15
0
30
60
90
+15
Time

compared to pre-exercise values.

Using the estimate that each gram of body tissue protein contains 590 μmol of leucine (Wolfe et al, 1982; Wolfe, 1984a; Wolfe et al, 1984b) the calculation of how much protein was utilized during the run can be made. It was calculated that females utilized 4.42 g protein during exercise, while males utilized 8.27 g protein during exercise. This amount of protein could account for 88.2% of the negative NBAL in the females and 90.8% of the negative NBAL in the males.

Non-oxidative leucine disposal (NOLD) is an approximation of protein synthesis (see section 1.3, chapter 1 for an explananation). Figure 11b shows the response of NOLD during the infusion protocol. Males and females had very similar responses in NOLD during the infusion protocol. Exercise did not have any significant effect on NOLD in males or females. Post exercise NOLD showed a significant ($p<0.01$) gender time interaction, with males showing a 40% increase in NOLD while females showed a 12% decrease.

Chapter 4

Discussion

The results of this thesis demonstrate that the RNI for protein is inadequate for persons habitually engaging in endurance exercise. When male and female athletes consuming their habitual energy intake and protein close to the Canadian RNI for protein (males = 0.94±0.01 g protein·kg^{-1} and females = 0.80±0.01 g protein·kg^{-1}) five of six of the female athletes and all of the male athletes were in negative nitrogen balance. Net N retention was -0.89±0.34 g·d^{-1} and -1.62±0.64 g·d^{-1} for the female and male subjects respectively. The lack of a statistically significant difference between the nitrogen balance of the male and female subjects is due to the high degree of variability in the balance estimates.

The estimates of sweat urea N losses during exercise, in the current study, are high compared to estimates from other investigations. This overestimate of sweat loss could lead to the conclusion that the estimates of balance were underestimated and that the conclusions regarding the adequacy of the RNI may be due to the magnitude of the sweat losses. Lemon and Mullin (1981) found that carbohydrate loaded subjects excreted approximately 600 mg sweat urea N·h^{-1}, whereas carbohydrate depleted subjects excretedalmost 1500 mg·h^{-1}. However, these estimates of sweat urea N excretion were

estimated from a single site (mid-back) and have subsequently been shown to be an overestimate of whole body sweat losses (Lemon et al, 1986). A pooled estimate of the sweat urea N losses from three studies of subjects running at comparable intensities (Lemon et al, 1986; Dolny and Lemon, 1988; Friedman and Lemon, 1989) showed that subjects excreted approximately 260 mg sweat urea $N \cdot h^{-1}$. The rate of sweat loss in this study was 626 mg sweat urea $N \cdot h^{-1}$ for the female athletes and 578 mg sweat urea $N \cdot h^{-1}$ for the male athletes. In the current investigation, the high estimates of sweat loss could have been due to the length of exercise studied (90 minutes). Compared to the previously mentioned studies (Lemon and Mullin, 1981; Lemon et al, 1986; Friedman and Lemon, 1989) which were only examining subjects running for 60 minutes. Since it is has been shown that serum urea increases sharply only after 60 minutes of endurance exercise (Lemon, 1987). That would result in a greater sweat urea N excretion after 60 minutes of exercise. In addition, the longer exercise bout of the current study would result in a greater muscle glycogen utilization (Hultman, 1989), which has been shown to increase sweat urea excretion (Lemon and Mullin, 1981). Since the current estimates of exercising sweat urea N loss were only obtained from three subjects per group the values may represent an overestimation due to the small sample size

studied.

It is not surprising that the athletes in this study were in negative nitrogen balance when consuming the low protein intakes provided. Other researchers have shown protein requirements for regularly exercising persons to be greater than the protein requirements of sedentary persons. The protein requirements of elite endurance athletes (runners and nordic skiers) were found to be 1.67 times greater than the protein requirements of sedentary persons (Tarnopolsky et al, 1988). The subjects studied by Tarnopolsky *et al.* (1988) were provided with pre-packaged, pre-weighed diets containing the subject's habitual energy intake and two defined protein intakes (both greater than requirement). A three day nitrogen balance was determined for each athlete consuming each protein intake. Regression analysis of nitrogen balance at each protein intake showed that when the protein intake to achieve zero nitrogen balance was adjusted for individual variability (by adding one standard deviation) the safe protein intake for the endurance athletes was 1.6 g protein$\cdot kg^{-1} \cdot d^{-1}$ (186% of the RNI for protein). The safe protein intake recommended by Tarnopolsky *et al.* (1988) is 1.7 and 2 times greater than the protein intakes consumed by the male and female athletes in this study respectively.

Athletes completing an intensive bout of exhaustive exercise may have even greater protein requirements, in the short term, than those who routinely exercise. Men completing two exhausting exercise days of cycle ergometry in a metabolic chamber - designed to simulate energy expenditures of similar magnitude to those that occur during the Tour de France bicycle race - were reported to have protein requirements in excess of 175% of the current RNI (Brouns et al, 1989a). The energy and protein intake of these cyclists during the experiment was *ad libitum* and included a high carbohydrate supplement of either maltodextrin or fructose. The intakes of the subjects were monitored and recorded by the investigators. During the two days of exhaustive cycling, the subjects were unable to maintain energy balance and excreted (urine, sweat and feces) a greater amount of N than they consumed (1.7 g protein$\cdot kg^{-1}$; Brouns et al, 1989c). The authors concluded that the high protein intake consumed by the cyclists was insufficient primarily due to the negative energy balance (-6.6 ± 0.5 MJ$\cdot d^{-1}$) experienced by the subjects. Nonetheless, the conclusions reached by Brouns *et al.* (1989a) are only applicable to athletes engaging in exercise of similar energy expenditures (26 MJ$\cdot d^{-1}$, 6200 Kcal$\cdot d^{-1}$). Since, the magnitude of these energy expenditures is unlikely to be duplicated by athletes on a consistent basis, the high intakes of protein

needed to maintain nitrogen balance reported by Brouns *et al.* (1989a) probably represent an extreme.

Other investigations of the protein requirements for athletes have shown that intakes close to the RNI were insufficient to maintain positive nitrogen balance (Friedman and Lemon, 1989). Friedman and Lemon (1989) examined runners consuming self-selected diets at a high (1.49±0.29 g protein$\cdot$kg^{-1}) and a low (0.86±0.23 g protein$\cdot$kg^{-1}) protein intake. The subjects consumed a meat-free diet for five days prior to completing a nitrogen balance experiment. Nitrogen balance of each subject was determined at each protein intake by collecting urine and sweat, and determining N content. Fecal and miscellaneous N losses were estimated and the total N was subtracted from the N intake (determined from dietary records). The net N retention was positive 2.41 g$\cdot$d^{-1} on the high protein diet and was negative -5.27 g$\cdot$d^{-1} on the low protein diet. Since the investigators did not measure all routes of N loss they estimated that the "hypothetical" protein requirements of this group were between 1.14 and 1.39 g protein$\cdot$kg$^{-1}\cdot$d^{-1} (132-161% of the protein RNI). The subjects studied by Friedman and Lemon were experienced long-distance runners (≥ 5 years) and were studied during their normal training routine. Thus, the results were not likely due to a transient training effect observed by some investigators

(Gontzea et al, 1975; Gontzea et al, 1975). However, since the subjects studied by Friedman and Lemon (1989) did not consume a controlled diet, the effects of excess energy and carbohydrate to fat ratio were not controlled. Analysis of the diets consumed at each protein intake showed that subjects consumed an extra 2052 kJ on the high protein diet. Since excess energy can increase protein retention (Göranzon and Forsum, 1985;) the estimates of nitrogen balance may be falsely increased.

Despite the fact that some studies have shown that the protein requirements of regularly exercising endurance athletes are greater than the RNI level, Meredith *et al.* (1989) reached a contrary conclusion. The protein requirements of endurance trained men were examined. The subjects studied by Meredith *et al.* were experienced (12.8 years training) habitually training endurance athletes (12.3 $h \cdot wk^{-1}$ of running). The protein requirements of these athletes were estimated from nitrogen balance at three levels of protein intake (0.6, 0.9, and 1.2 g $protein \cdot d^{-1}$). The protein intake needed to achieve zero nitrogen balance in these athletes was 0.92 ± 0.06 g $protein \cdot kg^{-1} \cdot d^{-1}$. This protein intake is roughly comparable to the protein consumed by the male subjects in this investigation. However, the results of this thesis have shown that this protein intake was not sufficient and resulted

in a negative nitrogen balance. This discrepancy could be due to the higher energy intakes consumed by the subjects studied by Meredith *et al.*, 18108 $kJ \cdot d^{-1}$ (254.7 $kJ \cdot kg^{-1} \cdot d^{-1}$), compared to the subjects of this study, 13319±889 $kJ \cdot d^{-1}$ (207.4±14.5 $kJ \cdot kg^{-1} \cdot d^{-1}$). The difference between the intakes consumed by the males in this study, and those consumed by the subjects of Meredith *et al.* (1989) is 4789 $kJ \cdot d^{-1}$. This difference could account for approximately 3.4 g $N \cdot d^{-1}$ (assuming the estimate by Calloway (1975) of an increase in N retention of 0.48-0.72 g $N \cdot 1000\ kJ^{-1}$ above the energy needed for weight maintenance is accurate). In the current study, regression analysis of the male subjects' weights, in the current study, measured three times during the 13 day study period, showed that the slope of the line joining successive weight measurements was not significantly different ($p=0.147$) from zero[3]. Thus, the diets consumed by the subjects in the current investigation contained sufficient energy to maintain weight and, therefore, the estimates of nitrogen balance are valid (FAO/WHO/UNU, 1985). The subjects of Meredith *et al.* (1989) were reported to be consuming sufficient energy to maintain body weight and were completing approximately 12 hours of endurance training per week. However, neither the intensity nor type of exercise

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[3] The regression equation joining the weights of the female subjects, measured on three separate occasions, also had a non-significant slope ($p=0.088$).

completed by the subjects was reported. The results of the current investigation and those of Meredith *et al.* (1989) could also have been different due to a type II error because of the small sample size. Meredith *et al.* (1989) reported that the safe intake of protein for endurance trained men would be 1.26 g protein$\cdot kg^{-1}\cdot d^{-1}$ of good quality protein[4]. Hence, it is possible that the six subjects in the present study, given the small sample size, had protein requirements that were closer to the 1.26 g protein suggested by Meredith *et al.* (1989).

Some investigators have found the protein requirements of persons who engage in habitual endurance activities to be no greater (Todd et al, 1984; Butterfield, 1987) and even less (Butterfield and Calloway, 1984) than the protein requirements of sedentary individuals. Butterfield and Calloway (1984) found that a protein intake of 0.57 g protein$\cdot kg^{-1}\cdot d^{-1}$ was sufficient to maintain nitrogen equlibrium (balance) in exercising males. Butterfield and Calloway (1984) interpreted their findings to suggest that the requirement for protein in chronically active individuals may be less than the protein requirements of inactive individuals. The protein

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[4] Good quality PRO defines PRO which has a high bioavailability. Since Meredith *et al.* (1989) used a mixed PRO mixture made up of milk and egg solids, which was of higher quality than the average North American diet (Pellet, 1990), then the values of nitrogen balance will be underestimated relative to the average North American diet (Pellet, 1990).

intake found to be sufficient by these investigators is well below the intakes consumed by the subjects in the present study, which were shown here to be inadequate. The finding of Butterfield and Calloway (1984) conflicts with the findings of this thesis primarily for the following reasons. The individuals studied by Butterfield and Calloway (1984) were beginning a comparatively mild exercise program consisting of a maximum of one hour of treadmill walking (3 miles$\cdot h^{-1}$ at 10% grade or approximately 25 ml $O_2\cdot kg^{-1}\cdot min^{-1}$) plus one hour of cycle ergometry (700 Kp at 60 r.p.m. or approximately 1.75 $l\cdot min^{-1}$). These exercise intensities represent approximately 35% of $\dot{V}O_{2max}$ which is lower than the exercise intensities that the subjects of this study were habitually engaging in: approximately 10 $km\cdot d^{-1}$ at 4 $min\cdot km^{-1}$ pace (see Table 1 for subjects' training characteristics). In addition, the subjects of Butterfield and Calloway (1984) completed the dietary treatment in the same order, starting with little or no exercise and increasing to the program described above. This would result in the exercises completed at the end of the study being of a lower relative intensity, due to the training effects on substrate metabolism such as decreased blood glucose turnover (Coggan et al, 1990), decreased muscle glycogen oxidation (Holloszy and Coyle, 1984), and decreased reliance on anaerobic glycolysis (Green et al, 1991). The